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Development of Novel Alkaloid Derivatives For the Treatment of Chronic Myeloid Leukemia

Lindsay Michelle Renn
Rowan University, Renn.lindsay@gmail.com

DEVELOPMENT OF NOVEL ALKALOID DERIVATIVES FOR THE TREATMENT OF CHRONIC MYELOID LEUKEMIA

by

Lindsay M. Renn

A Thesis

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Dedications

To my father,

Without you, I would not be where I am today. You have taught me right from wrong and that in the end hard work pays off. Thank you for your continuous love, support and encouragement while I was completing my education. I do what I do to make you proud and hope to one day repay all of the favors and support you have given to me.

To my brother and sister,

I am so proud of the adults you have become. I strive to be the best role model and sister I can be, for you. You push me to work harder and do the right thing. I don't know where I would be, if I did not have both of you. Thank you for the constant motivation and support throughout this entire process.

Acknowledgement

To my mentor,

I would like to acknowledge my mentor for the time, hard work and patience she has given me. I truly do not know how I can ever thank you enough. You have allowed me the opportunity to be the best scientist I can be. You have taught me so much personally and educationally and I will carry this knowledge throughout my career and life. I am forever grateful that I was a part of your lab.

Abstract

Lindsay M. Renn
DEVELOPMENT OF NOVEL ALKALOID DERIVATIVES FOR THE TREATMENT OF CHRONIC MYELOID LEUKEMIA
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Gregory A. Caputo, Ph.D.
Master of Science in Pharmaceutical Sciences

The majority of chronic myeloid leukemia (CML) patients can be treated with and respond to imatinib mesylate (Gleevec). Imatinib is known to inhibit BCR-ABLl kinase activity, and is effective for the treatment of the majority of CML patients. Multiple mutations have been found in patients resistant to imatinib treatment, including many located in the BCR-ABLl tyrosine kinase domain (e.g. E255K and T315I). Matrine is a bioactive alkaloid from *Sophora flavescens* and has been shown to inhibit several types of cancers and is used in Chinese medicine. The goal of this study is to develop new matrine derivatives that inhibit growth or induce apoptosis of imatinib-resistant CML cells. *In vitro* toxicity assays were performed using CML cells and various compounds, including matrine derivatives. Initial results indicate that there is approximately a 70% increase in apoptosis within 72 h of treatment in CML cells when treated with matrine, open matrine, and AMA3. After testing a second-round of derivatives, 156 and 159, it was determined that there is a significant increase in apoptosis of CML cells, especially when treated in combination with Imatinib. However, all derivatives require a concentration of at least 40 μM and induce apoptosis in NIH3T3 cells (fibroblast control cells). It appears though that 156 and 159 combination treatment with Imatinib is effective against E255K cells, but not T315I cells. Therefore, refinement of these inhibitors is warranted.

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Chapter 1

General Introduction

Chronic Myeloid Leukemia

Cancer occurs when abnormal cells in the body begin to grow uncontrollably and avoid apoptotic and senescent signals, among other traits (Hanahan & Weinberg, 2011). Abnormal cell growth may lead to formation of a detectable mass of tissue known as a tumor, but other non-solid cancers such as leukemia do not. Cancer affects more than one million people each year and can develop from any cell type in the body; however, some cancer types are more common than others. There are more than 100 different types of cancer which are categorized by the properties of the cell from which the cancer arises (What is cancer?; Chronic myelogenous leukemia treatment.). Cancers are categorized into five main categories; carcinomas, sarcomas, leukemias, lymphomas, and central nervous system cancers (What is cancer?). Carcinomas are found in or on the lining of internal organs, sarcomas develop in bone or connective tissues, leukemia originates in the bone marrow and blood cells, lymphoma involves abnormalities of the immune system, and central nervous system cancers originate in the brain and the spinal cord (What is cancer? - cancer treatment centers of america | CTCA.).

Leukemic cells do not form solid tumors because they originate in the blood and bone marrow. However, large numbers of abnormal white blood cells will accumulate and eventually outnumber the healthy blood cells. There are four major types of leukemia: acute myeloid, chronic myeloid, acute lymphoblastic, and chronic lymphoblastic. The difference between acute and chronic leukemia is that acute

leukemia is a rapid progression of cells that are not fully developed, whereas chronic leukemia is a slow progression with mature cells which have some normal function (Arceci, R., 2014). Lymphoblastic leukemia originates from lymphocyte progenitors and forms abnormal lymphocytes (B- or T-cells), and myeloid leukemia forms from myeloid progenitors.

Chronic myeloid leukemia (CML) is a myeloproliferative disorder of multipotent hematopoietic stem cells (Gorre et al., 2001). CML is prevalent in adults and rare in children and adolescents because the disease develops due to acquired mutations in the genomic DNA of a bone marrow stem cell, which will then continue to produce CML cells. Therefore, only a portion of the bone marrow stem cells is abnormal, so normal cells are also produced. CML accounts for 10-20% of all adult cases of leukemia and has a low annual mortality rate (1-2%) (Yun et al., 2014). The prevalence is predicted to reach 180,000 new cases per year by the year 2030 (Yun et al., 2014). When a patient is diagnosed with CML they typically do not have any symptoms because the chronic phase does not completely interfere with red blood cell development and is less severe than acute leukemia (Arceci, R., 2014). If symptoms are present they usually are tiredness, shortness of breath, unexplained weight loss, pain in abdomen, anemia, night sweats, and intolerance to warm climates (Arceci, R., 2014). There are three different phases of CML - chronic, accelerated, and blast crisis. CML in the chronic phase is mild and treatable because less than 10% of the cells in the bone marrow are immature white blood cells (blasts). In the accelerated phase, the blast percentage is higher than normal levels and patients will begin to feel fatigued or develop splenomegaly (enlarged spleen). During blast crisis phase, the number of blasts increases in the blood and bone marrow. This

increase in blast cells causes a decrease in red blood cells, platelets and neutrophils and the patient will feel fatigue, bone pain, abdominal pain due to spleen enlargement, bleeding, and infections.

The cause of CML is attributed to the Philadelphia (Ph) chromosome which is a reciprocal translocation of chromosomes 9 and 22, t(9;22) (q34;q11) (Yun et al., 2014). The translocation allows for the expression of a 210 kDa chimeric fusion protein known as p210 BCR-ABL which leads to constitutive activity of the tyrosine kinase (Puissant et al., 2008). The unregulated tyrosine kinase activity in CML patients plays a key role in the pathogenicity and the progression of this disease (Yun et al., 2014), which is why the BCR-ABL tyrosine kinase is an effective drug target for CML patients.

Tyrosine Kinase Inhibitors

Imatinib (Gleevec) is a tyrosine kinase inhibitor (TKI), developed by Novartis in 1993. Imatinib was a breakthrough discovery because of its selectivity and specificity; it was the first targeted drug to be used as a cancer therapy (Lamontanara, Gencer, Kuzyk, & Hantschel, 2013). This drug specifically targets the BCR-ABL tyrosine kinase in patients diagnosed with CML. It is only an initial therapy for chronic phase and helps control diseases progression in most patients for at least 10 years (Arceci, R., 2014). Patients that are treated with Imatinib may experience a variety of side effects, including edema, nausea, vomiting, muscle cramps, chronic fatigue, and possible cardiac events. Although the development of Imatinib was ground-breaking for CML treatment, it is not a cure. A portion of the patients (12-15%) are refractory to Imatinib treatment and more than 50-85% of the resistance to treatment is attributed to point mutations that occur in

the BCR-ABL tyrosine kinase domain (Arceci, R., 2014; (Soverini et al., 2011). The T315I mutation accounts for 15-20% of mutations in the ABL kinase domain, and the E255K mutation is also highly prevalent (17%) (Yun et al., 2014). These two mutations disrupt the binding interactions of the ABL kinase with Imatinib (Figure 1), making it nearly impossible for Imatinib to interact with the kinase resulting in resistance to Imatinib treatment in CML cells carrying these mutations.

The E255K mutation occurs in the glycine (Gly)-rich loop of the ABL kinase. The degree of resistance of mutations that occur in the Gly-rich loop is relatively mild, however, this change is sufficient to restore BCR-ABL kinase activity in patients treated with Imatinib carrying BCR-ABL with clones of this mutation. In addition, increased kinase activity has been shown for the E255K mutation. So, in addition to mediating Imatinib resistance, E255K may make BCR-ABL more 'aggressive', which would lead to a poorer prognosis for patients containing this mutation (Lamontanara et al., 2013).

The T315I mutation occurs in the gatekeeper residue of BCR-ABL and was the first mutation to be identified in relapsed CML patients (Lamontanara et al., 2013). The T315I mutation affects the threonine in the ABL kinase domain which allows for hydrogen bonding to Imatinib due to the hydroxyl side chain interactions. The mutation of a threonine to isoleucine removes the hydroxyl group and, therefore, removes hydrogen bonding when Imatinib is interacting with the ABL kinase domain. Figure 1 shows a schematic demonstrating the ABL kinase interacting with Imatinib (red) and the location of the hydrophobic site 2 which displays the interaction of Imatinib with the gatekeeper residue. The hydrogen bonding is depicted by the dotted line and is critical for the binding of Imatinib to ABL.

In order to overcome the resistance to Imatinib, other TKIs such as Nilotinib (Tasigna) and Dasatinib (Sprycel), were developed (O'Hare et al., 2008) and approved by the FDA in 2010 (Arceci, R., 2014). Both of these drugs treat chronic and accelerated phases of CML in the adults that were resistant to Imatinib. The side effects of these TKIs include diarrhea, headache, edema, low blood calcium, and possible cardiac events. Patients experienced less side effects from treatment with these second-generation drugs compared to patients treated with Imatinib. Fortunately, these inhibitors were able to overcome the resistance of most ABL mutations, including the E255K mutation, but are not effective against the T315I mutation. Therefore, the search for inhibitors effective against cells expressing BCR-ABL with the T315I mutation is still ongoing.

Matrine as a Cancer Treatment

A possible new therapy could be a compound known as matrine. Matrine is a natural product that is purified from the Chinese herbal plant *Sophora flavacens* and has been shown to have pharmacological effects on several types of cancer. It has induced apoptosis of murine hepatoma cells *in vitro* and *in vivo*, inhibited tumor growth, inhibited metastasis of human malignant melanoma cells, reduced HeLa cell adhesion, and induced apoptosis in gastric cancer cells (Biaoxue, Shuxia, Wenlong, & Shuanying, 2015). Matrine is used in Chinese medicine to treat multiple disorders such as hepatitis, inflammation, fibroids, and cancer; it was approved for human treatment in 1992 by the China State Food and Drug Administration (Biaoxue et al., 2015). Matrine has shown effectiveness in treating solid-tumors such as breast, lung, and pancreatic cancers (Li et al., 2010; Zhang et al., 2009), but to date it has not been demonstrated whether matrine is efficacious against leukemia. Matrine is administered for treatment in China as

oxymatrine in the form of an injection. To date there have not been any side effects reported other than rare reactions at the injection site (Dharmananda, Subhuti., 2002).

Therefore, the goal of this study was to treat CML cell lines with matrine, particularly cells harboring the T315I BCR-ABL mutation, and determine if matrine is efficacious against Imatinib-resistant CML cells. In addition, modifications to the structure of matrine by the addition and/or removal of one or more functional groups were performed to generate matrine derivatives. CML cell lines were to be treated with the derivatives to determine if these new compounds may be potential therapeutics for CML and/or candidates for further modification.

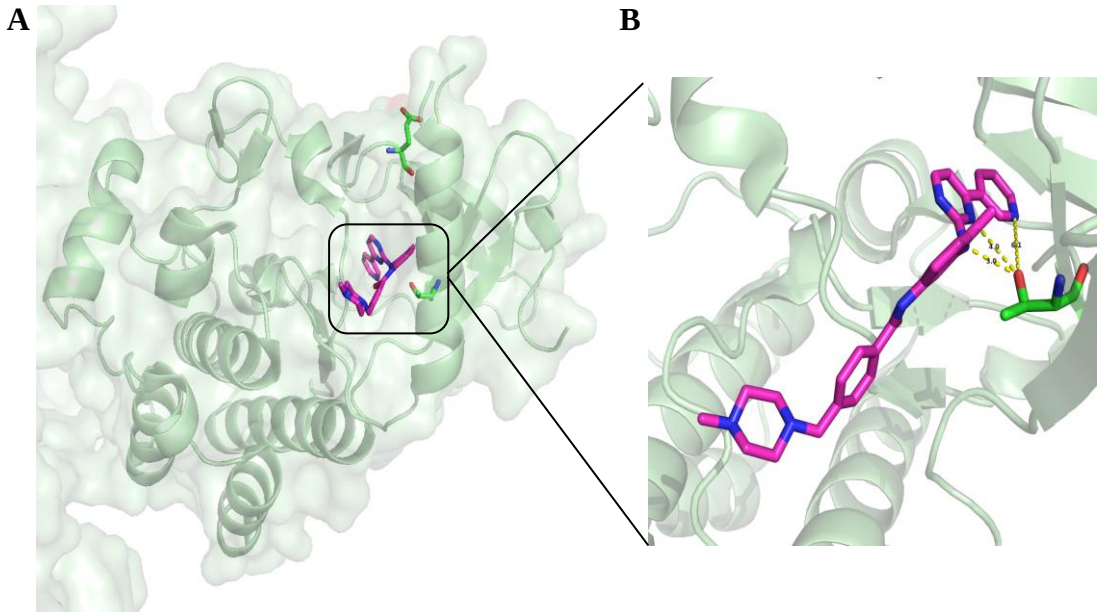


Figure 1. ABL kinase domain interacting with Imatinib. Schematic of ABL kinase bound to imatinib with important E255 and T315 residues identified (A) and imatinib hydrogen bonding interactions with the T315 residue (B).

Chapter 2

Materials and Methods

Reagents

Cell lines: The CML cells lines (p210, E255K, and T315I) were a kind gift from Dr. Brian Druker, (Druker et al., 2001).

RPMI-1640 medium, FBS and Penicillin-Streptomycin was purchased from ThermoFisher Scientific (Waltham, MA).

Compounds

Extracted and purified matrine was a gift from collaborators in China (Dr. Jin, Department of Hematology and Institute of Hematology, the First Affiliated Hospital, Zhejiang University College of Medicine) and the derivatives, thiomatrine, open matrine, AMA3, 156, and 159 were synthesized by Dr. Gustavo Moura-Letts' laboratory at Rowan University, Glassboro, NJ.

Imatinib was purchased from BioVision Inc. (Milpitas, CA) and MTT was purchased from Alfa Aesar (Ward Hill, MA).

Antibodies

Antibodies were purchase from the following companies: Cell Signaling Technology (Danvers, MA): phosho-c-Abl (Tyr^{412}) (247C7), c-Abl, β-Tubulin (D3U1W); Thermo Fisher (Waltham, MA): Caspase 3 Antibody (3CSP03 (4.1.18)); Santa Cruz Biotechnology, Inc. (Dallas, TX): Caspase-8 p18 (D-7): sc-393776. All primary

antibodies were used at 1:1000 dilutions in 5% non-fat milk casein blocking solution made with TBST.

Tissue Culture

CML cells were passaged every two to three days using RPMI supplemented with 10 % FBS and 1% penicillin-streptomycin at 37°C, 5% CO_2, and maintained between 2 x 10^5 and 1 x 10^6 cells/ml.

Sequencing of BCR-ABL Point Mutations in CML Cell Lines

Total RNA was isolated from each cell line (P210, E355K, T315I) and cDNA was generated through reverse transcription. BCR/ABL cDNA was amplified via high fidelity PCR primers with SalI/EcoRI overhangs (5' BCR – CTA GTCGAC ACAGCATTCCGCTGACCATCAATAAG, 3' ABL – CTA GAATTC AGACGTCGGACTTGATGGAGAACT, Rejali et. Al, 2015). PCR products were resolved by agarose gel electrophoresis, visualized, isolated, and ligated into the pUC19 vector. Plasmids were transformed into Max efficiency STBL2 cells and colonies generated and screened. Positive clones were sent to GeneWiz for Sanger Sequencing. Sequence alignments were performed using Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA6).

MTT Assay

Cells were seeded at a density of 1.5 x 10^5 cells/ml in a 96-well plate and incubated for 24 hours. Cells were treated with derivatives at the indicated concentrations for 24 hours and 3- (4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide MTT was added. Absorbance was measured at 570 nm.

Western blot

Cells were lysed using lysis buffer (25 mM HEPES pH=7.5, 0.1% Triton X-100, 300 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 protease inhibitor tablet, 1 mM Na_2VO_4, 50 mM NaF) and approximately 30 µg of total protein was resolved by SDS-PAGE gels in Tris-Glycine Running Buffer (25 mM Tris, 192 mM glycine, 1% SDS). Gels were resolved at 150V for approximately 1 hour and then transferred to PVDF membrane for 2.5-5 hours at 30V in Tris-Glycine/MeOH Transfer Buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membrane was blocked using 5% non-fat milk in TBS-T for 30 minutes and incubated with the indicated primary antibody (1:1000 in 5% milk/TBS-T) overnight at 4°C. Membrane was probed with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature.

Annexin-V Assay

Cells were seeded in RPMI 1640 at a density of $5x10^4$ cells/mL in 24 well plates and treated with desired drug for the indicated times at 37°C, 5% CO2. Cells were collected and stained with 125µL (1:1 ratio) of Annexin V and PI mixture from Takara (Mountain View, CA) and analyzed on an Attune NxT Flow Cytometer

Chapter 3

Results and Conclusion

Results

Synthesis of derivatives. The structure of matrine and the novel derivatives are shown in Figure 2. Thiomatrine contains sulfur instead of oxygen compared to matrine. In order to synthesize open matrine, the bond between the carbonyl carbon and the tertiary amine was broken to give the open structure. AMA3 contains a cyanide group and an additional hydrocarbon chain compared to open matrine. Open matrine 2 is generated by breaking the bond between a carbonyl and the amine, however, it is hydrolyzed to produce the carboxylic acid instead of an ether. The derivatives 159 and 156 were synthesized using open matrine 2 as the starting material.

Confirmation of CML cell lines. The CML cells that were used for these experiments were Ba/F3, p210, E255K and T315I. Ba/F3 is a murine pro-B cell line that is IL-3-dependent and typically used in testing potency and signaling of oncogenic kinases, the p210, E255K, and T315I cell lines were generated by the Druker Lab by genetically modifying Ba/F3 cells. p210 cells are Imatinib-sensitive and contain the BCR-ABL translocation but do not have a mutation. E255K cells are partially Imatinib-resistant and contain the BCR-ABL translocation with a mutation from glutamic acid to lysine at residue 255. T315I cells are completely Imatinib-resistant and contain the BCR-ABL translocation and with a mutation from threonine to isoleucine at residue 315. Confirmation of the BCR-ABL translocation and the appropriate mutation was confirmed for each of the cell lines received from the Druker Lab. The presence of the BCR-ABL

translocation was confirmed via RT-PCR. Total mRNA was isolated from each of the cell lines and cDNA generated. PCR was then performed using the newly generated cDNA and primers specific for the BCR-ABL translocation. Sequencing reactions were then performed to confirm the ABL mutations. Genomic DNA was isolated and PCR performed to clone the appropriate region of ABL into the circular pUC-19 vector. Sanger sequencing was then performed by GENEWIZ using Primers to confirm the ABL mutations. The DNA sequence results from GENEWIZ were analyzed using MEGA software. The mutation in E255K occurs at residue 255 and is a switch from a glutamic acid (GAG) to a lysine (AAG) (Figure 3A). The T315I mutation occurs at residue 315 switching from a threonine (ACT) to isoleucine (AUU) (Figure 3B). Therefore, it was determined that the CML cell lines we received from the Druker Lab contained the correct BCR-ABL mutations.

Toxicity studies of matrine and derivatives. After confirmation of the cell lines, toxicity studies were performed on p210 and T315I CML cells using matrine and two derivatives, thiomatrine and open matrine. Toxicity was assessed using an (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay determines cell viability by using a yellow dye called MTT, which is converted to purple MTT formazan in viable cells. This color change is caused by an enzyme called mitochondrial reductase that is present in living cells. If there are viable cells while processing the assay, the color of the MTT will be converted from yellow to purple and will vary in intensity depending on how many cells are viable in the culture. This color difference is quantified by absorbance at 570 nm.

The CML cell lines were treated for 24 hours with the indicated drug and MTT assays performed. There is a noted decrease in the viability of T315I cells at 0.8 mM. The results show that matrine and open matrine are the most efficacious compounds tested; these derivatives decreased the percent survival of the T315I CML cells by approximately 50% and 80%, respectively. Thiomatrine decreased percent survival by approximately 40% in the T315I cells. Once reviewing these results, the study was continued and matrine, open matrine and AMA3 were tested while imatinib was utilized as a positive control.

After the preliminary results using the MTT assay, conformation of compound activity was warranted. A complementary assay, Annexin V/propidium iodide (PI) apoptosis assay, was used to confirm apoptosis. PI was used in conjunction with Annexin V to determine cell viability. This is possible because Annexin V and PI allow for detection of differences in plasma membrane integrity and permeability which help to determine if the cell is viable, apoptotic, or necrotic by flow cytometric analysis. This assay will allow cells to be measured individually into viable, early apoptotic, late apoptotic, and necrotic cells as seen in Figure 5A. This technique was utilized to collect all of the data for each specific CML cell line and derivative concentration from this point forward. Percent survival was then quantified and represented graphically. As a positive control, Imatinib treatment of p210 cells was used to verify that the assay was performing properly and the cell lines responded as noted in the literature (Druker et al., 2001). p210 cells were treated with vehicle (Figure 5B) or 4 µM Imatinib (Figure 5C). Figure 5B shows little to no apoptosis and necrosis with vehicle treatment, however,

treatment with 4 µM Imatinib resulted in an increase in early and late apoptotic events by approximately 70%.

Additionally, Figure 6 shows the results for the quantification of percent survival for the CML cells tested at 24, 48, and 72 hours with Imatinib using flow cytometric analysis. It was confirmed that the indicated CML cells were responding as previously published in the literature (Druker et al., 2001). (See Appendix A for more information on Imatinib and preparation of the stock solutions.)

Matrine, open matrine, and AMA3 were treated for 72 hours with Imatinib, matrine, open matrine, or AMA3 (Figure 7). Matrine and open matrine treatment markedly decreased the percent survival of all CML cells tested at 4 mM. AMA3 begins to decrease percent survival of CML cells at 400 µM. Promisingly, the matrine derivatives inhibit the survival of CML cells harboring mutations in the BCR-ABL kinase domain, however, the required inhibitor concentration is very high. Therefore, new set of matrine derivatives was generated and tested.

Combinatorial studies. Since open matrine, the parent analog of AMA3, also showed a decrease in percent survival at 4 mM, combinatorial experiments were performed. This included open matrine and imatinib administered together to determine if there was a synergistic effect that may help increase the efficacy of open matrine and allow for treatment at lower doses (Figure 8). There was a decrease in the viability of CML cells at 72 hours when treated with 40 nM open matrine and 4 µM Imatinib together, including the T315I cell line. The assay was repeated (Figure 9) and a normal murine fibroblast cell line, NIH3T3, was included to evaluate toxicity. Unfortunately, the

same reduction in percent survival in CML cells with 40 nM open matrine and 4μM Imatinib treatment was not observed. Of note, the characteristic marked decrease in survival of the p210 cell line with 4μM Imatinib treatment was not observed (see Appendix A); typically, 70% reduction in survival is observed with treatment, whereas there was only approximately 30% reduction in these experiments (Figure 9). (We later determined that the reduced response with Imatinib treatment was due to the sample preparation and storage conditions.) The assay was repeated as shown in Figure 10. This time an additional analog, open matrine 2, was included. Analysis of the data revealed that the results shown in Figure 8 were not replicable.

Analogs of open matrine 2, 156 and 159 were tested using the same Annexin-V apoptosis assay to check for activity (Figure 11). The combination treatment of derivative with 4 μM Imatinib was included to determine if the inhibitors were synergistic. There was approximately a 50% and 85% reduction in viability in the T315I mutant cell line beginning at 400 μM treatment with 156 or 159, respectively. There was also a remarkable decrease in percent survival with the combinatorial treatments at 400 μM 156 or 159 combined with 4 μM Imaitinib. However, toxicity in NIH3T3 cells was also observed with 400 μM treatment of both 156 and 159; 159 appears to be more toxic than 156. Since minimal and high toxicity was observed at 40 nM and 400 μM treatment, respectively, dosing was refined between these two concentrations in the attempt to find a dose toxic to CML cells and not NIH3T3 cells. As seen in Figure 12, T315I toxicity was increased compared to NIH3T3 cells beginning at 100 μM 156 + 4 μM Imatinib treatment and 40 μM 159 + 4 μM Imatinib treatment. Refinement assays were not performed in triplicate due to a limitation in materials. However, assays needed

to be performed in triplicate in order to perform statistical analysis. Due to the limited amount of derivative, only one concentration of 156 and 159 was used for the assay performed in triplicate. The concentrations of 100 µM 156 and 40 µM 159 were chosen for single agent and combination assays because these were the concentrations at which a decrease in T315I cell viability higher than that seen with NIH3T3 cells is first observed. Of note, it has been emphasized that Imatinib treatment was not consistently reducing cell viability properly in the controls of the indicated experiments; p210 cells should decrease by approximately 70% with 40 µM Imatinib treatment for 72 hours (Figure 6). Tests experiments were performed and it was found that if Imatinib is stored, even briefly, at -20°C or 4°C potency is lost (Appendix A). Therefore, Imatinib was prepared fresh for each assay from this point forward and potency was restored. This additional information was also taken into account when choosing the concentrations of 156 and 159 for the triplicate experiment; it was anticipated that Imatinib activity would be higher than what was observed in Figure 11. Figure 13 shows the results of the triplicate experiments; Ba/F3 cells were also added to this assay as a second toxicity control. Treatment with 156 (100 µM) or 159 (40 µM) alone or in combination with 4 µM Imatinib resulted in significant reduction in cell viability in CML cell lines. Significant reduction in CML cell lines was observed both with single and combinatorial treatment. Interestingly, combinatorial treatment with 156 or 159 increased the potency of Imatinib for E255K cells comparably to Imatinib treatment of p210 cells alone. This suggests that 156 and 159 may be somehow enhancing the interaction of Imatinib with BCR-ABL. Unfortunately, the reduction in cell viability in T315I cells was not increased above the toxicity in Ba/F3 and NIH3T3 cells with single agent or combinatorial treatment.

Western blot analysis. c-Abl protein is present in the nucleus and the cytoplasm of cells and plays a role in the regulation of cell proliferation, adhesion, apoptosis, and stress response. Phosphorylation of c-Abl at multiple residues regulates activity, and inhibition of BCR-ABL activity by Imatanib leads to dephosphorylation of certain residues, including Tyr^{412}. Therefore, it was investigated whether the reduction in cell viability observed in CML cell lines was due to inhibition of BCR-ABL activity and dephosphorylation of Tyr^{412}. There is a decrease in phosphorylation of BCR-ABL in p210 cells treated with Imatinib, as has been previously published (Druker et al., 2001). A decrease in BCR-ABL phosphorylation was also observed in E255K cells treated with Imatinib, 156, or 159. Of note, a slight decrease in the phosphorylation of BCR-ABL was observed in the T315I cells treated with Imatinib alone, although T315I is known to be completely Imatinib resistant to treatment according to the literature. A decrease was also observed with 156 and 159 treatments; however, it was not reduced greater than what was observed with Imatinib treatment. Although cell lines were checked by sequencing to confirm, it is possible there could have been some trace contamination from an Imatinib-sensitive cell line. If a small proportion of the cells were sensitive to Imatinib treatment, then a reduction in BCR-ABL phosphorylation may be detectable by western blot analysis.

In order to determine the apoptotic mechanism induced by 156 and 159 treatment of cells, activation of caspase-8 and caspase-3 was determined. Caspase-8 is an initiator caspase which will activate effector caspases, such as caspase-3; caspase-8 is the upstream protease that begins the cascade responsible for apoptosis (Elmore, 2007; Liu et al., 2017). No increase in active caspase-8 or cleaved caspase-3 was observed with

treatment for E255K or T315I cells (Figure 15 & 16, respectively). These results do not mean that apoptosis is not occurring, but that apoptosis is occurring via a caspase-independent mechanism. Further experiments are warranted to determine this mechanism.

Conclusion

Imatinib is an effective treatment for approximately 85% of CML patients. However, approximately 15% of patients are resistant to Imatinib treatment. Of these resistant cases, 85% are due to mutations in the BCR-ABL kinase domain resulting in constitutive activation that cannot be inhibited by Imatinib. Second and third generation inhibitors have been developed which are effective for treating patients harboring most Imatinib-resistant mutations, including E255K. However, patients harboring one mutations in particular, T315I, which occurs in 15% of the resistant patients has proven very difficult to treat. One third generation inhibitor, ponatinib, has been shown to inhibit cell growth in T315I CML cells, however, toxicity is very high, including severe cardiac events and fatalities in 10% of treated patients (FDA approval for ponatinib hydrochloride.). Therefore, the goal of this project was to identify inhibitors that are effective for treatment against CML cells harboring the E255K and T315I mutation with less toxicity.

Matrine is isolated from the Chinese herbal plant *Sophora flavacens* and is used for treatment in Chinese medicine, including for certain cancers (Liu et al., 2017). Therefore, we attempted to determine whether matrine or matrine derivatives might be

effective for treatment of CML cells harboring Imatinib-resistant mutations in BCR-ABL.

MTT assay results showed that there is a decrease in viability of CML cells when treated for 24 hours with matrine and open matrine at 3.2 mM. Thiomatrine also had some effect on the viability of the CML cells but not as drastic as matrine and open matrine. The Annexin V/PI apoptotic assay was used to confirm and refine the results of the MTT analysis. Matrine and open matrine were shown to decrease the percent survival of CML cells, specifically the E255K and T315I cell lines at 4 mM, which confirmed the MTT assay results. An analog of open matrine, AMA3, was found to decrease the percent survival of CML cells between 40 nM and 400 µM. Open matrine 2 did not appear to increase apoptosis in CML cells. However, two of its analogs, 156 and 159, appeared to be the most efficacious against CML cells when coupled with Imatinib treatment, especially on the E255K cells. The T315I cells do decrease in viability when treated with 156 and 159, but not above the toxicity observed with control cells, NIH3T3 and Ba/F3.

Western blot analysis showed that 156 and Imatinib combinatorial treatment resulted in a decrease in phosphorylation of Tyr^{412} on BCR-ABL in E255K cells, which was not observed in treated T315I cells. These data are consistent with the flow cytometric analysis results showing that E255K, not T315I, decrease in viability with this treatment. Caspase-8 and caspase-3 activation were not detectable. Therefore, further analysis to determine whether caspase-independent apoptotic mechanisms are induced is required.

New compounds derived from matrine, open matrine, and open matrine 2 will be analyzed to identify an analog effective for treating cells with the T315I BCR-ABL mutation. The concentrations of inhibitors utilized in this paper are high and will need to be reduced in order to be considered as candidate compounds for treatment. Therefore, further refinement of the already identified inhibitors, such as 156 and 159, will have to be completed. The toxicity of these compounds will continue to be analyzed using NIH3T3 cells and Ba/F3 cells. Once the most efficacious compounds are identified and western blot analysis confirms dephosphorylation of BCR-ABL, further analysis using *in vivo* assays will be performed to determine toxicity in rodents.

A BCR-ABL kinase assay should be performed in order to perform high-throughput screening and identify inhibitors that are specific for BCR-ABL. This would allow for testing to be specific and more effective. Once all the mechanistic information is known and understood, then *in vivo* studies can be performed. Injection of the CML cells into immunocompromised mice would allow them to develop leukemia. Once the mice have developed CML, treating with the desired drug will allow for analysis of survival and death of the CML cells, as well as toxicity to the animal, to determine the efficacy of the compound.

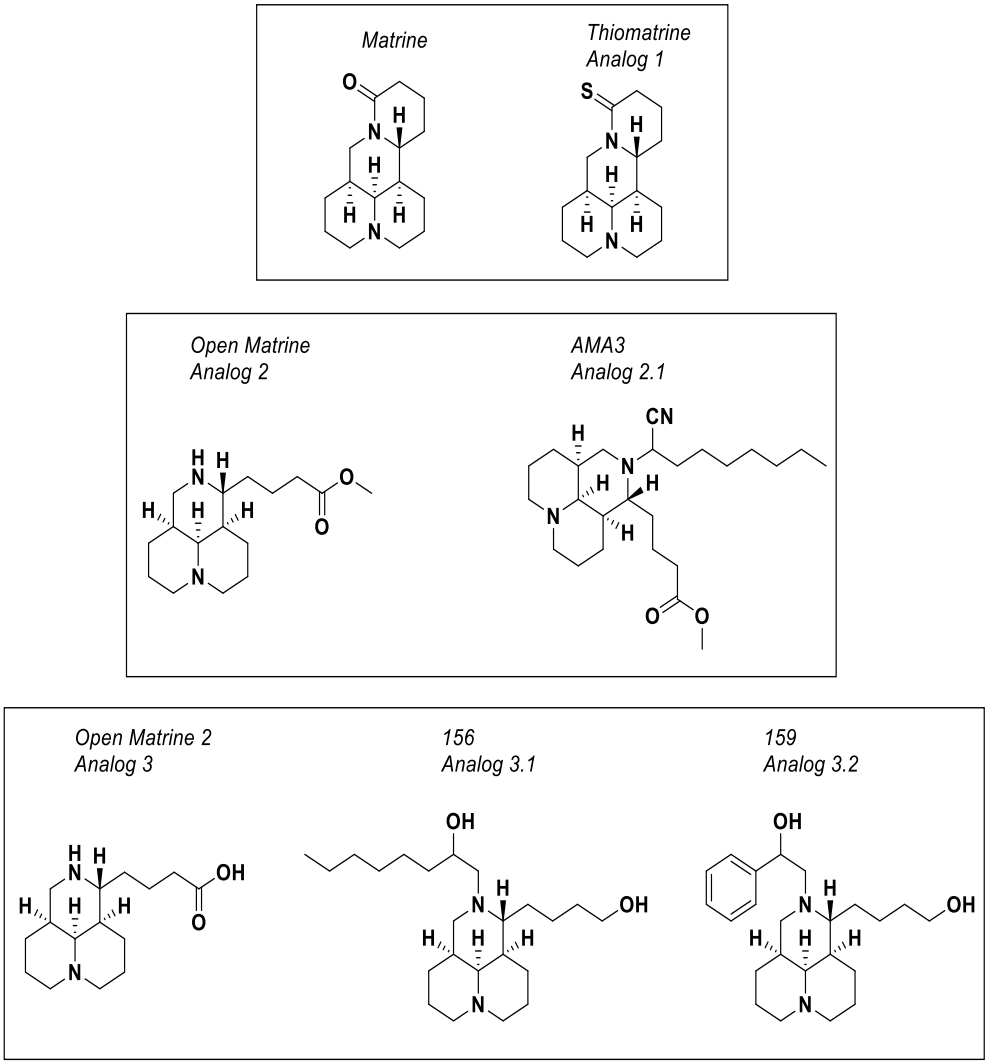
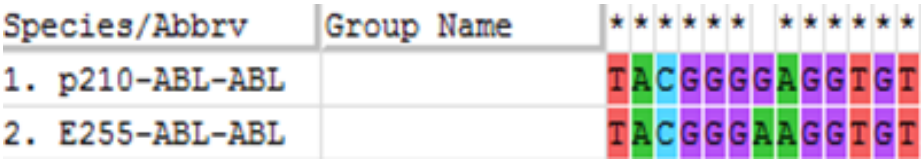


Figure 2. Structures of matrine and matrine derivative tested on CML cells. These analogs were synthesized from matrine and tested on CML cells at various concentrations to determine if apoptosis is induced.

A



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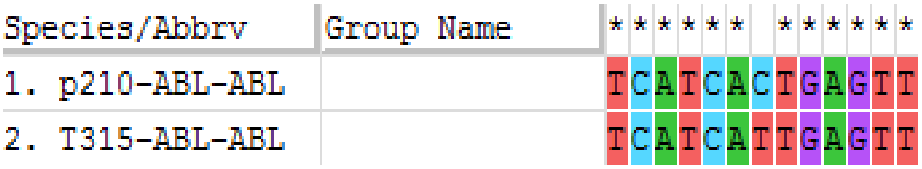

Figure 3. Confirmation of BCR-ABL mutations in p210, E255K, and T315I cells. Genomic DNA was isolated from the indicated cells and Sanger sequencing performed. Results were analyzed using MEGA6.0 software. The DNA sequence of p210 and E255K (A) and p210 and T315I (B) indicating the position of the mutation is shown.

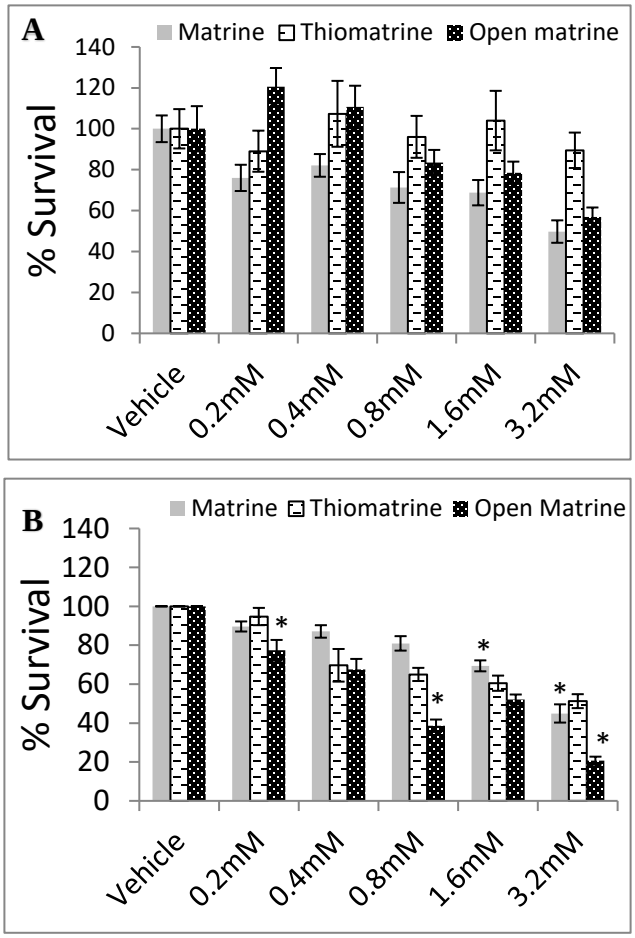

Figure 4. Determination of p210 and T315I cell viability after treatment with matrine and matrine derivatives by MTT assay. CML cell lines, p210 (A) and T315I (B), were treated with matrine, thiomatrine, and open matrine for 24 hours. MTT assays were performed and percent survival determined.

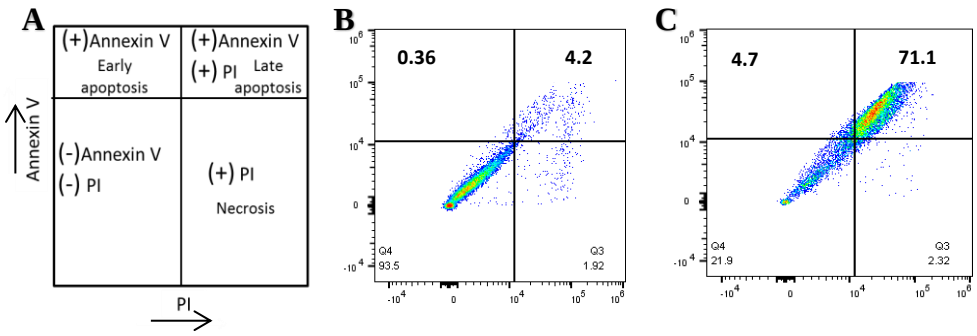

Figure 5. Example flow cytometric analysis assay. (A) A schematic of the quadrants shows how the viable, early apoptotic, late apoptotic, and necrotic cells from the assay are visualized. p210 cells were treated vehicle (B) or 4 µM Imatinib (C) for 72 h. Cells were stained with Annexin V antibody and propidium, iodide (PI) to detect viability. The results of the assay were analyzed and dot plots generated using FlowJo software.

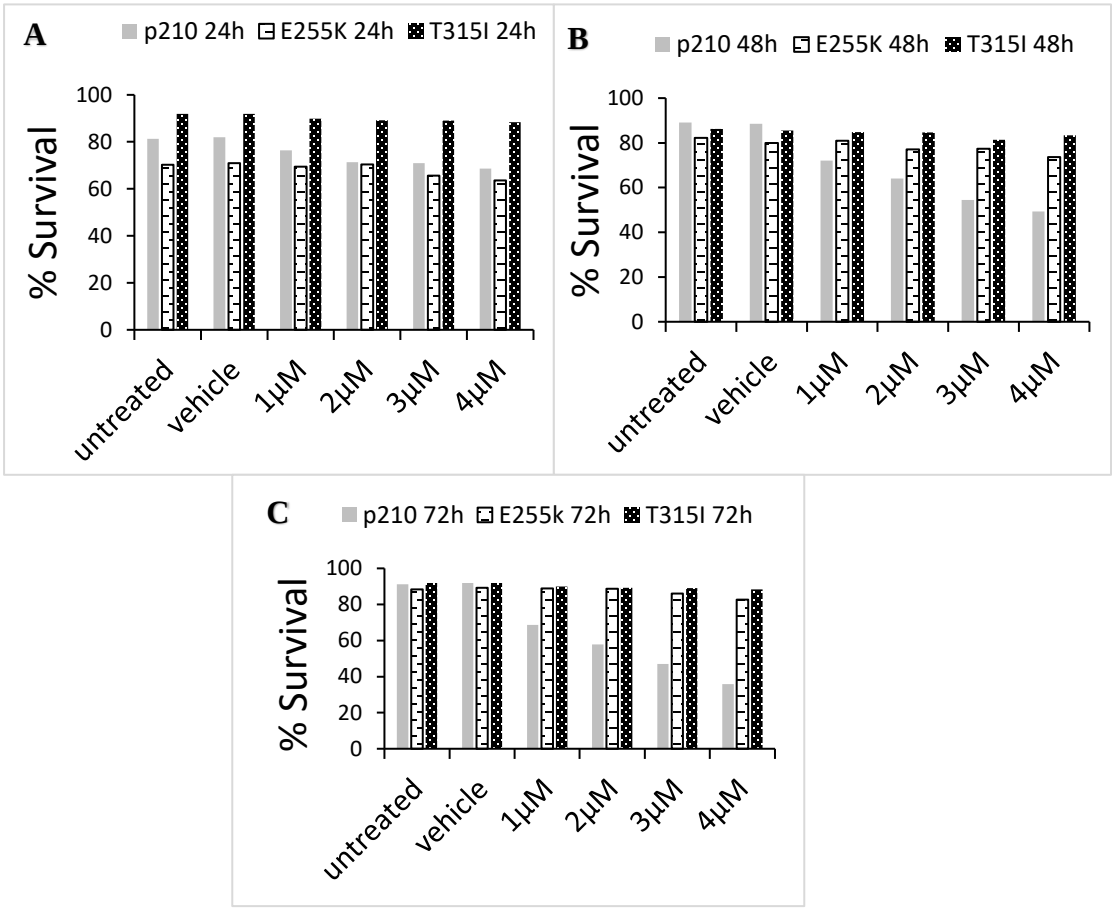

Figure 6. Imatinib treatment time course. P210, E255K, and T315I cells were treated with Imatinib at the indicated concentration for various time points to determine optimal performance: 24 hours (A), 48 hours (B), and 72 hours (C).

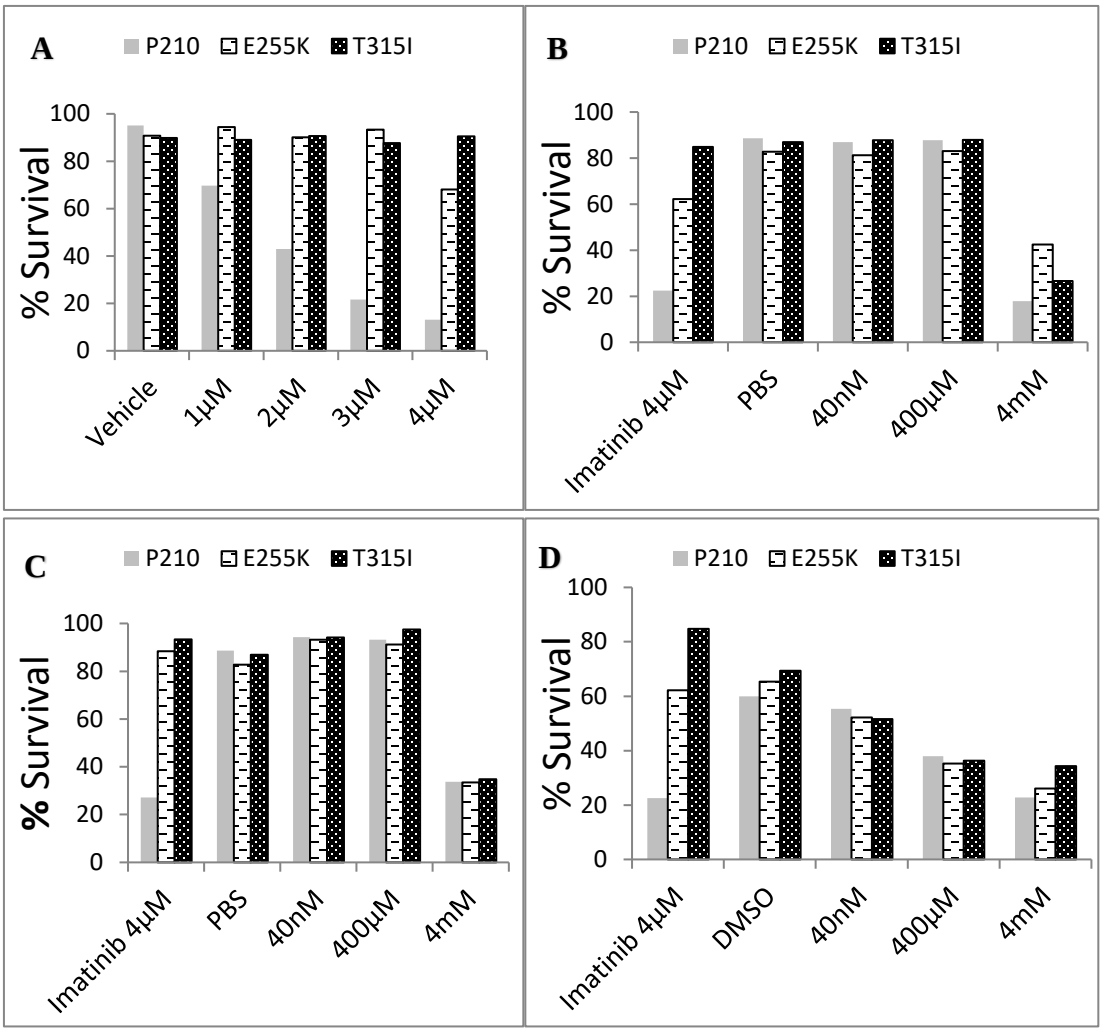

Figure 7. Treatment of CML cell lines with Imatinib, matrine, open matrine, and AMA3. The indicated CML cells were treated with Imatinib (A), matrine (B), open matrine (C), or AMA3 (D) at various concentrations for 72 h at 37°C. Annexin-V assay was then performed. Data was analyzed using FlowJo software and percent survival graphed.

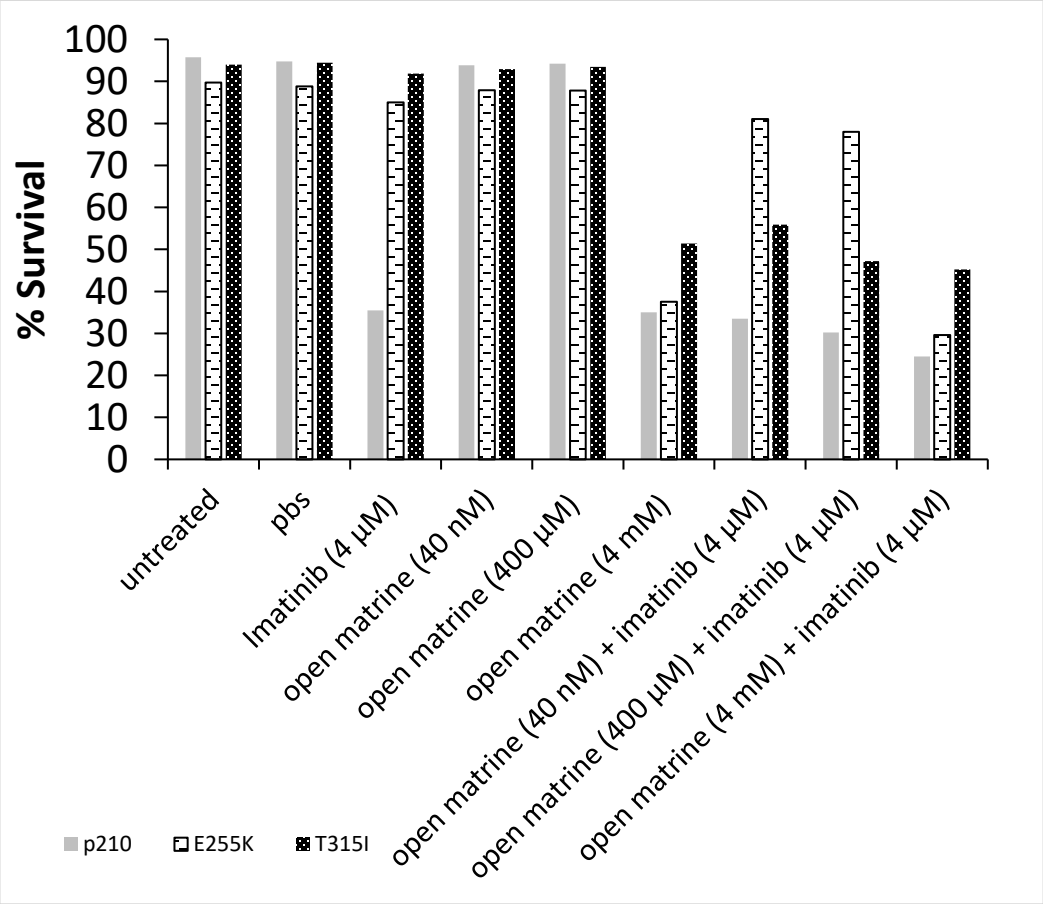

Figure 8. Combination treatment with open matrine and Imatinib induced apoptosis of CML cells. CML cell lines, p210, E255K, and T315I were treated with the indicated concentrations of open matrine for 72 hours. Annexin-V apoptosis assay and flow cytometric analysis was performed and percent survival determined.

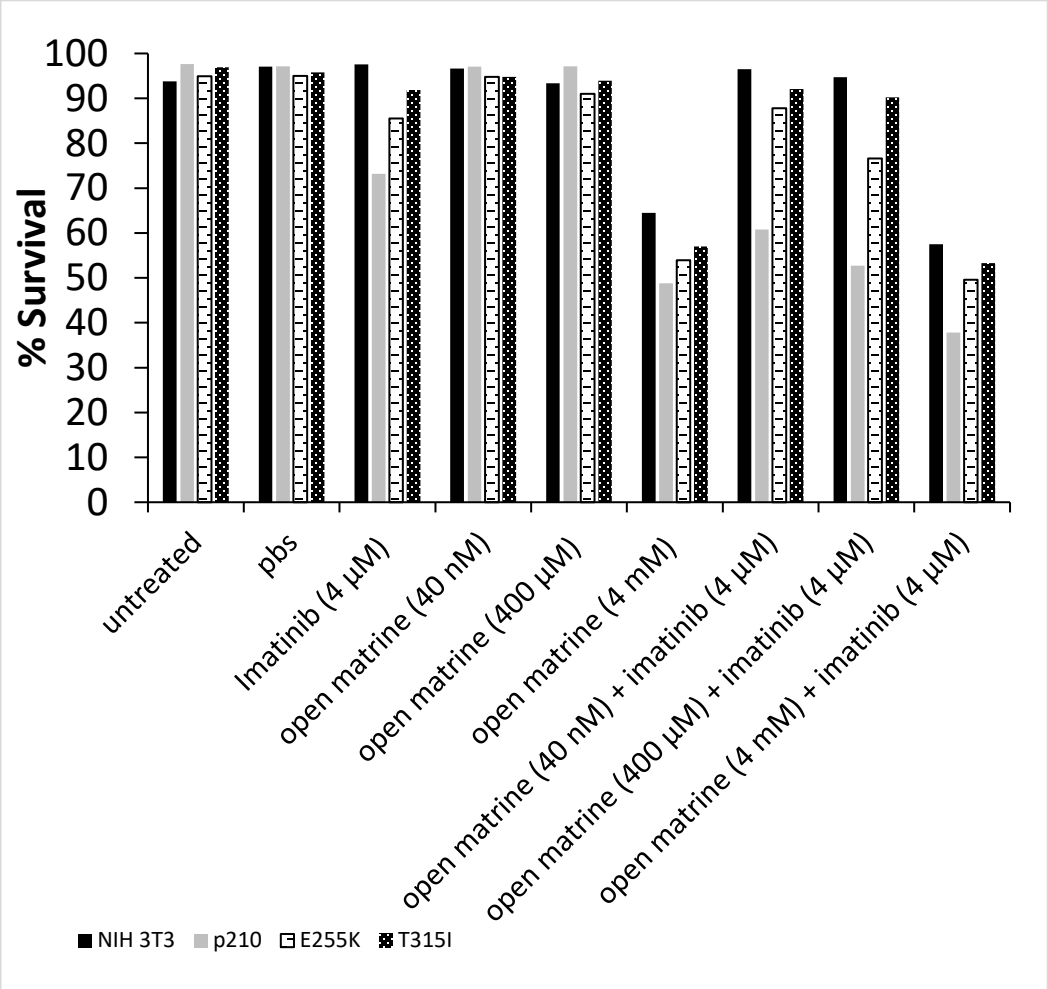

Figure 9. Treatment of NIH3T3 cells and CML cell lines with open matrine and Imatinib. NIH3T3, p210, E255K, and T315I cells were treated with the indicated concentrations of open matrine and/or Imatinib for 72 hours. Cells were stained with Annexin V antibody and propidium iodide (PI) to detect viability. The results of the assay were analyzed using FlowJo software and dot plots were visualized and data graphed.

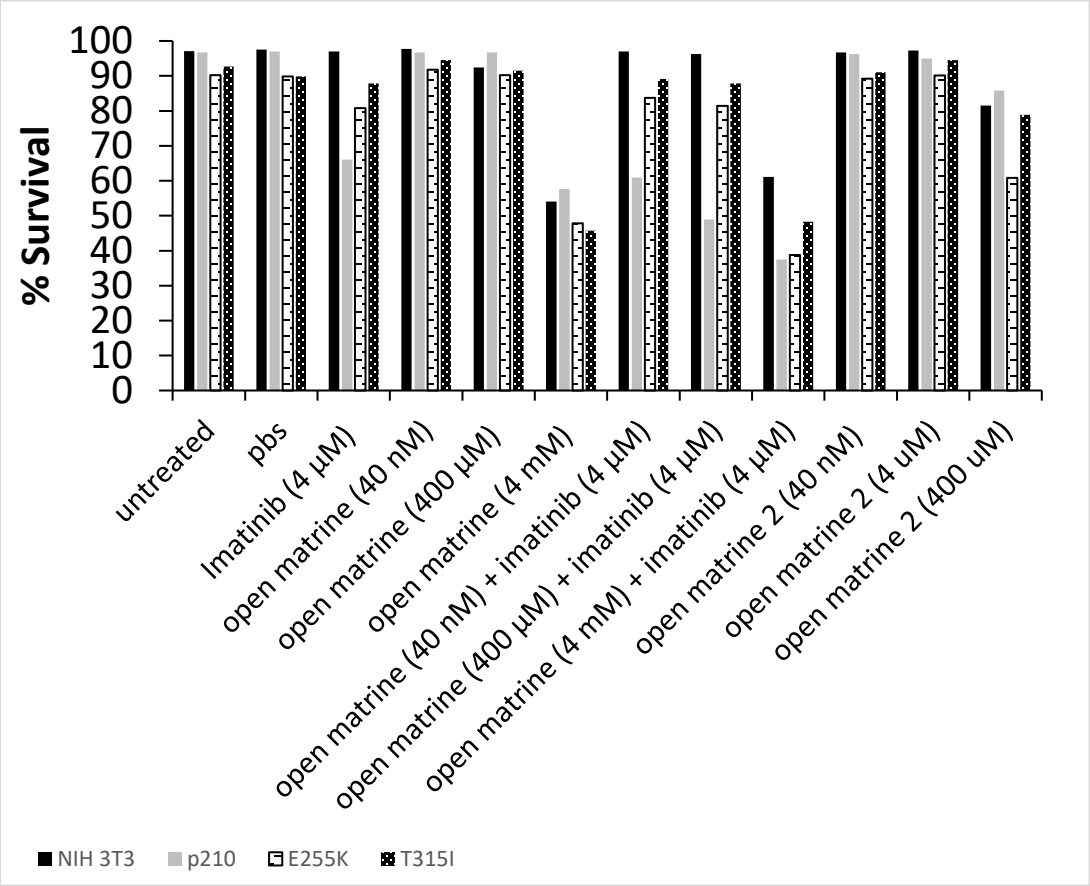

Figure 10. Treatment with open matrine, open matrine 2, and Imatinib. NIH3T3, p210, E255K, and T315I cells were treated with the indicated concentrations of open matrine, open matrine 2 and/or Imatinib for 72 hours. Annexin V apoptosis assay was performed and results were analyzed using FlowJo software and percent survival plotted.

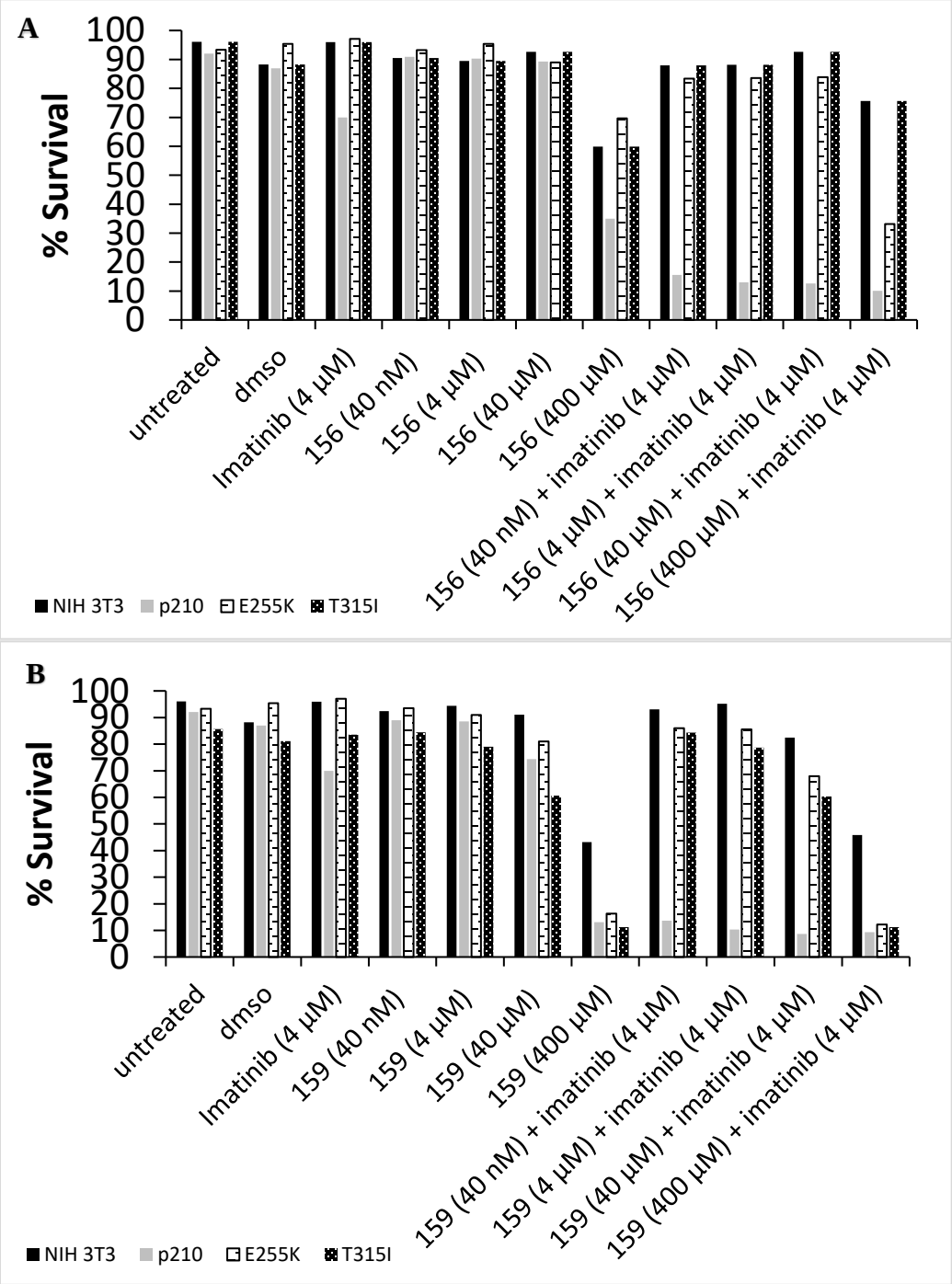

Figure 11. First-round treatment - 156 and 159 induce apoptosis in CML cells. NIH3T3, p210, E255K, and T315I cells were treated with the indicated concentrations of 156 (A) or 159 (B) with or without 4 µM Imatinib for 72 hours. Annexin-V apoptosis assay and flow cytometric analysis was performed and percent survival determined.

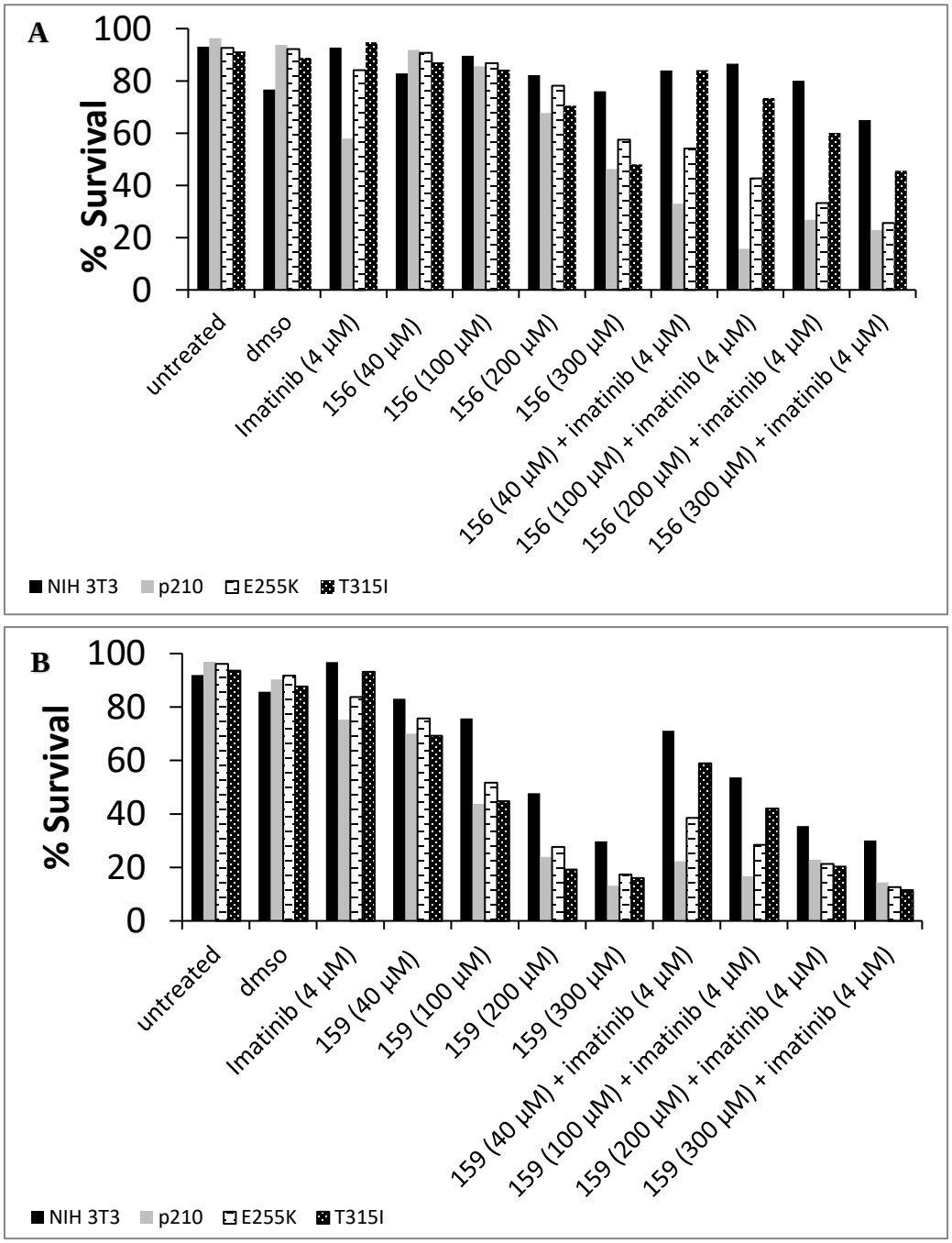

Figure 12. Second round treatment - 156 and 159 induce apoptosis in CML cells. NIH3T3, p210, E255K, and T315I cells were treated with the indicated concentrations of 156 (A) or 159 (B) with or without 4 µM Imatinib for 72 hours. The Annexin-V apoptosis assay was performed. Data was analyzed using FlowJo software and percent survival plotted.

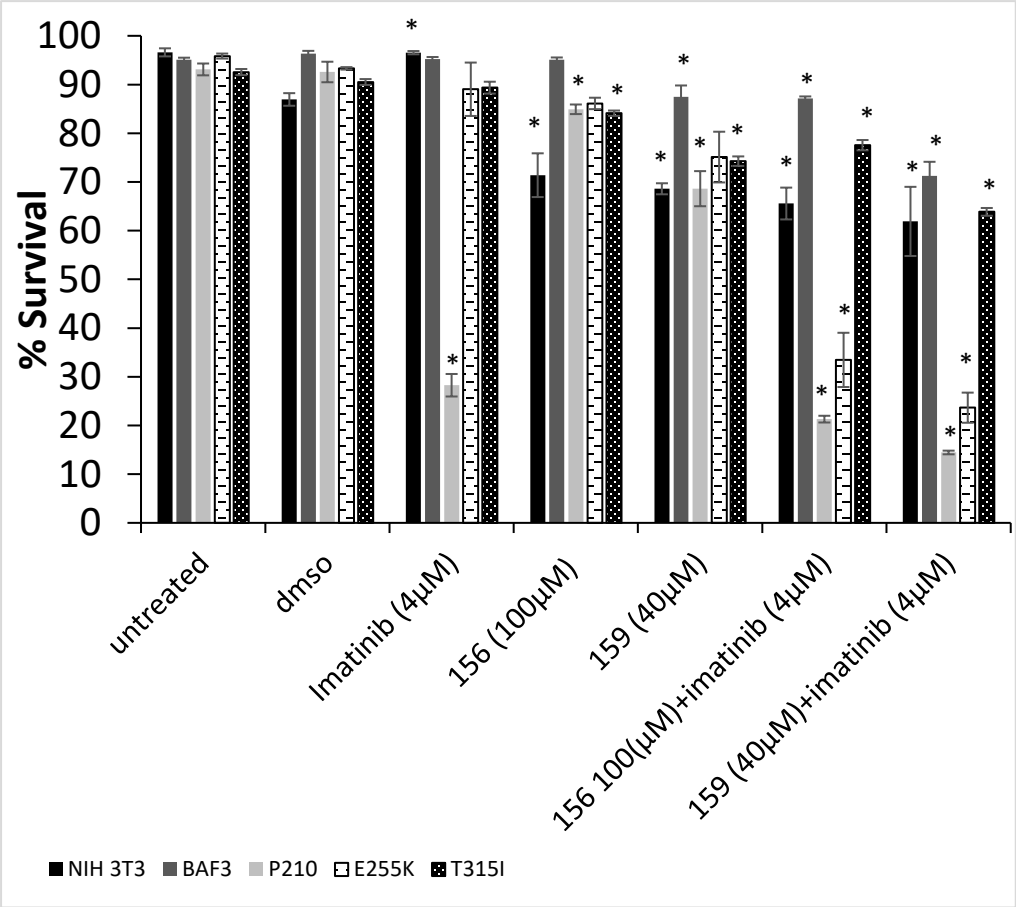

Figure 13. Treatment with 156 and 159 Induce Apoptosis of E255K and T315I cells. The indicated cell lines were treated with the indicated concentrations of derivative 156 or 159 in triplicate for 72 hours. Cells were stained with anti-Annexin V antibody and PI. Data was analyzed using FlowJo software and percent survival plotted (Annexin-V negative cells) and standard deviation determined.

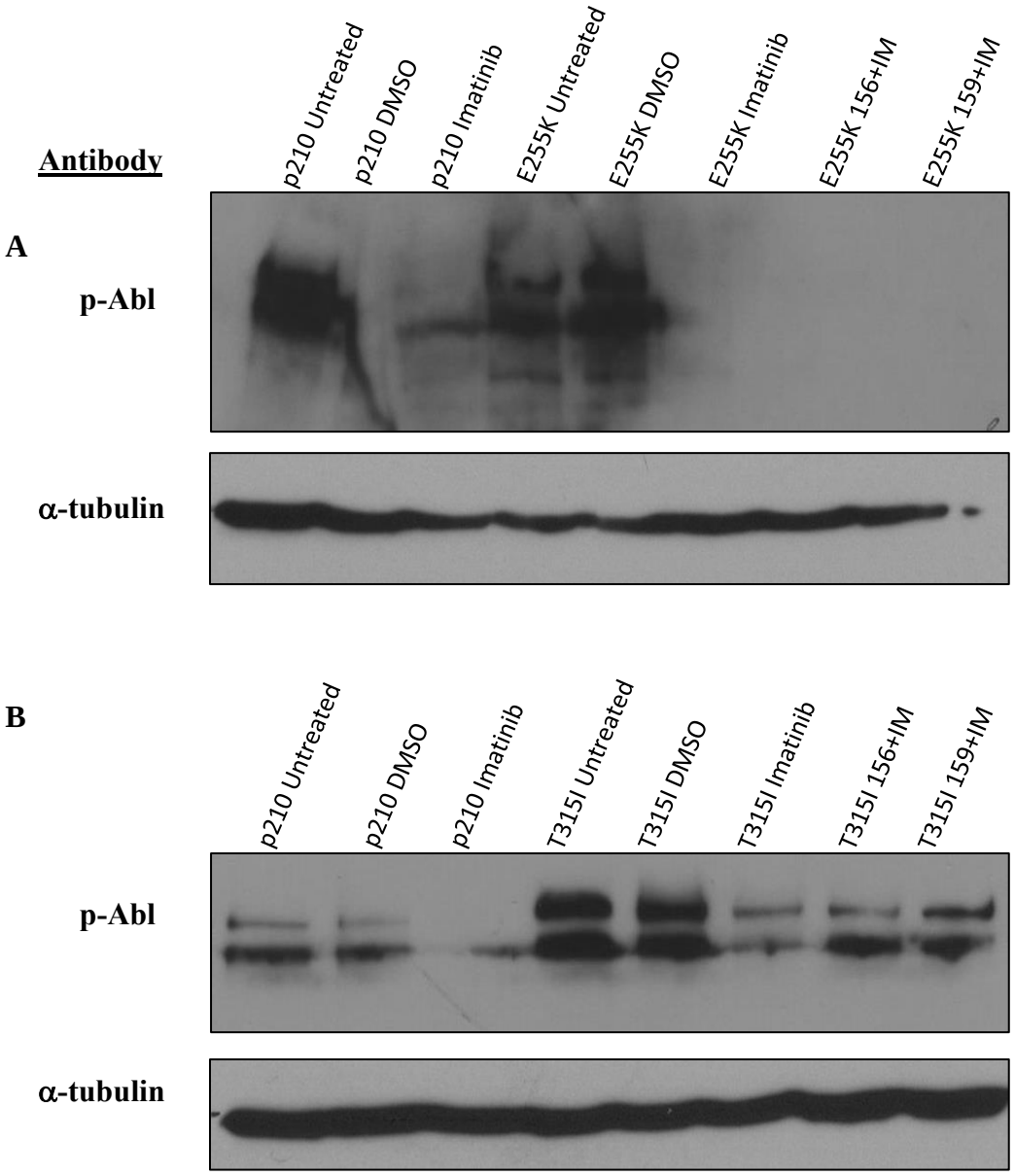

Figure 14. Analysis of ABL phosphorylation at Tyr^{412} after treatment. The indicated CML cells were treated with Imatinib (4 μM), or Imatinib with 156 (100 μM) or 159 (40 μM) for 24 hours. Western blot analysis was performed using anti-pABLTyr^{412} antibody.

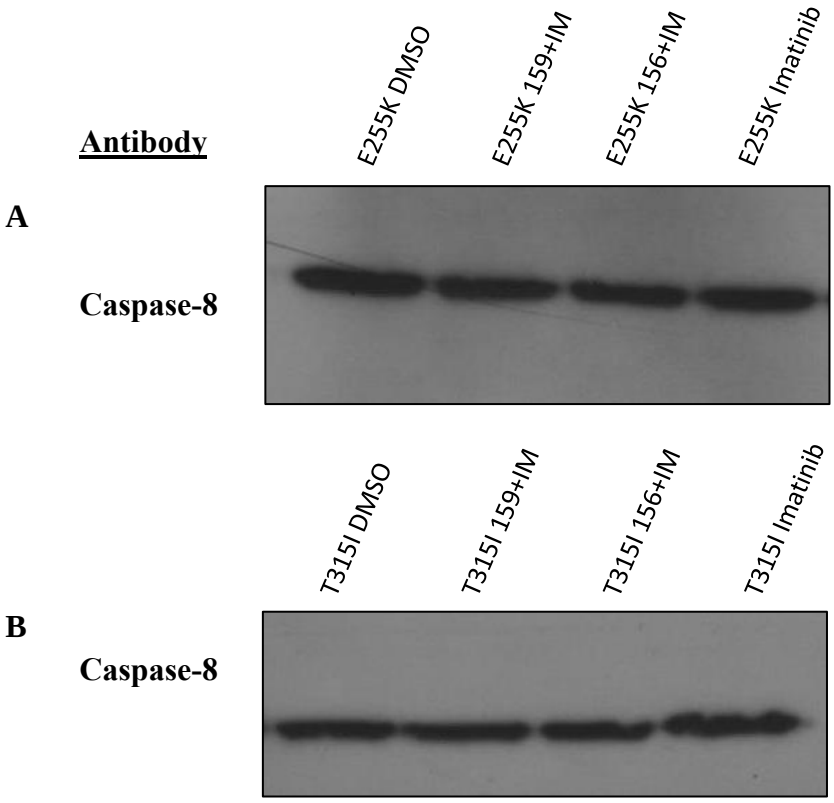

Figure 15. Caspase-8 is not activated in treated CML cells. The indicated CML cells were treated with Imatinib (4 µM) or Imatinib plus 156 (100 µM) or 159 (40 µM) for 48 hours. Western blot analysis was performed for active caspase-8.

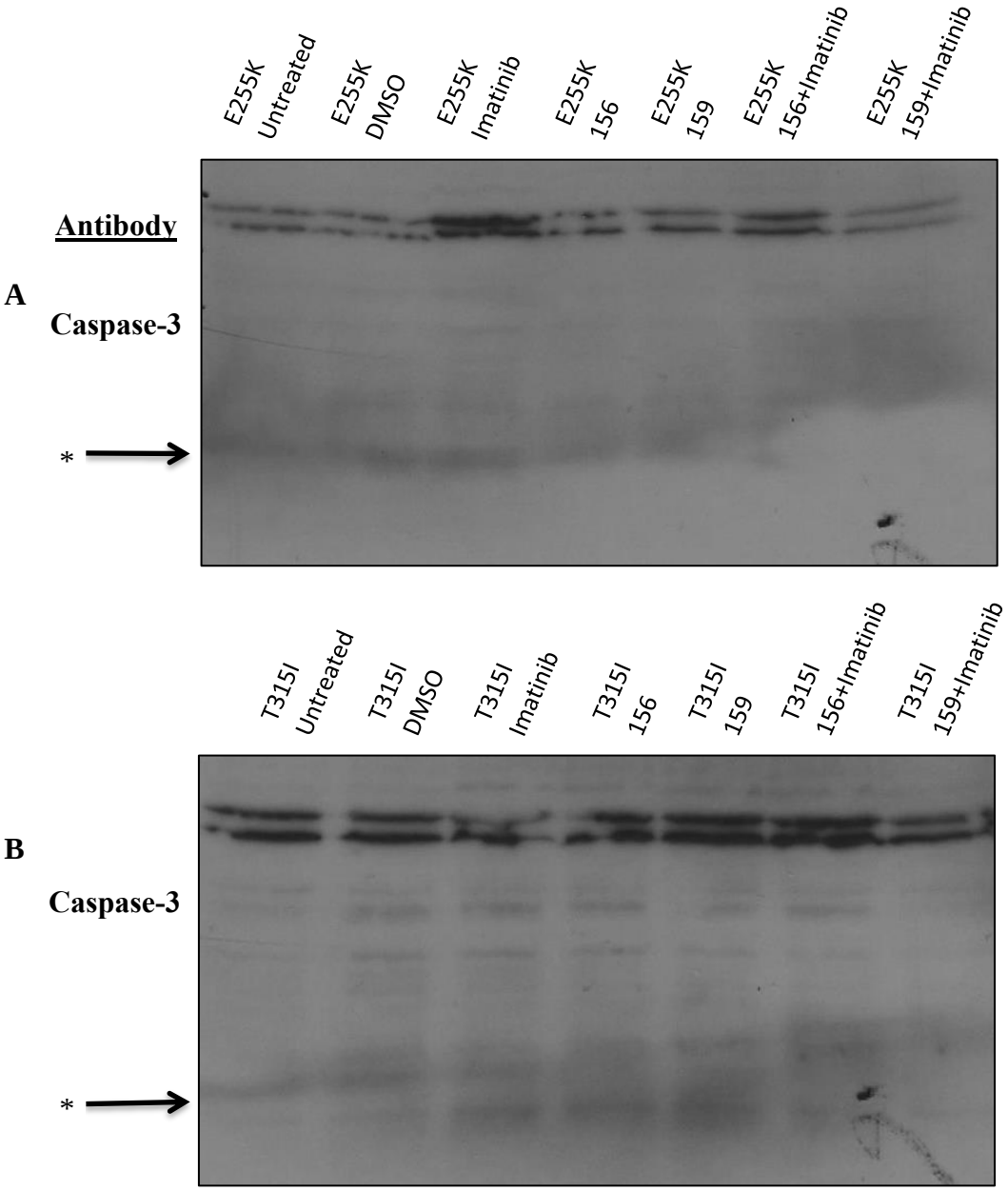

Figure 16. Cleavage of pro-caspase-3 is not observed in treated CML cells. CML cells, E255K (A) or T315I (B), were treated with Imatinib (4 µM), 156 (100 µM), and 159 (40 µM) alone or in combination for 48 hours, and western blot analysis performed for caspase-3. Upper doublet is pro-caspase 3, *indicates cleaved caspase-3.

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Yun, S., Jung, K. H., Kim, S. J., Fang, Z., Son, M. K., Yan, H. H., . . . Hong, S. (2014). HS-438, a new inhibitor of imatinib-resistant BCR-ABL T315I mutation in chronic myeloid leukemia. *Cancer Letters, 348*(1-2), 50-60. doi:10.1016/j.canlet.2014.03.012

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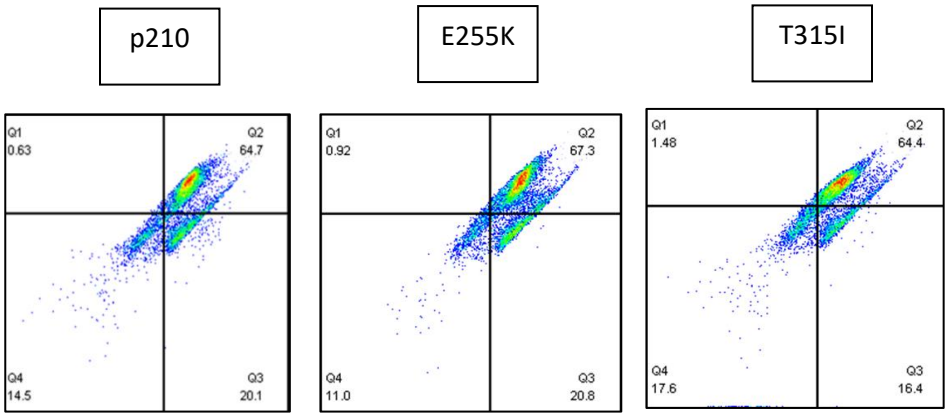
Appendix A

Additional Open Matrine and Imatinib Data

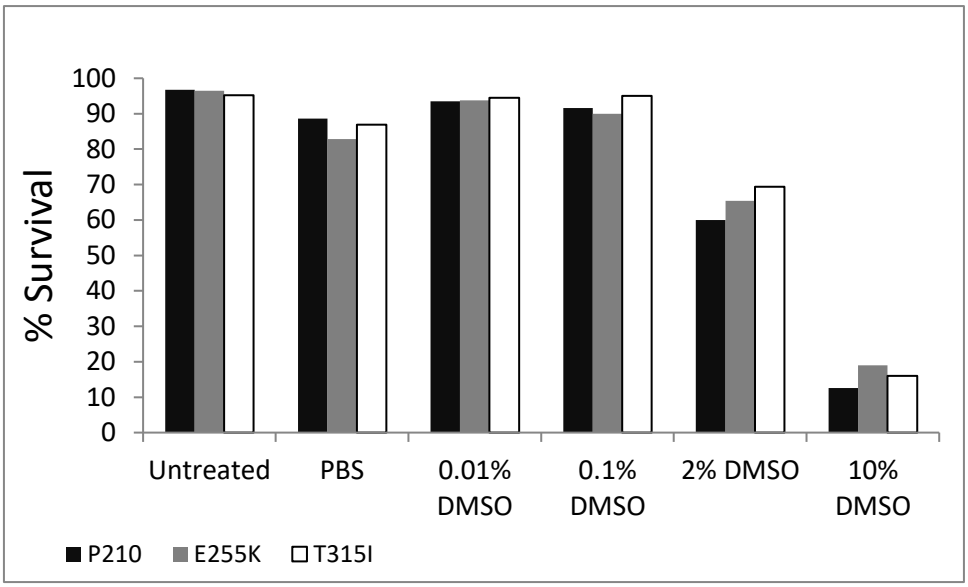
Potential necroptotic pathway induced by open matrine treatment. A unique pattern was observed during flow cytometric analysis of the all CML cells treated with open matrine (4 mM). There is a second population of cells in quadrant three of the dot plot which appear to be necroptotic (Supplemental Figure 1). This potentially necroptotic mechanism is characteristic of only open matrine treatment and was not seen with treatment of any other compound tested on these cells under the same conditions.

CML cells were treated with Imatinib at various time points to determine the effectiveness of the compound. Shown is Supplemental Figure 3, Imatinib only shows efficacy after 72 hours of incubation at a concentration of 4 µM. Due to the results that were obtained in Supplemental Figure 3, CML cells were treated with Imatinib a second time but only at the highest concentration of 4 µM. The results were not as expected, which is likely due to storage temperature. Shown in Supplemental Figure 4, the p210 cells treated with Imatinib at 4 µM for 72 hours do not lose more than 10% cell viability, which does not correlate with the known response for p210 CML cells. Due to the lack of consistency and reproducibility of the Imatinib treatment results, CML cells were treated with Imatinib that had been stored in various conditions after preparation. From the results obtained, it was determined that the Imatinib stock solution works best when made fresh the same day Imatinib treatment is performed. After determining that Imatinib solutions must be made fresh the day of treatment, the CML cells began to

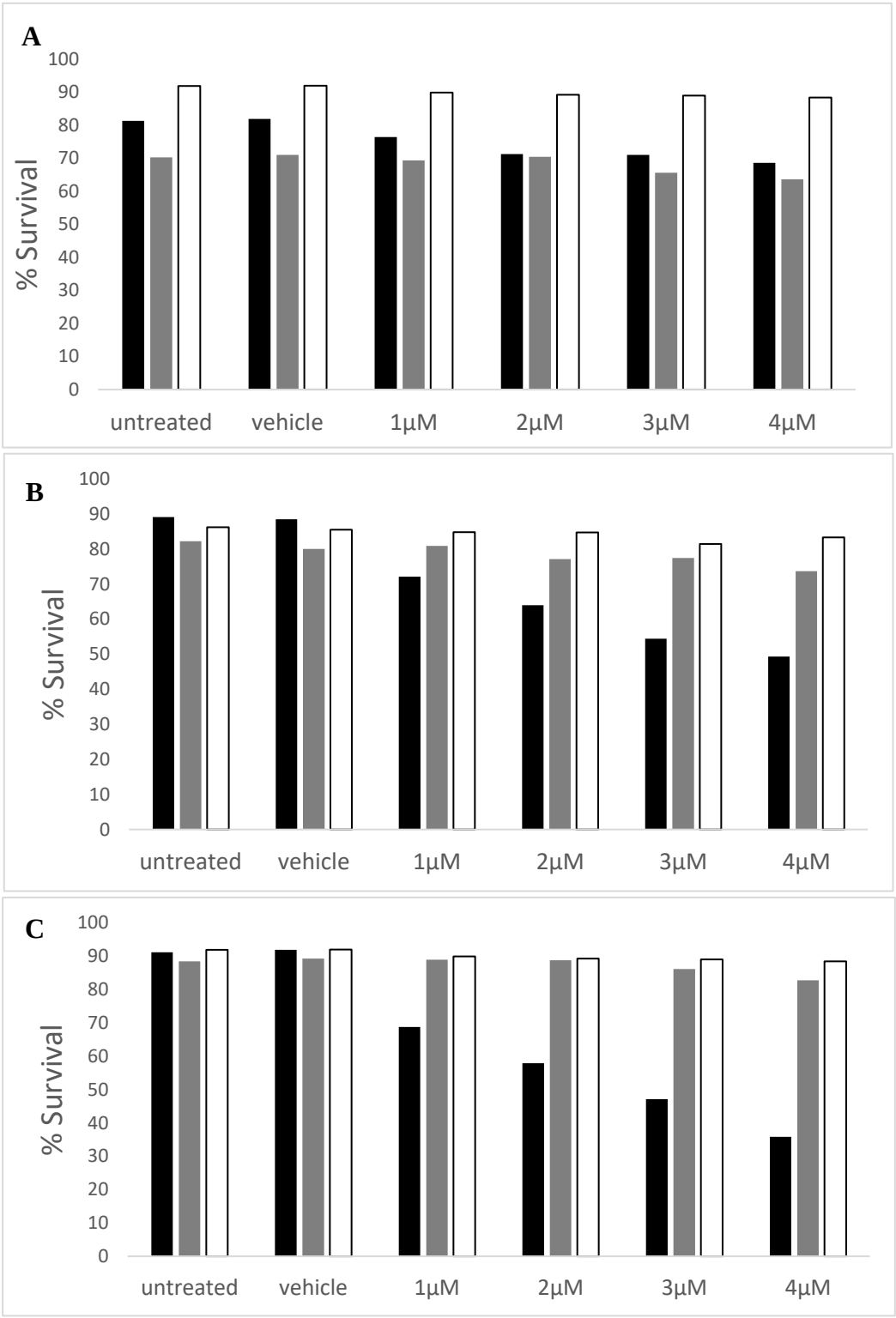
respond as described in literature and allowed for the completion of the apoptosis experiments by using flow cytometry.



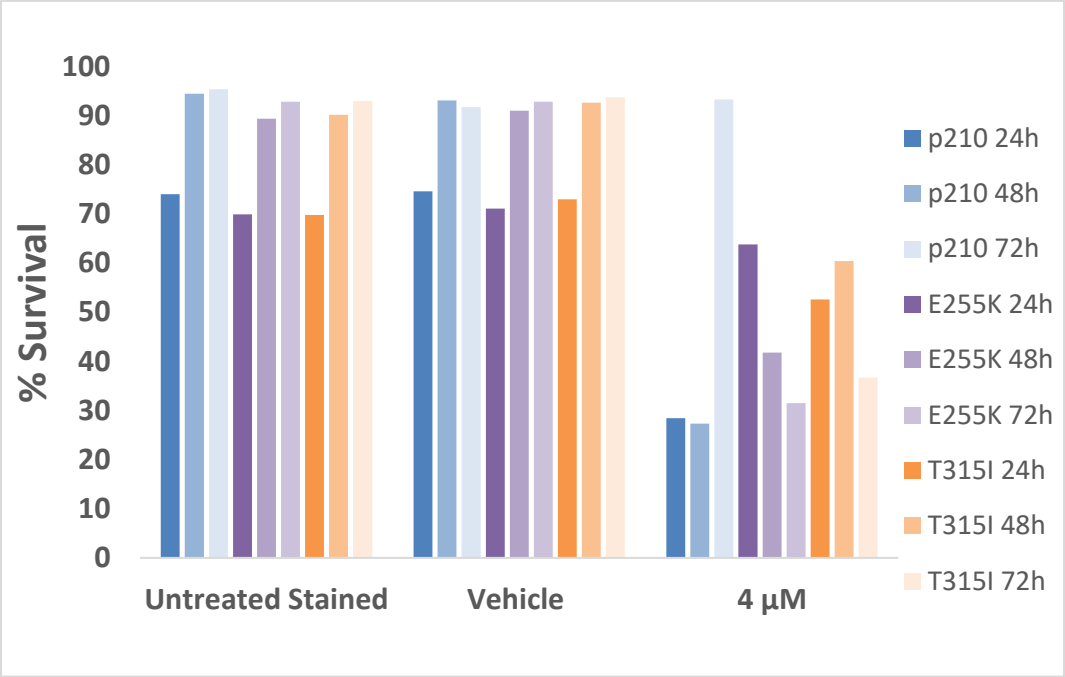
Supplemental Figure 1. Open matrine treatment results in a potential necroptotic mechanism. Dot plot of p210, E255K, and T315I cells treated with 4 mM open matrine for 72 h. Note the two populations of PI-positive cells indicating late apoptosis and a possible necroptotic mechanism.



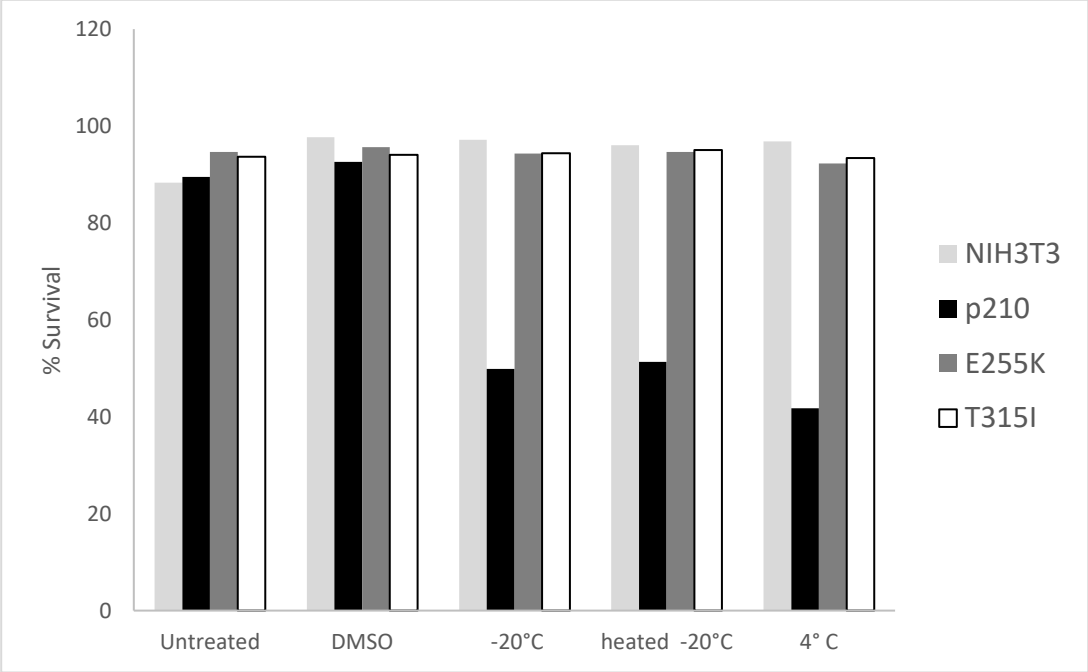
Supplemental Figure 2. Apoptosis of CML cell lines with DMSO at various concentrations. The indicated concentration of DMSO was incubated with CML cells for 72 hours to determine the maximum concentration of DMSO allowable to resuspend inhibitors.



Supplemental Figure 3. CML cells, p210 (black), E255K (grey) and T315I (white), were treated with various concentrations of Imatinib at 24 hours (A), 48 hours (B) and 72 hours (C).



Supplemental Figure 4. CML cells treated with Imatinib only at 4 µM for 24, 48 and 72 hours.

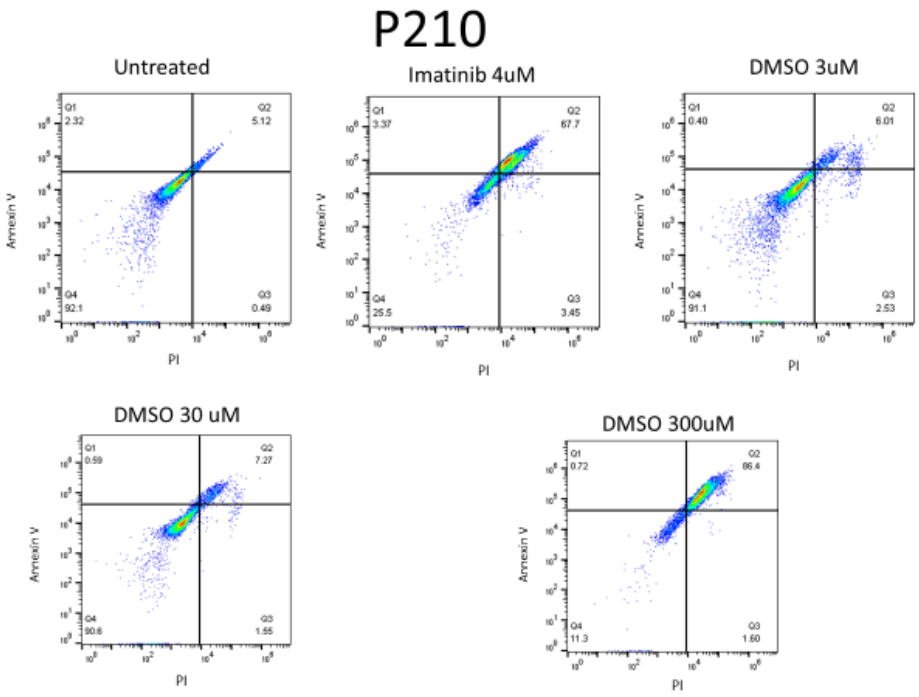


Supplemental Figure 5. CML cells treated with Imatinib stored at various temperatures.

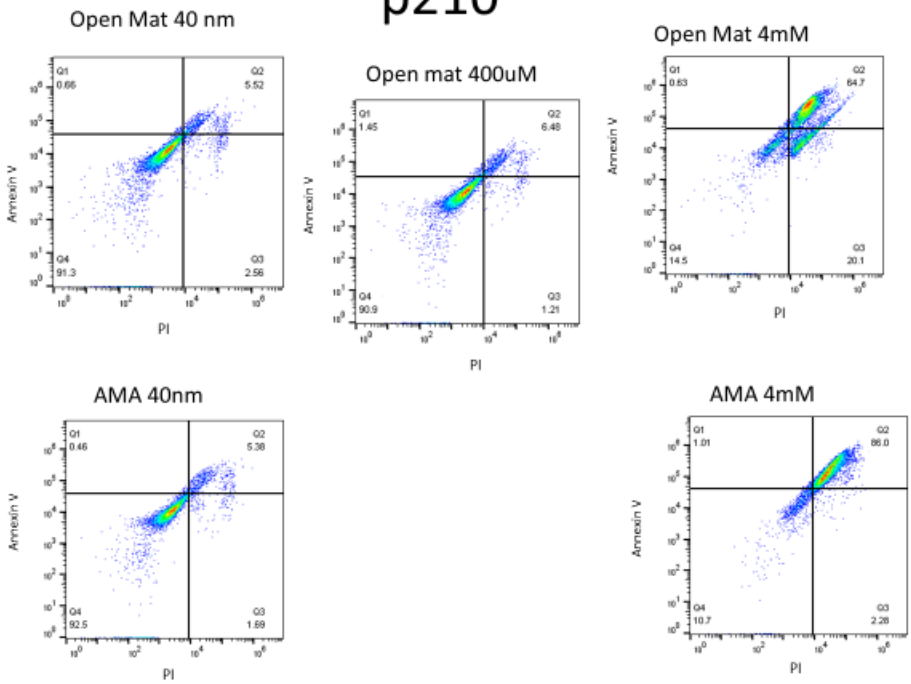
Appendix B

Flow Cytometry Dot Plots

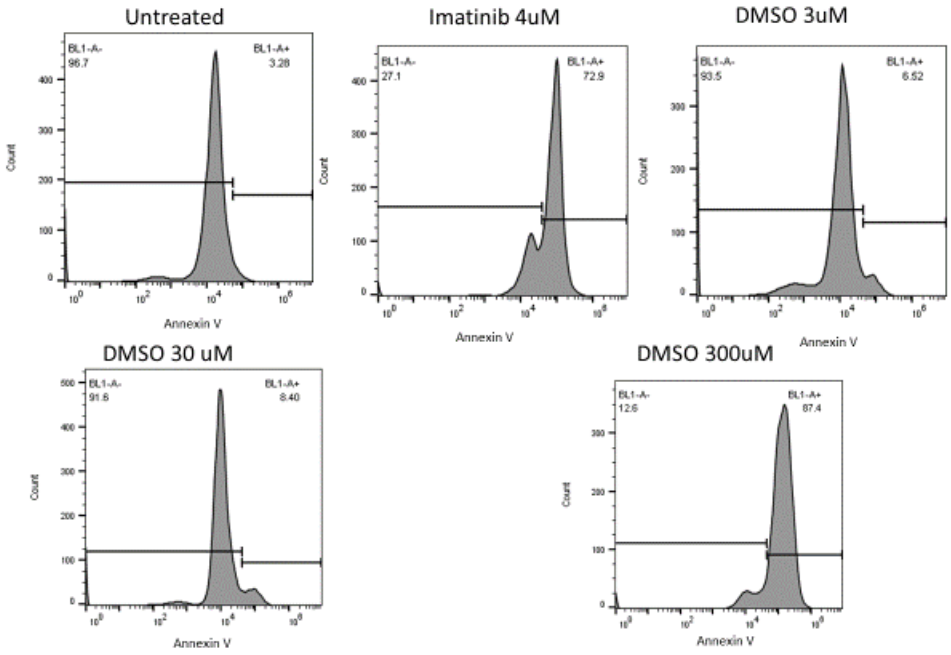
Attached are all the flow cytometric dot plots collected during the apoptosis experiments which are shown after data was analyzed via FlowJo Software. Histogram images are also provided of the data. The quantification of the dot plot data was used to produce the graphs presented throughout the thesis document. Plots are labeled with the cell line treated and the concentration of inhibitor(s). Time of treatment was 72 hours. Y-axis represents annexin-v positive cells and X-axis represents PI positive cells.



p210



P210



P210

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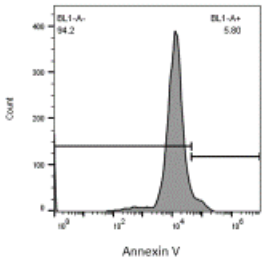
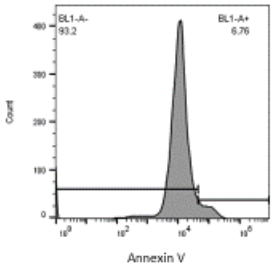
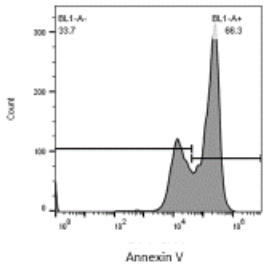


Open mat 400uM



Open Mat 4mM



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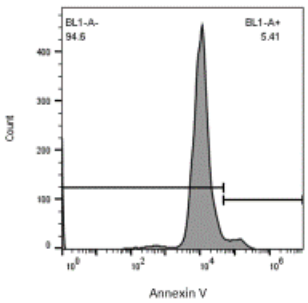
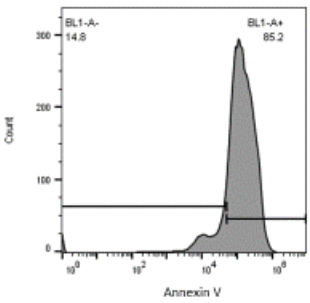
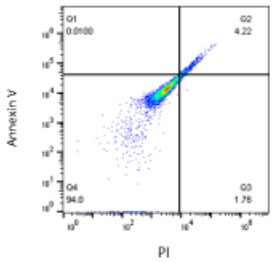


AMA 4mM

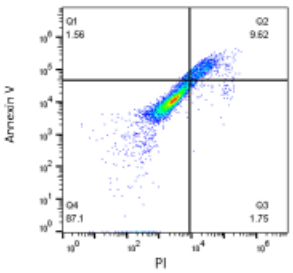


E255K

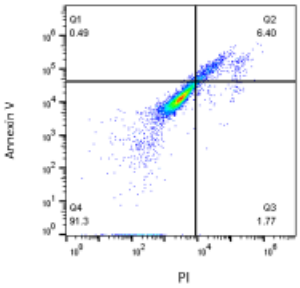
Untreated



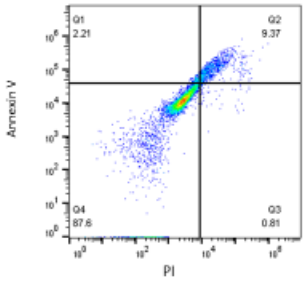
Imatinib 4uM



DMSO 3uM



DMSO 30 uM



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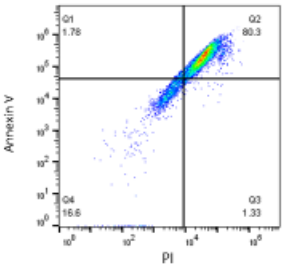

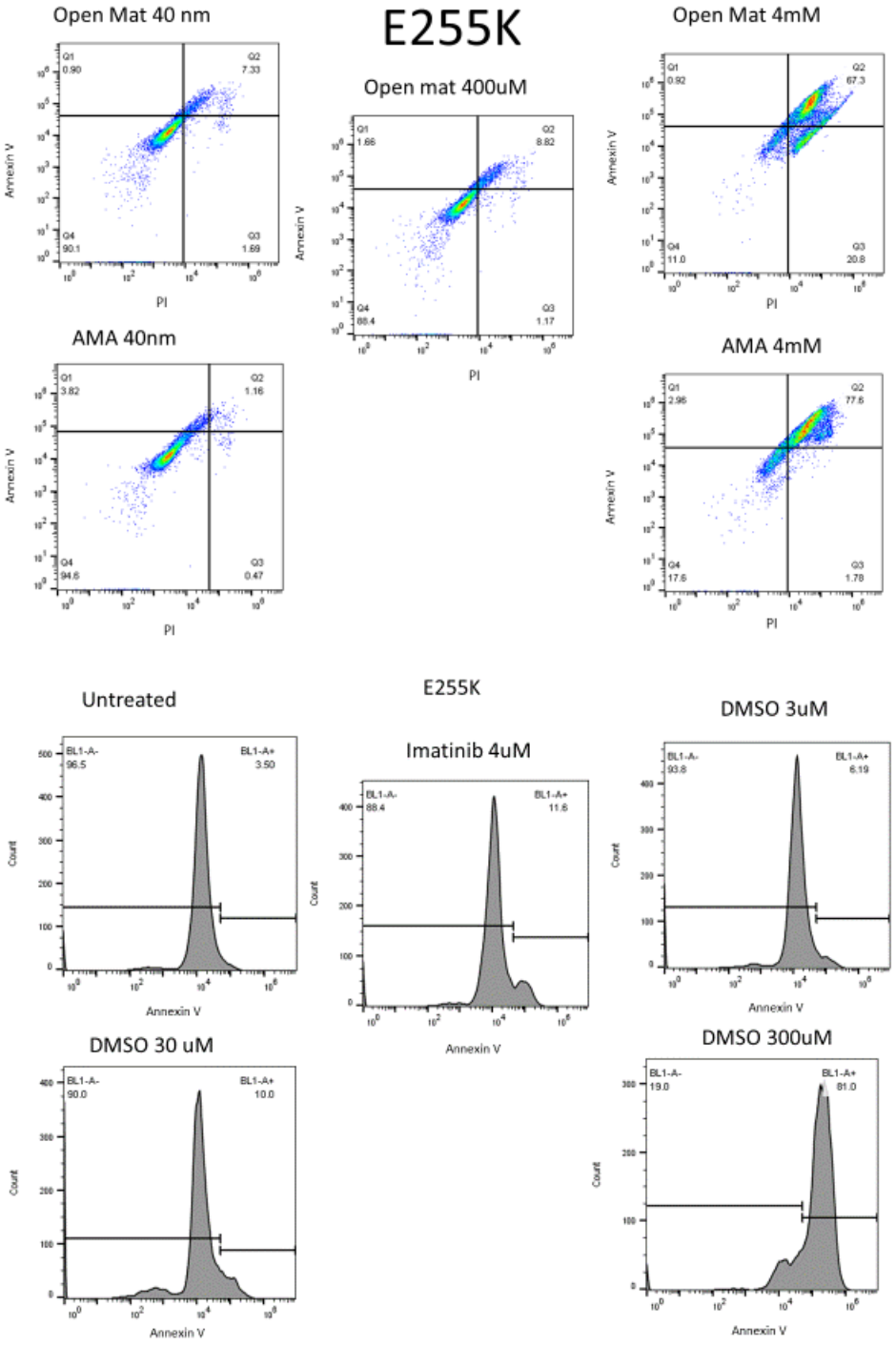

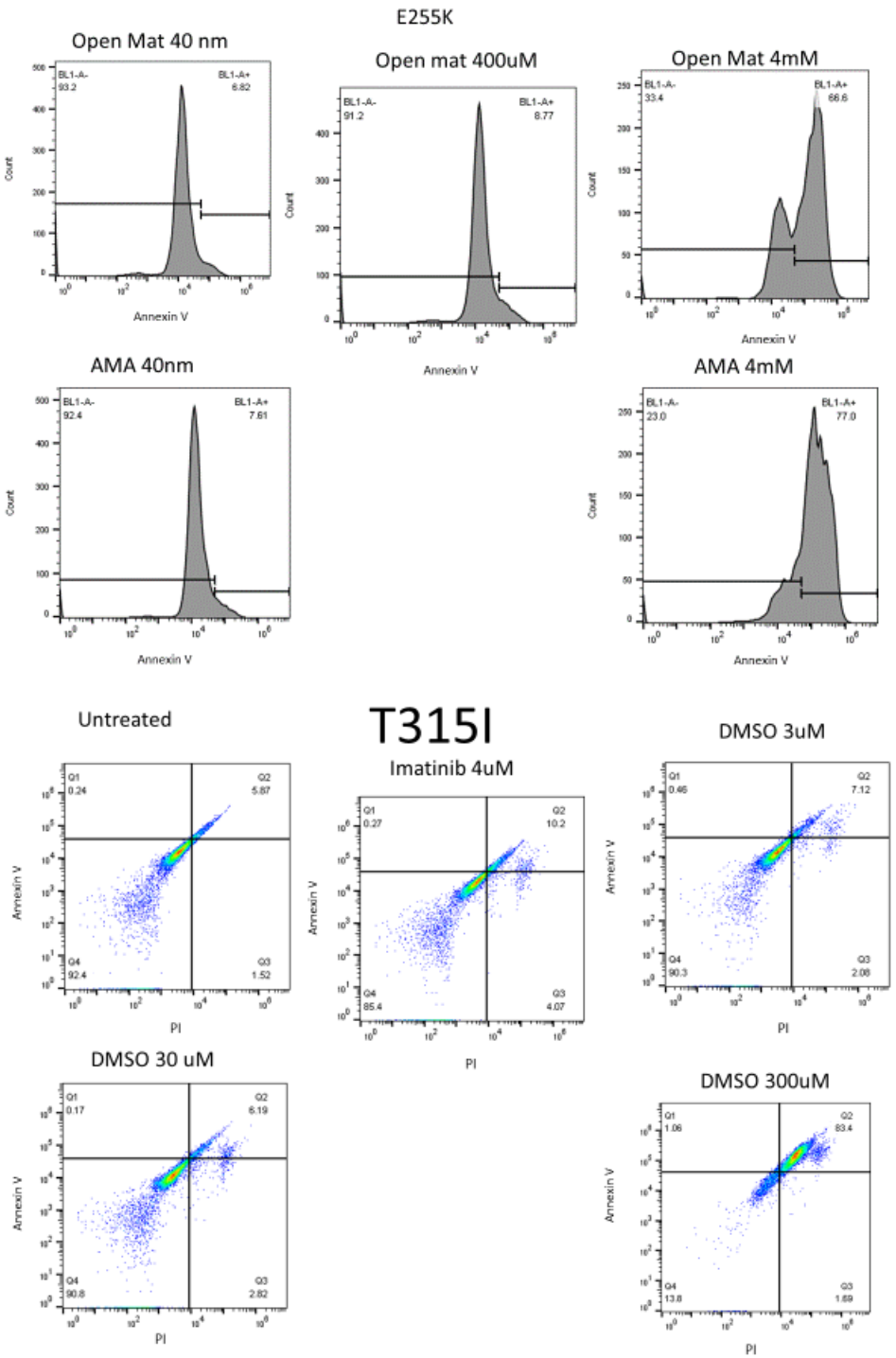

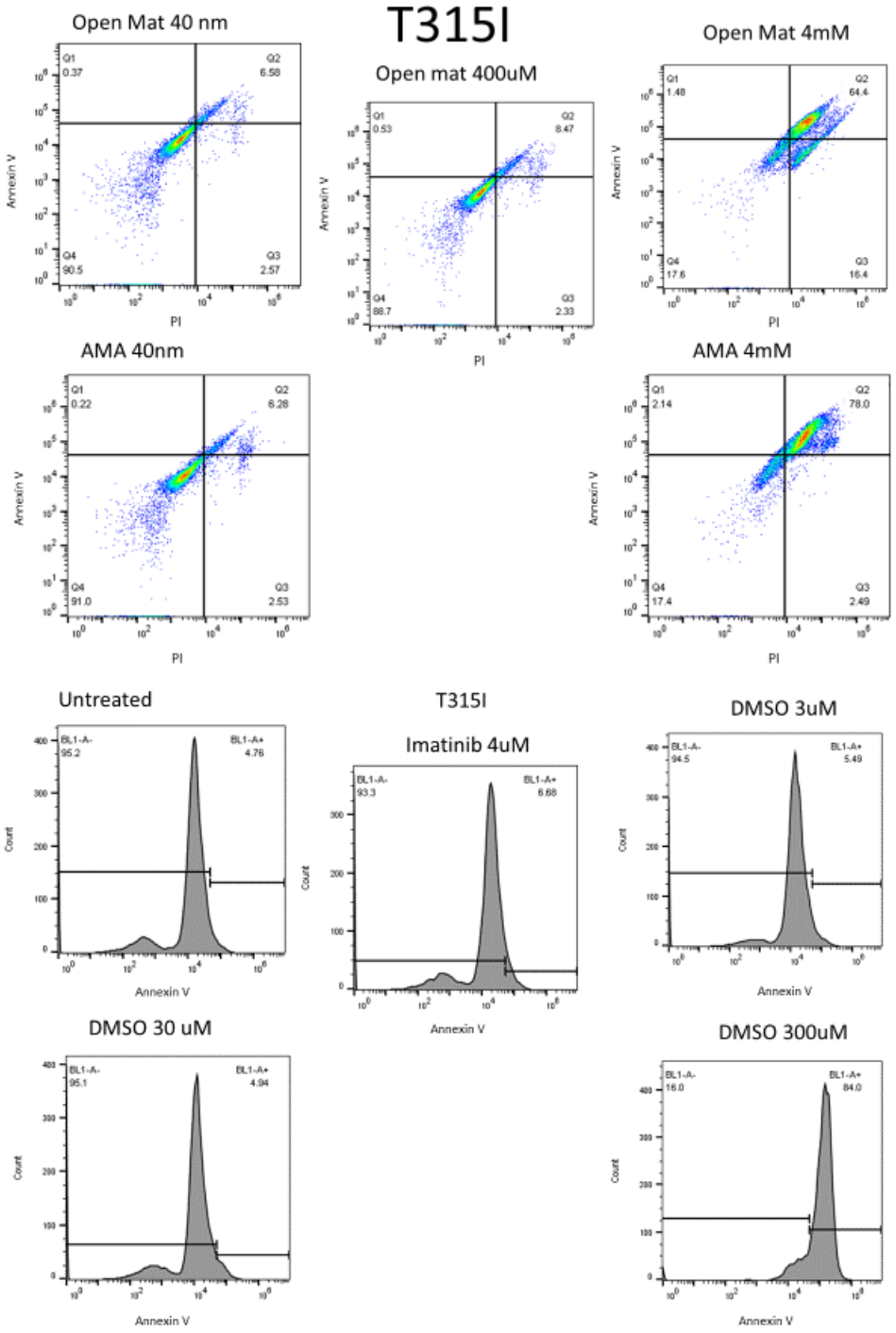

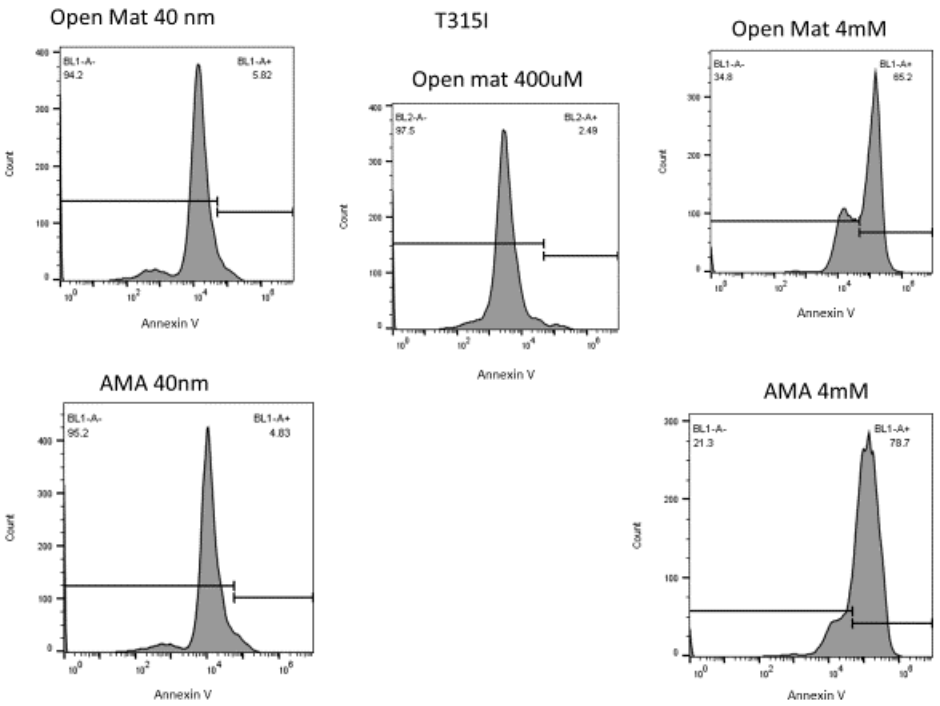

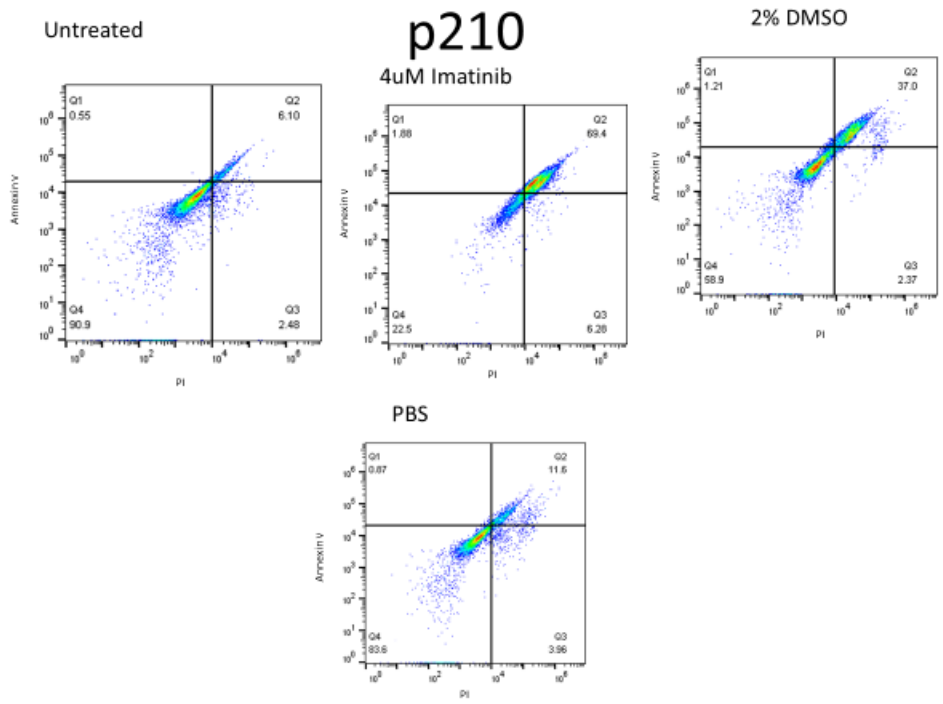

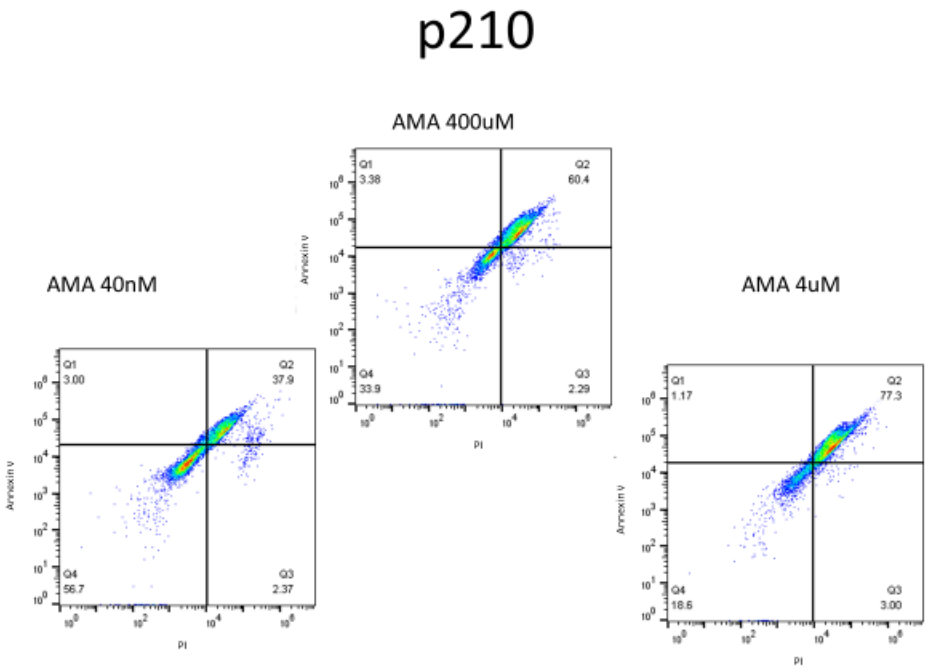

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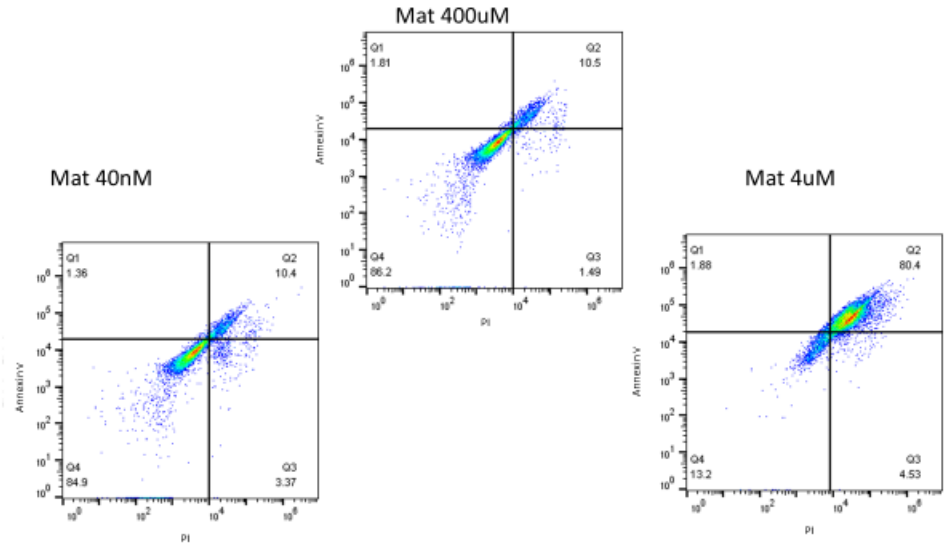

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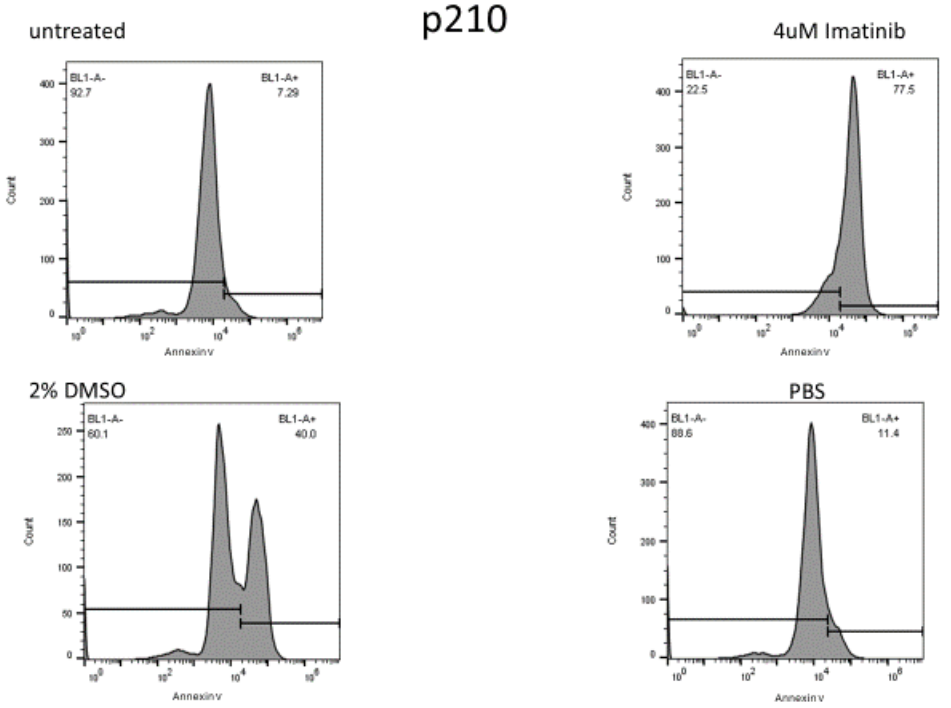

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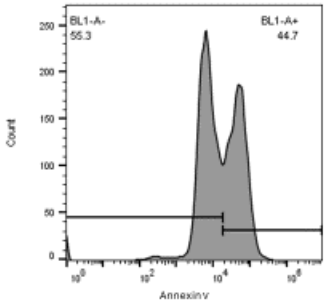
AMA 40nM



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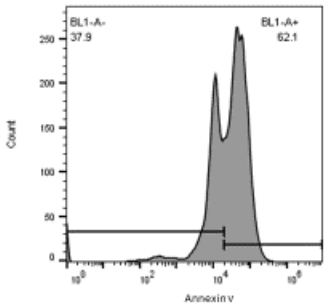

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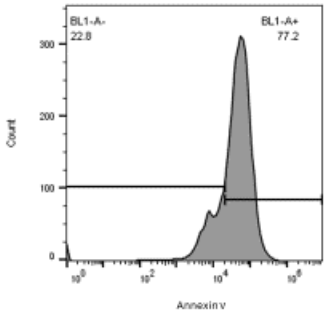

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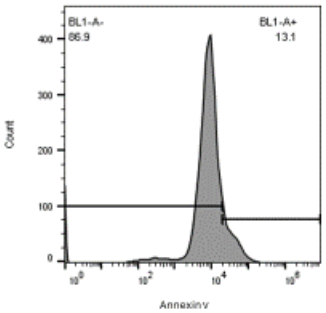

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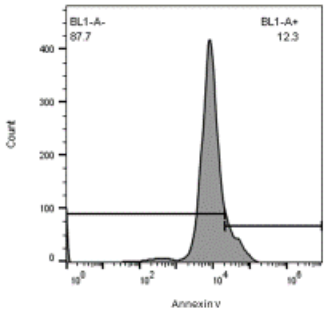

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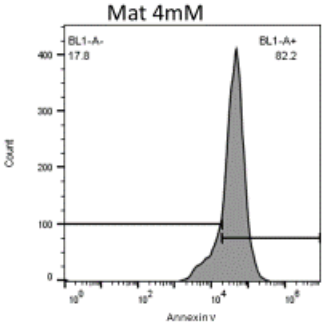

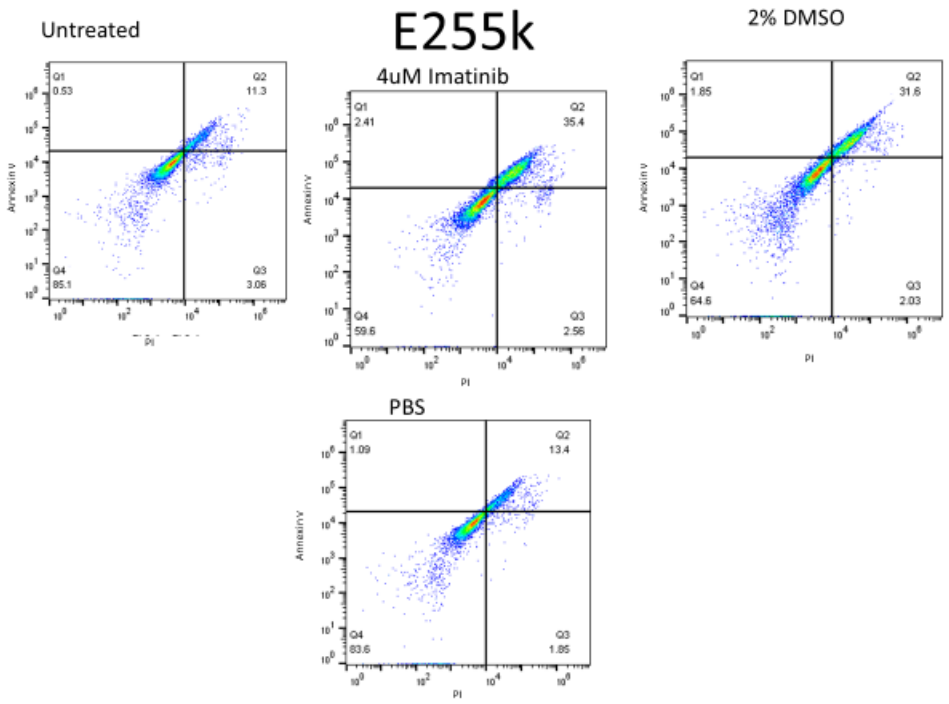

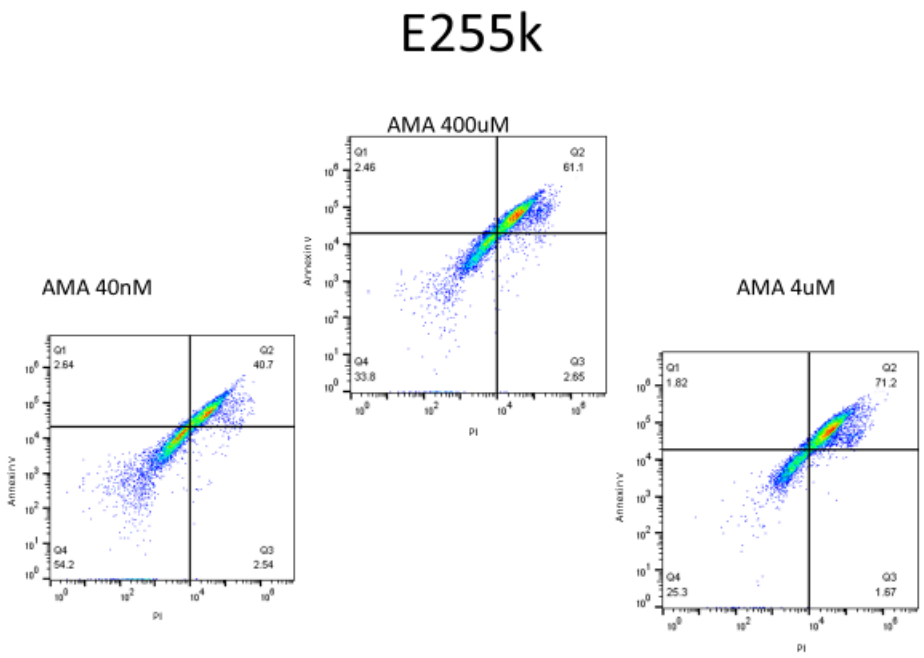

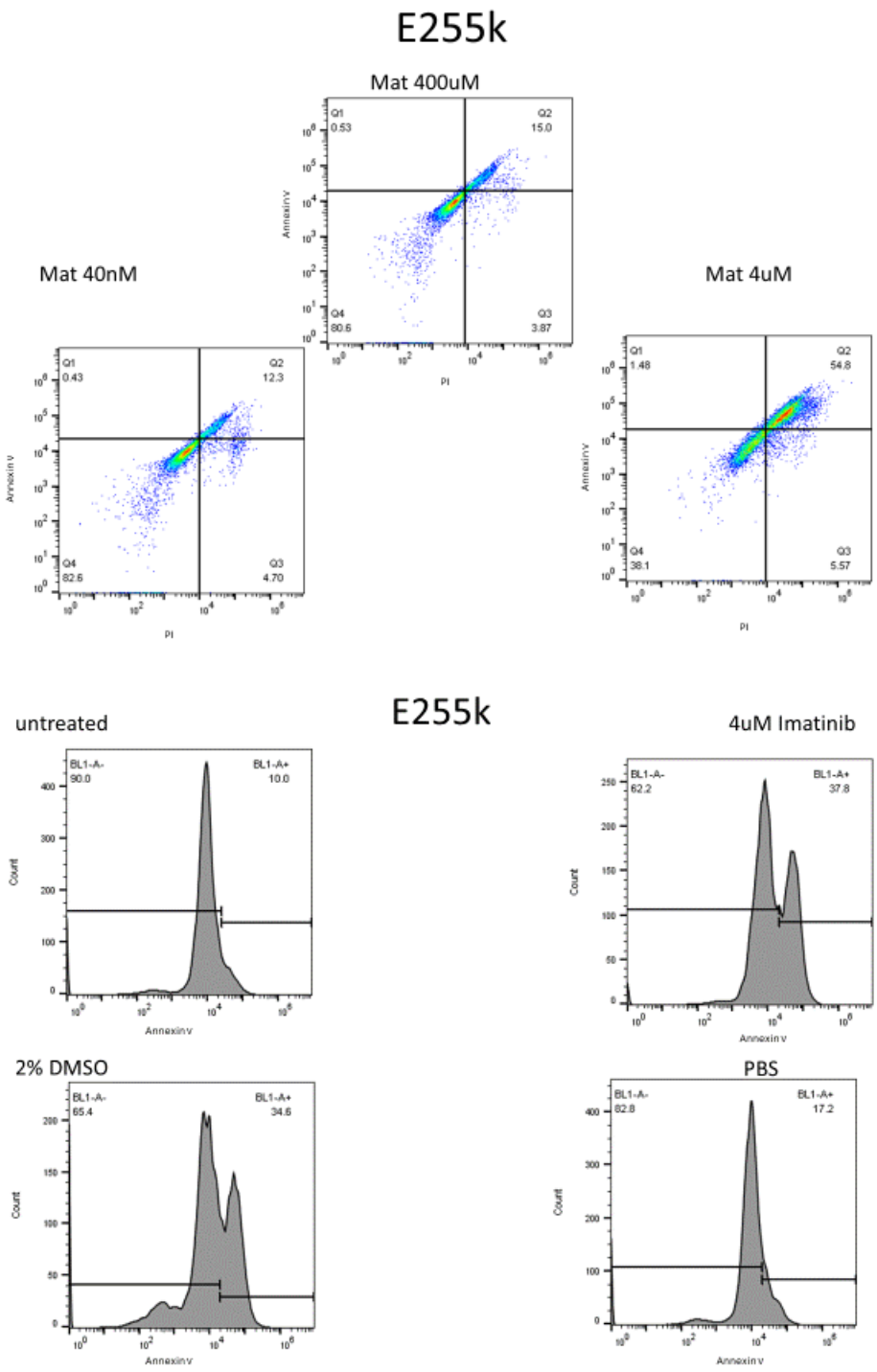

E255k

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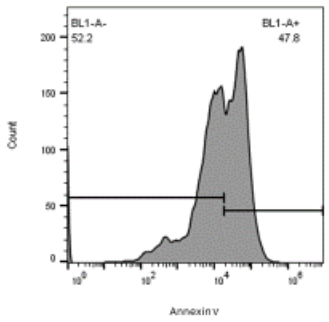

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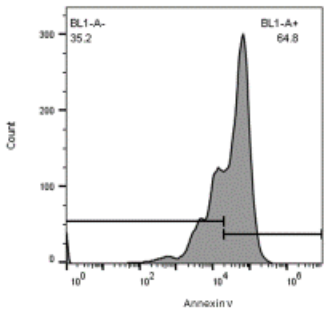

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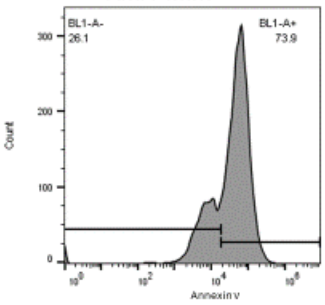

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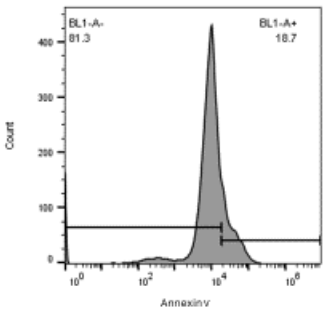

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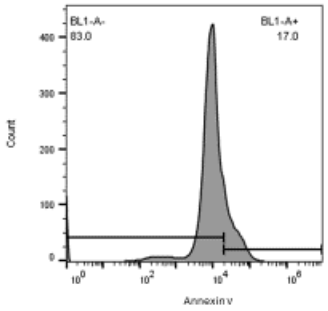

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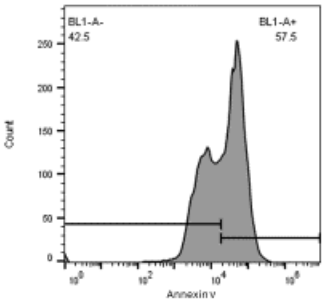

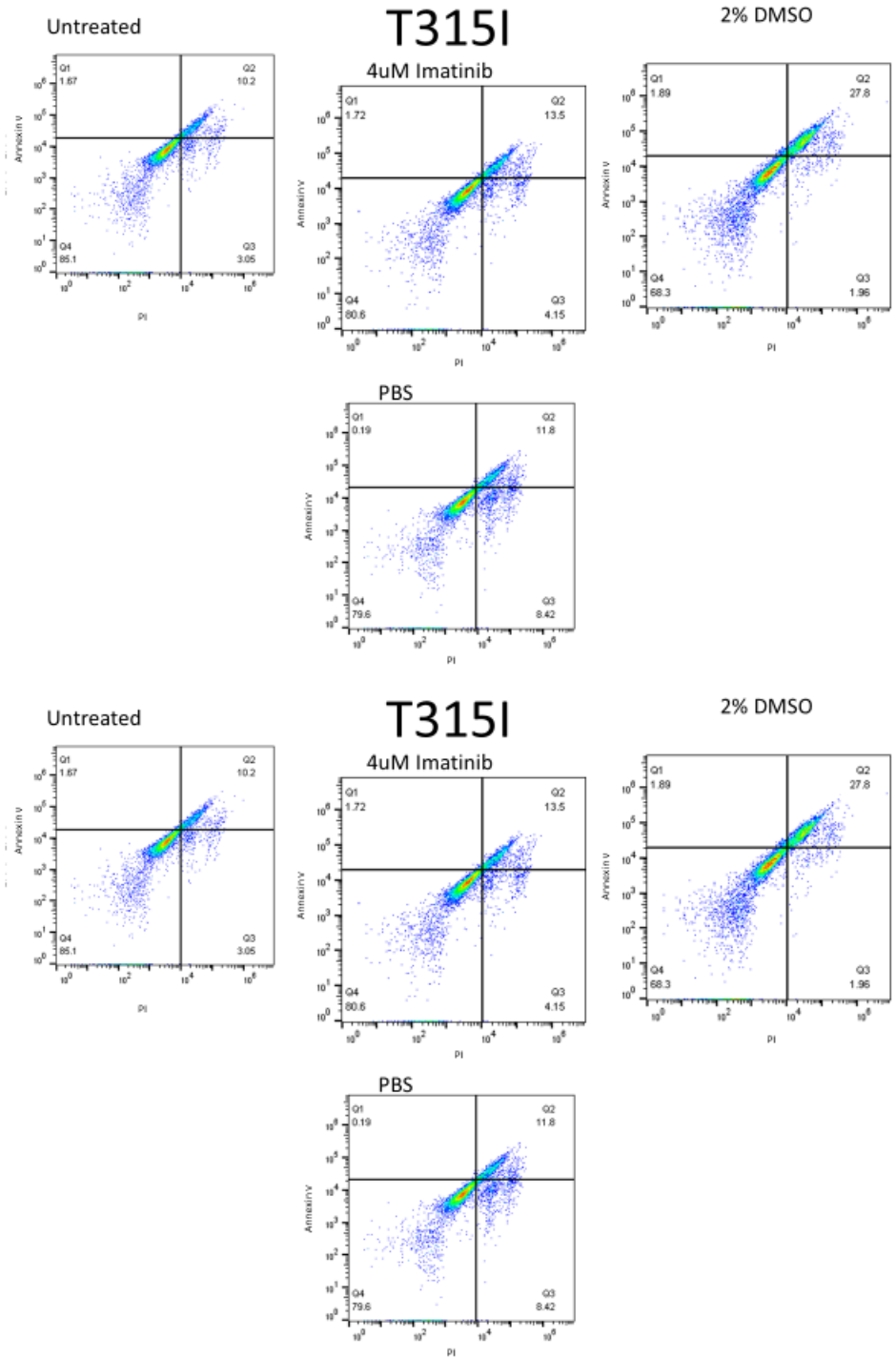

T315I

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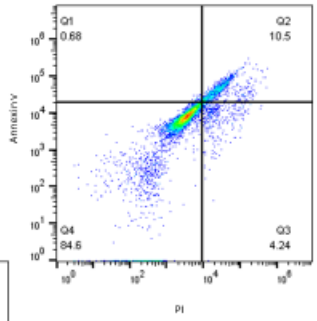

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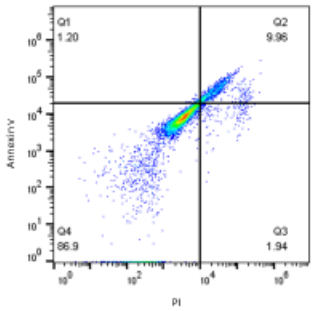

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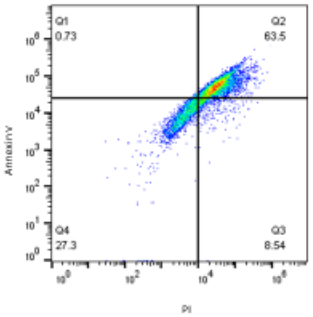

T315i

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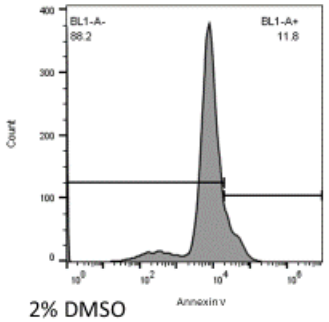
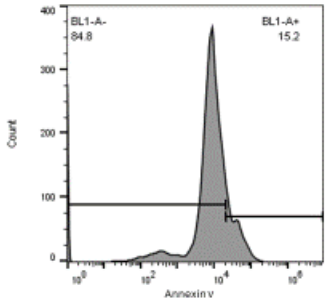


4uM Imatinib



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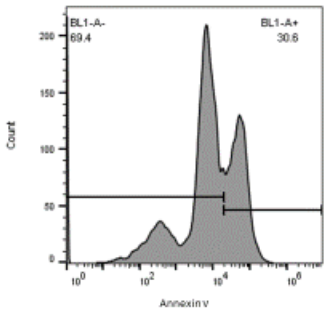

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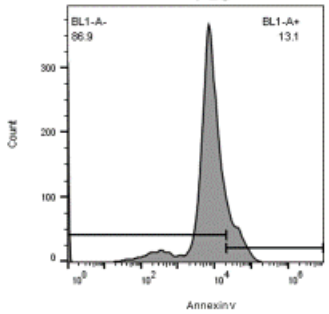

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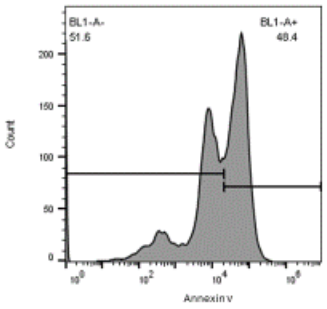

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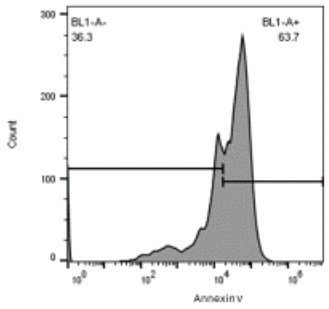

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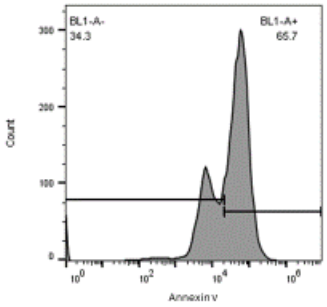

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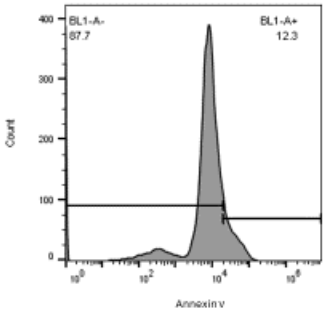

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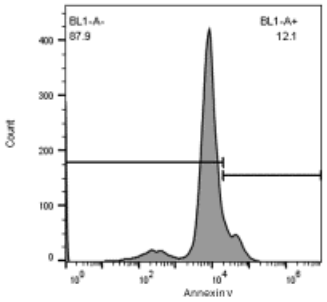

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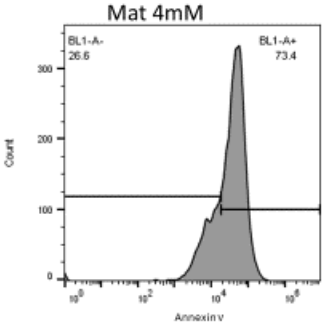
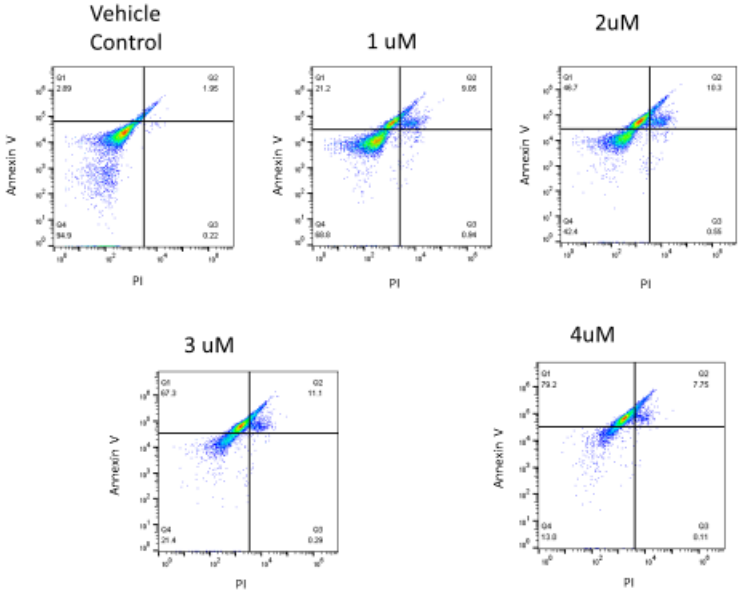


P210 Imatinib



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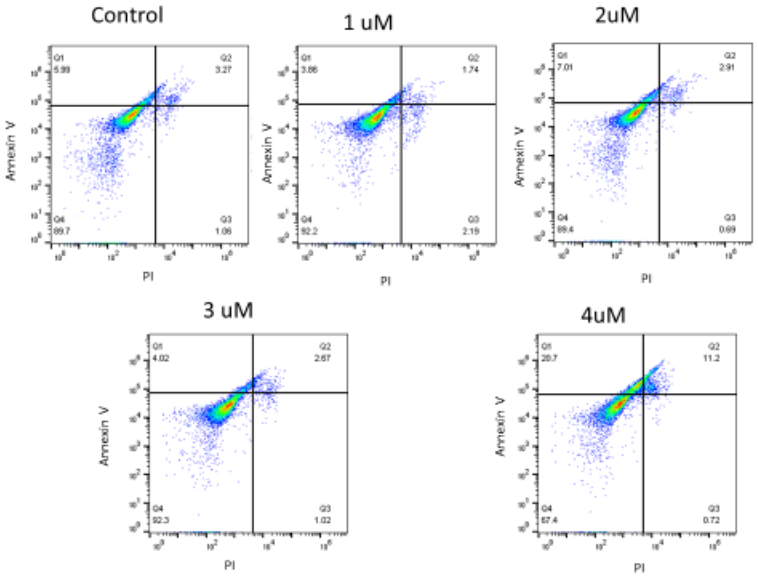
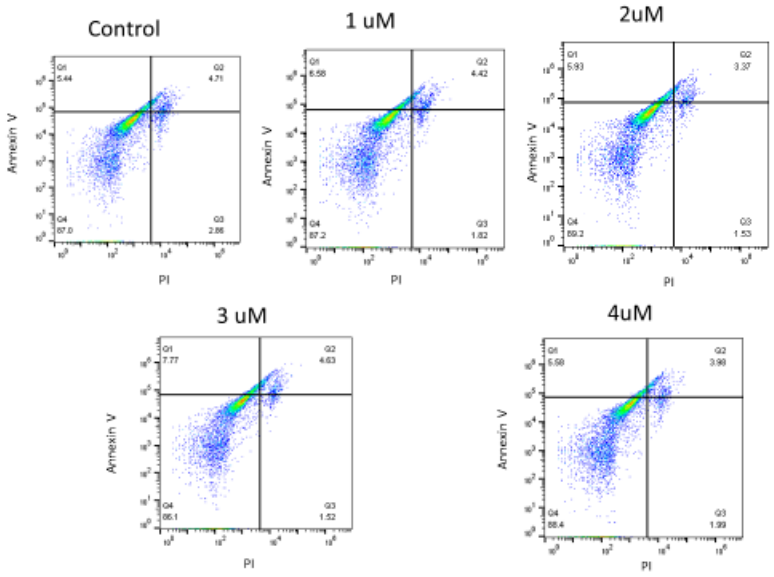
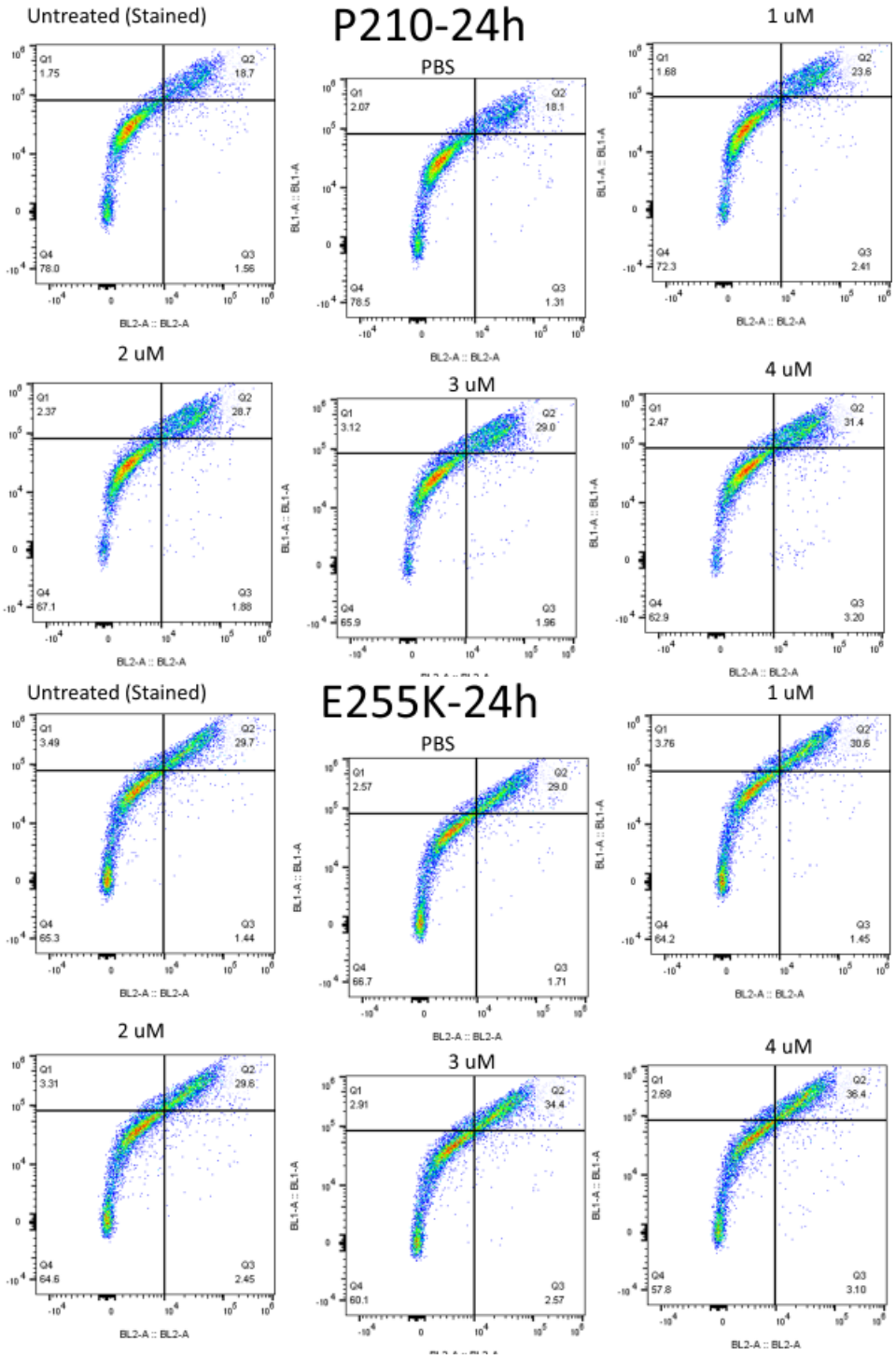
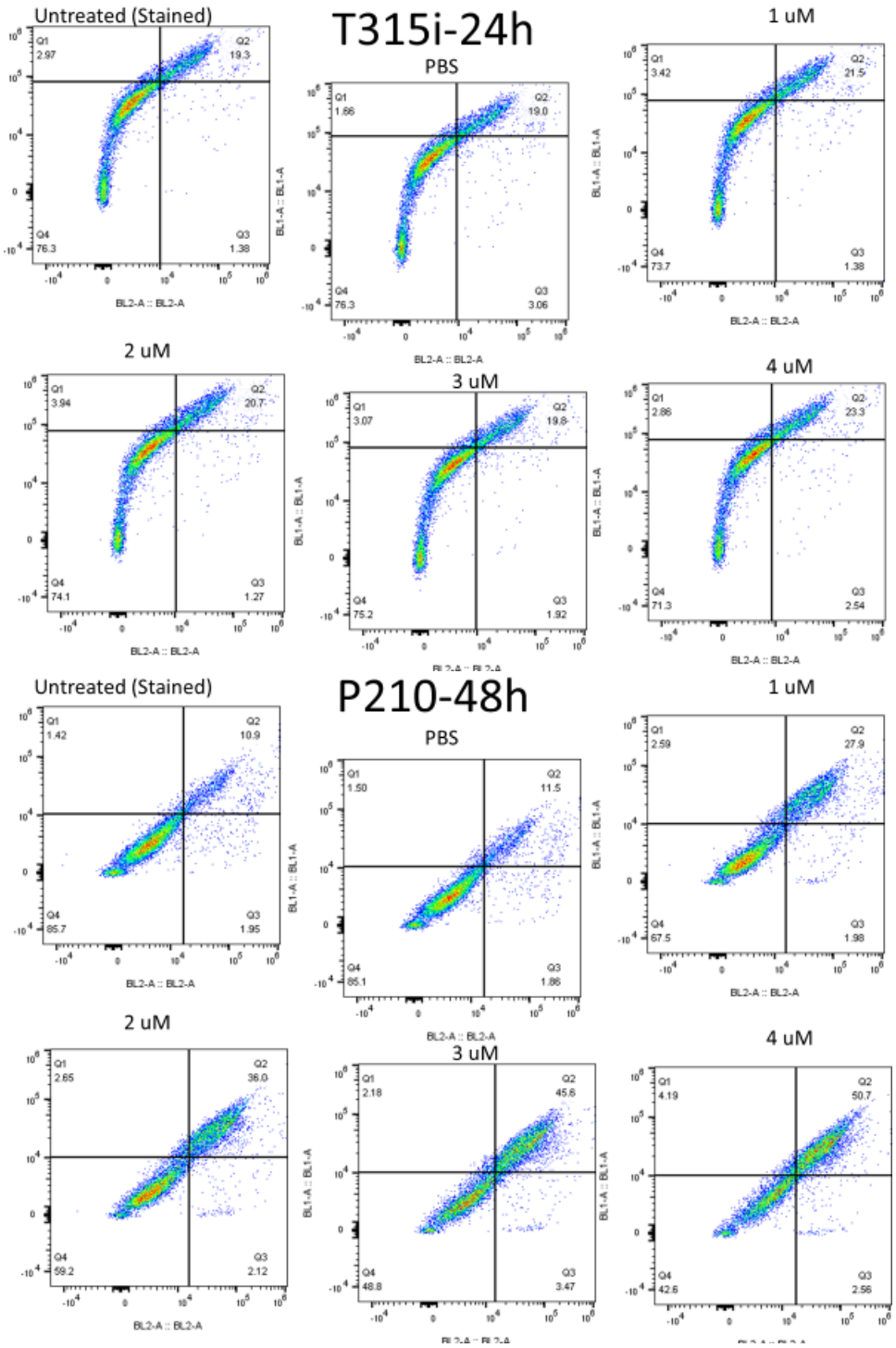
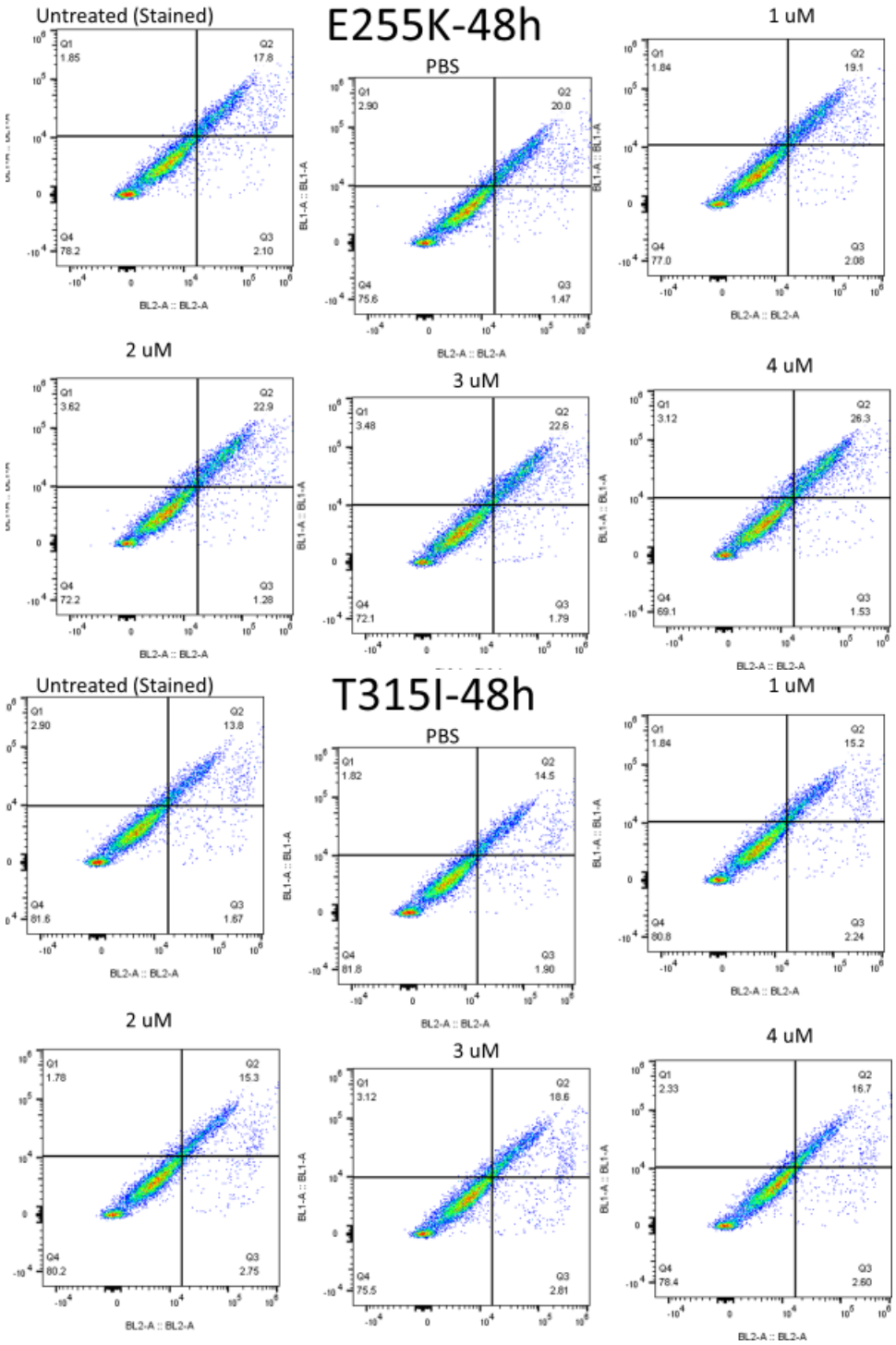


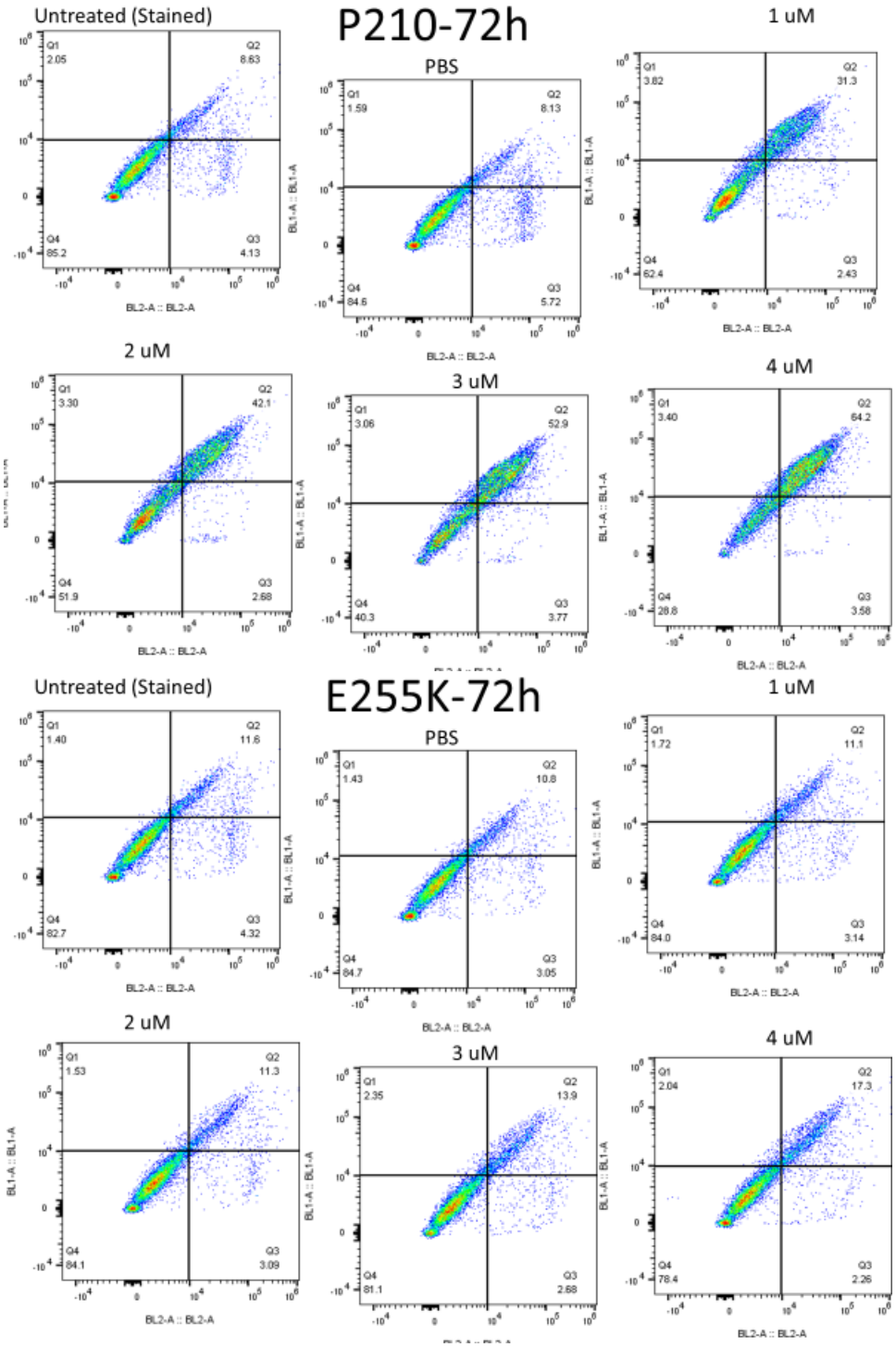
T315I Imatinib

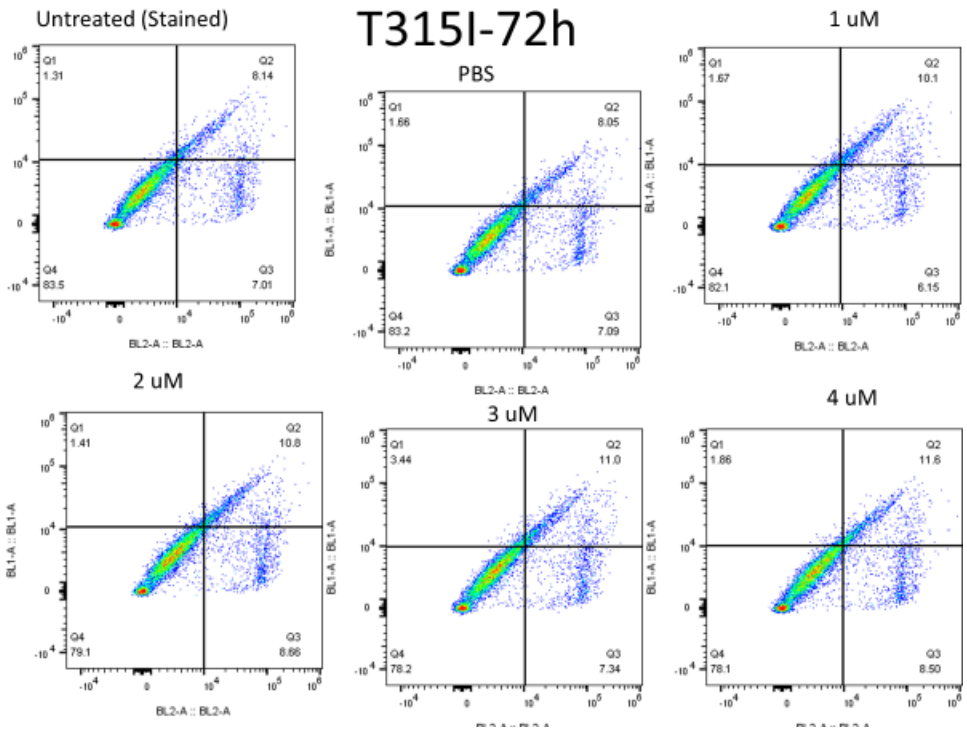


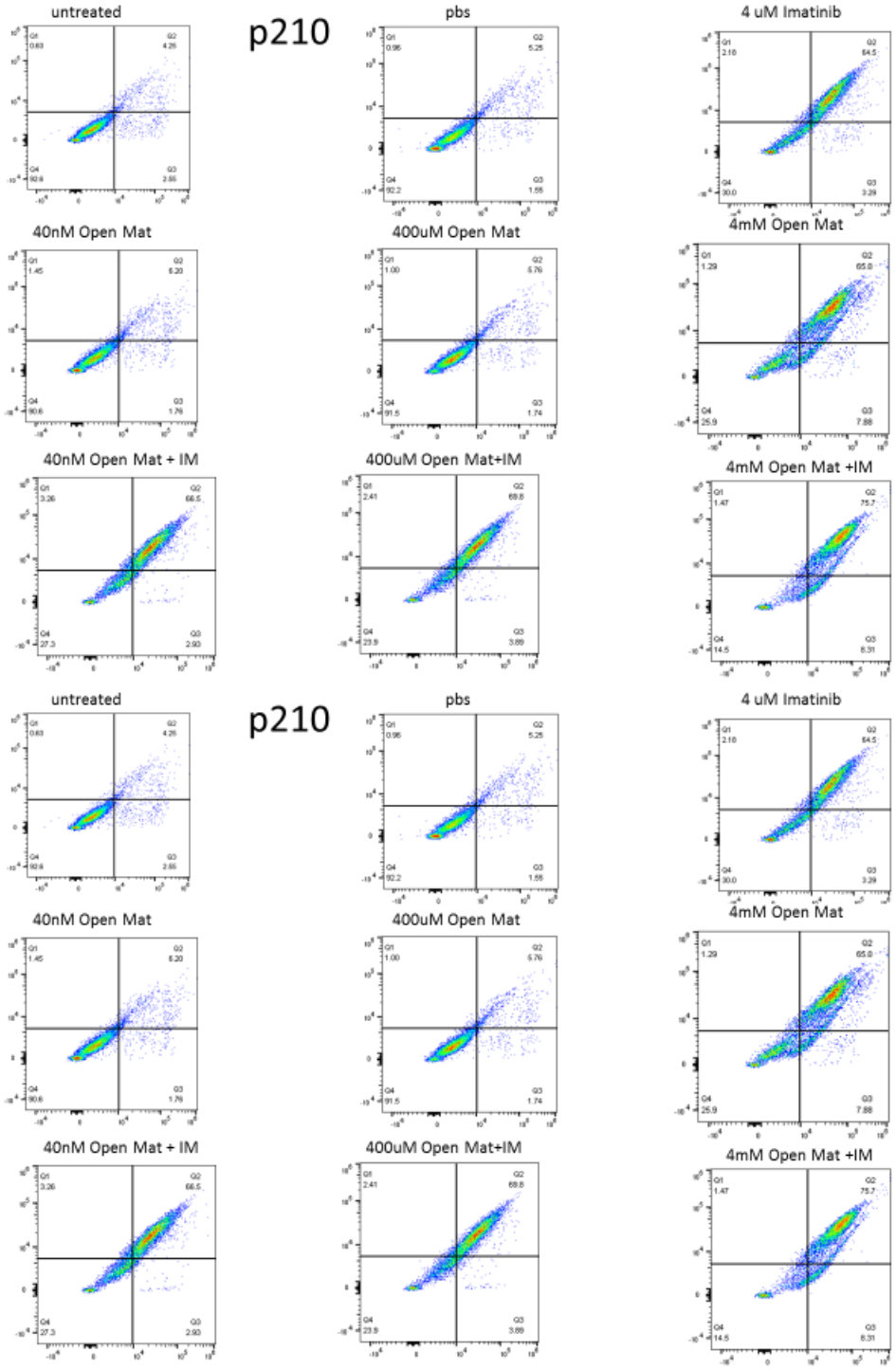


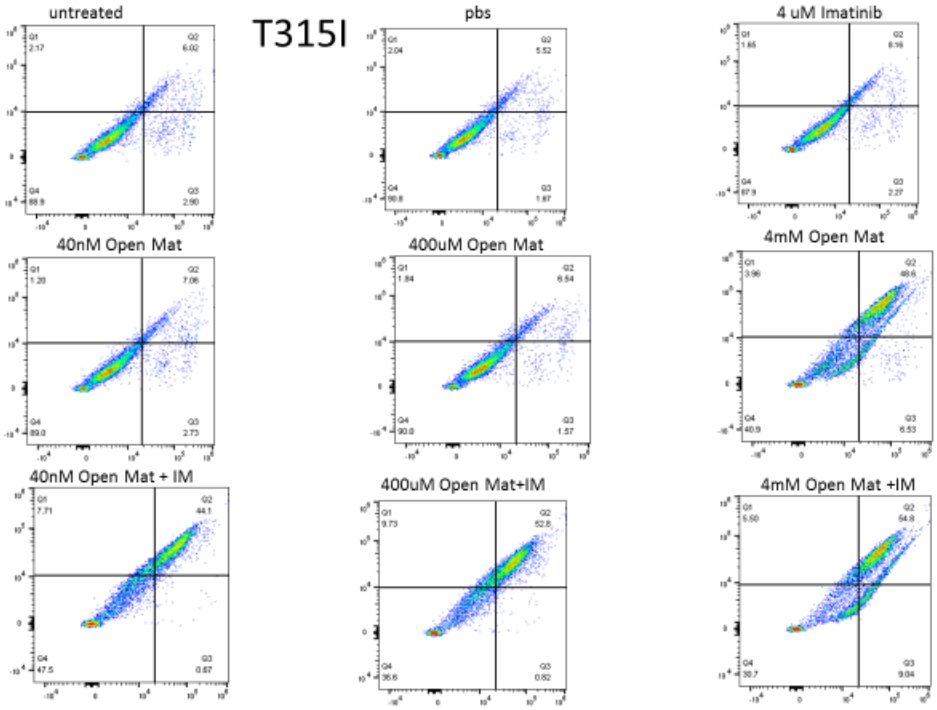


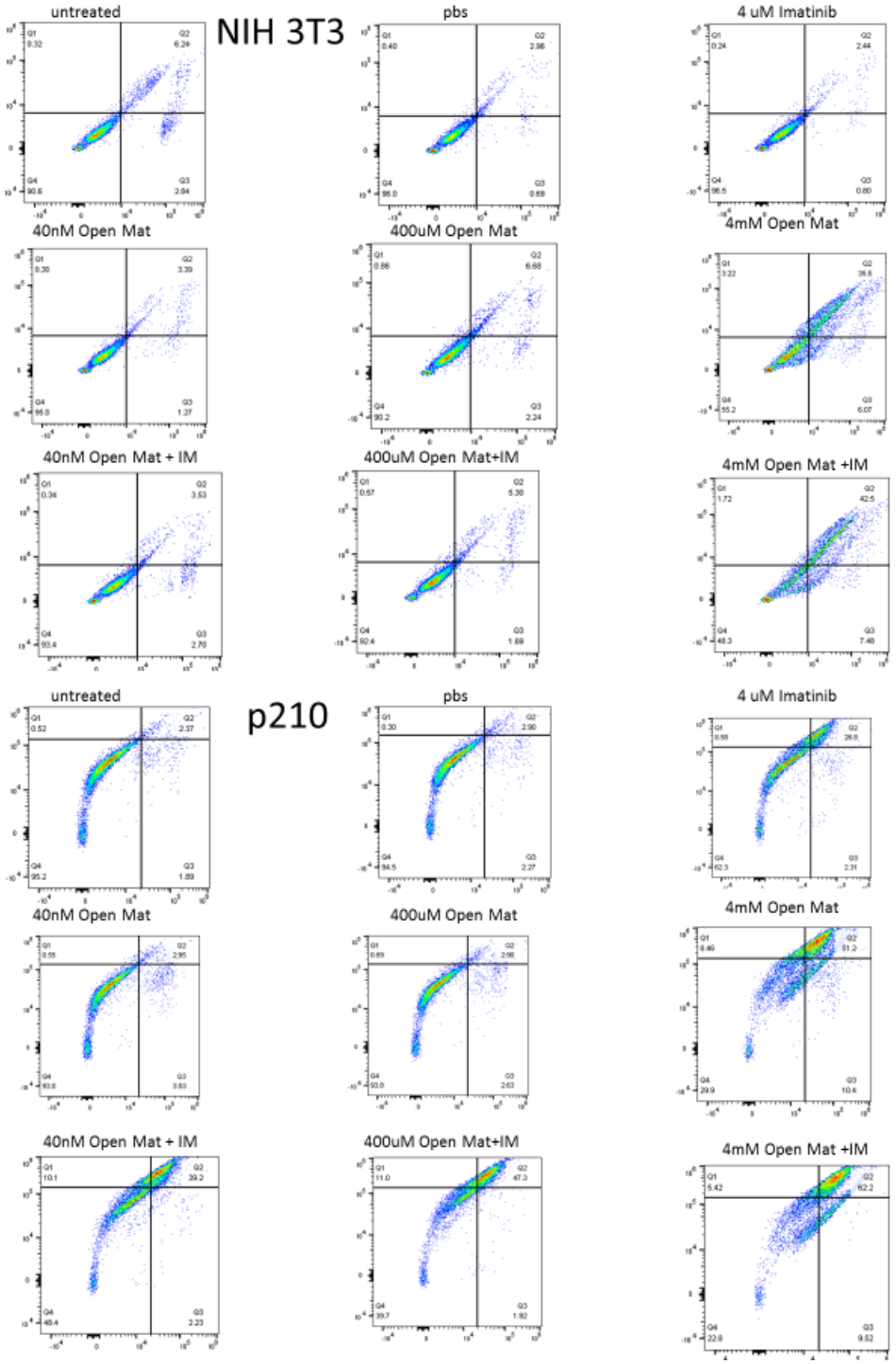


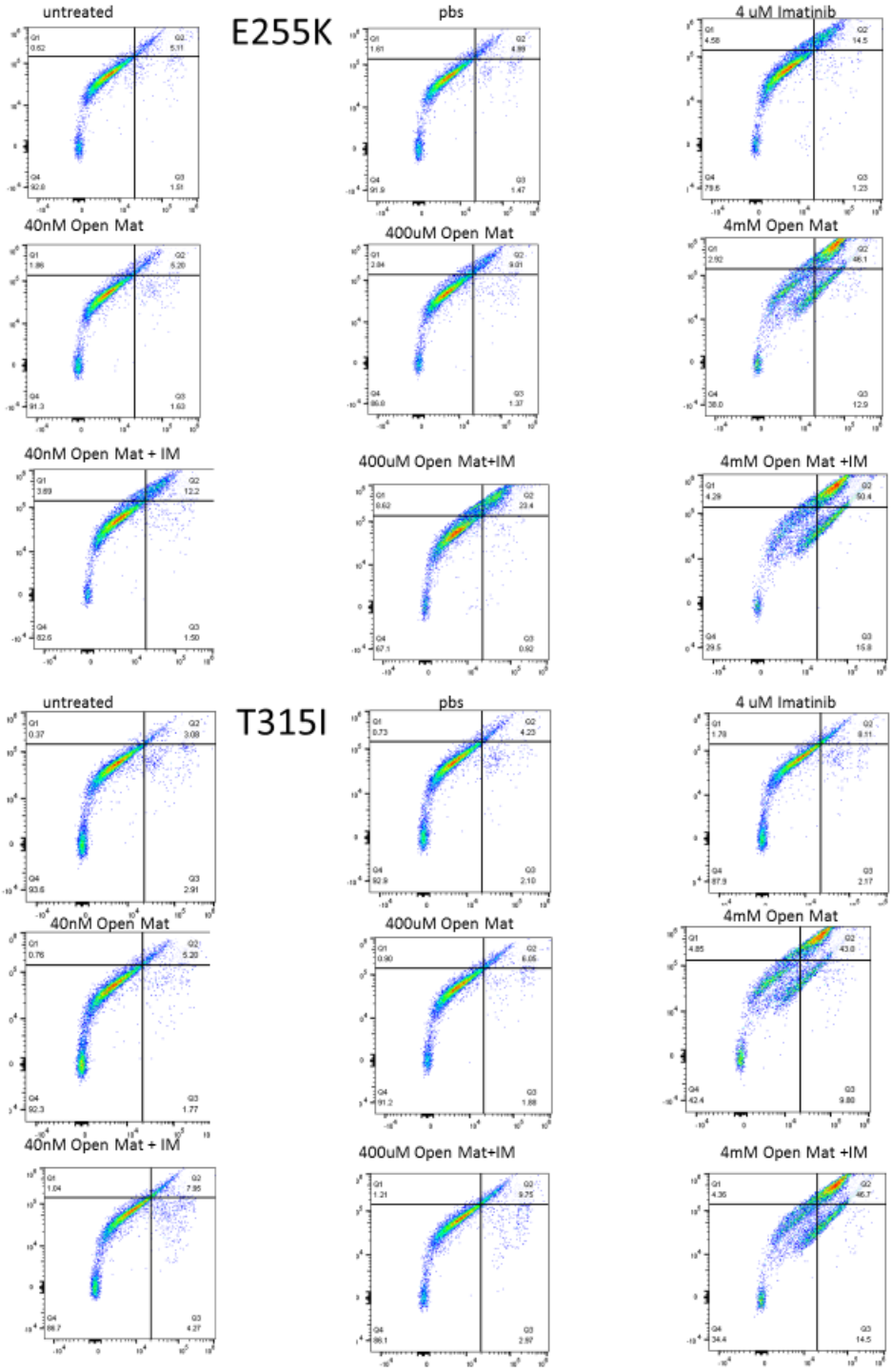


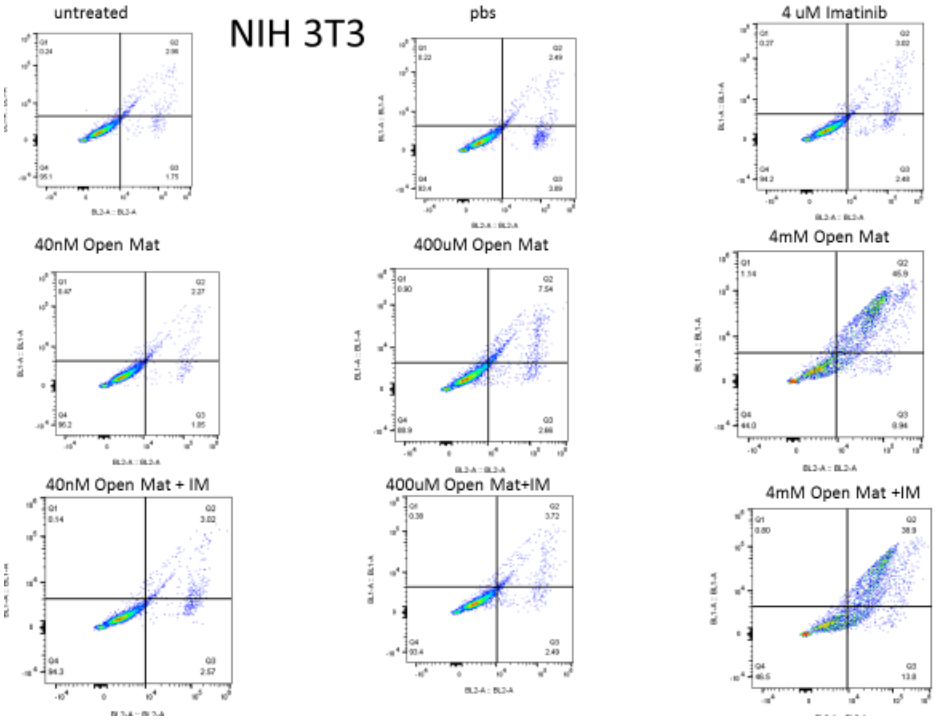




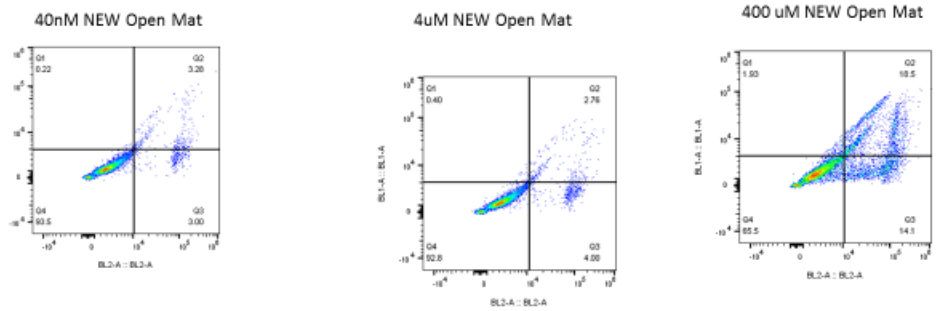


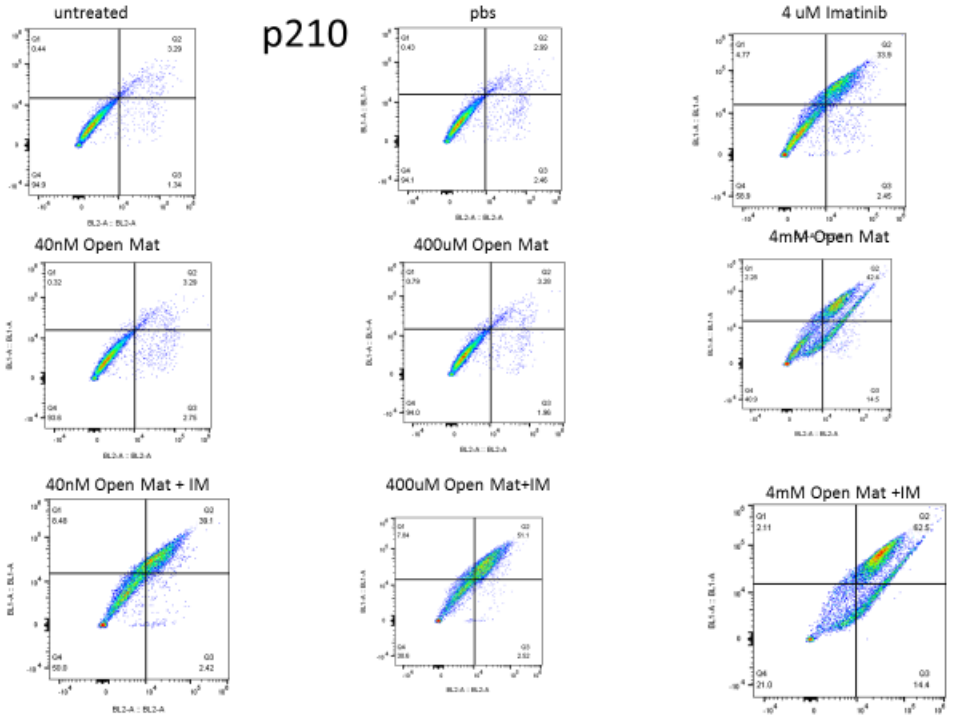






NIH 3t3





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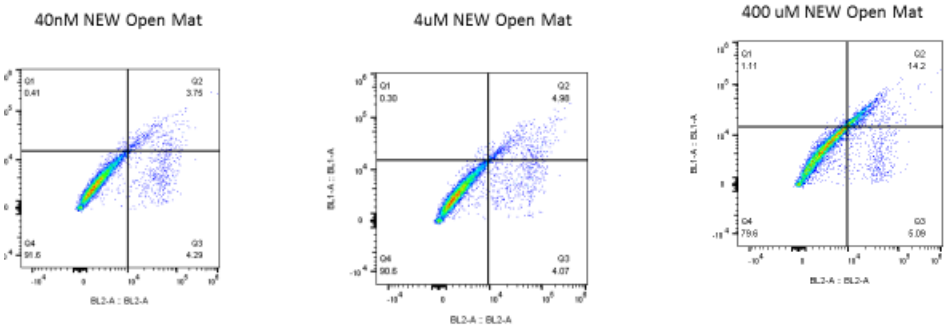

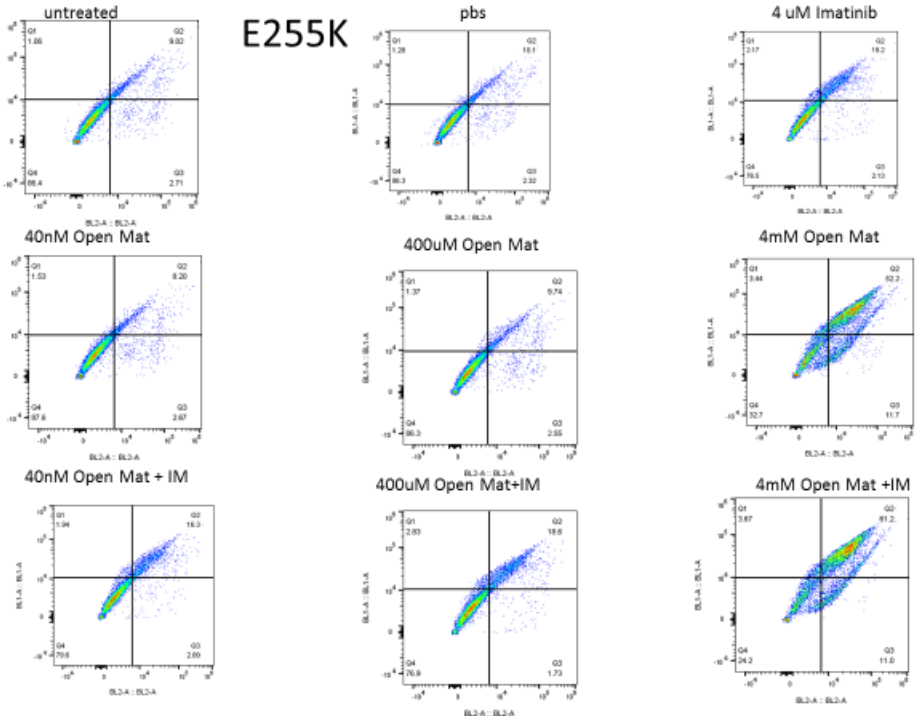

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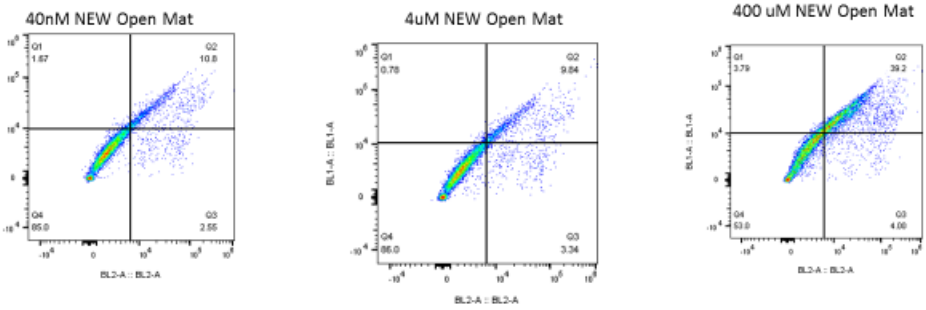
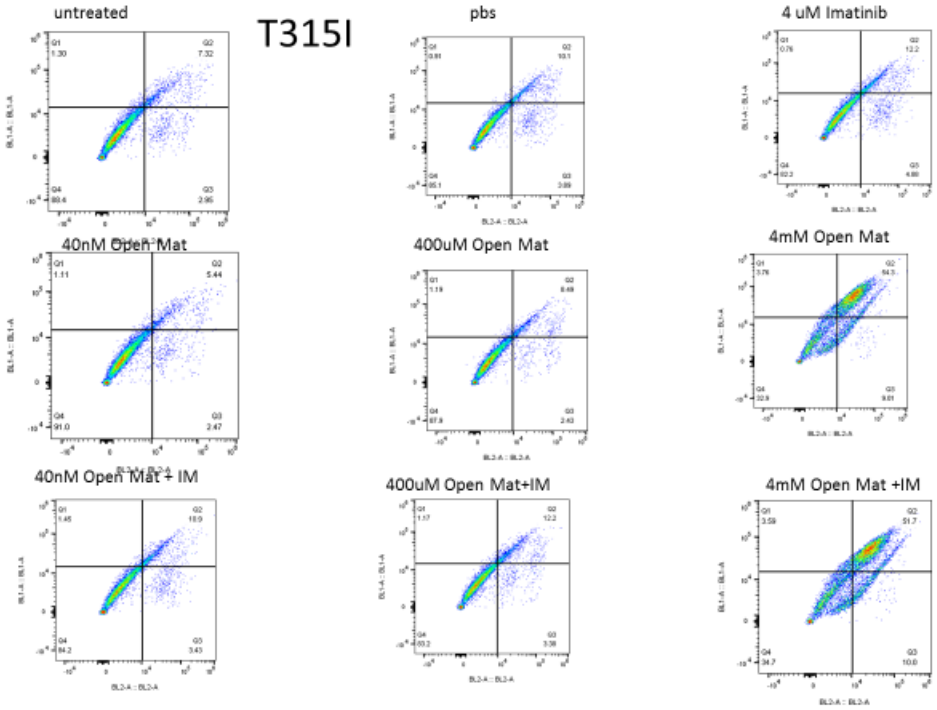
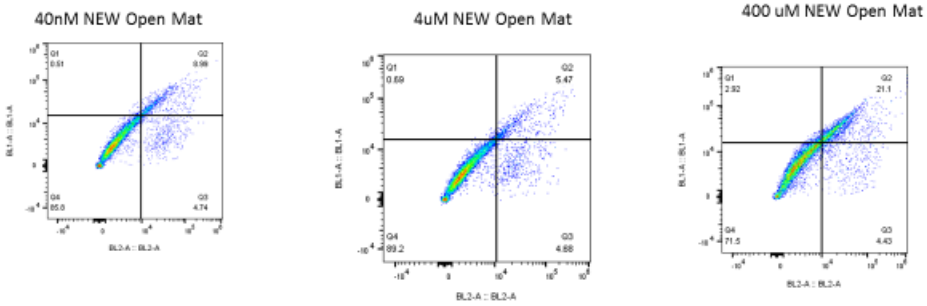




T315I



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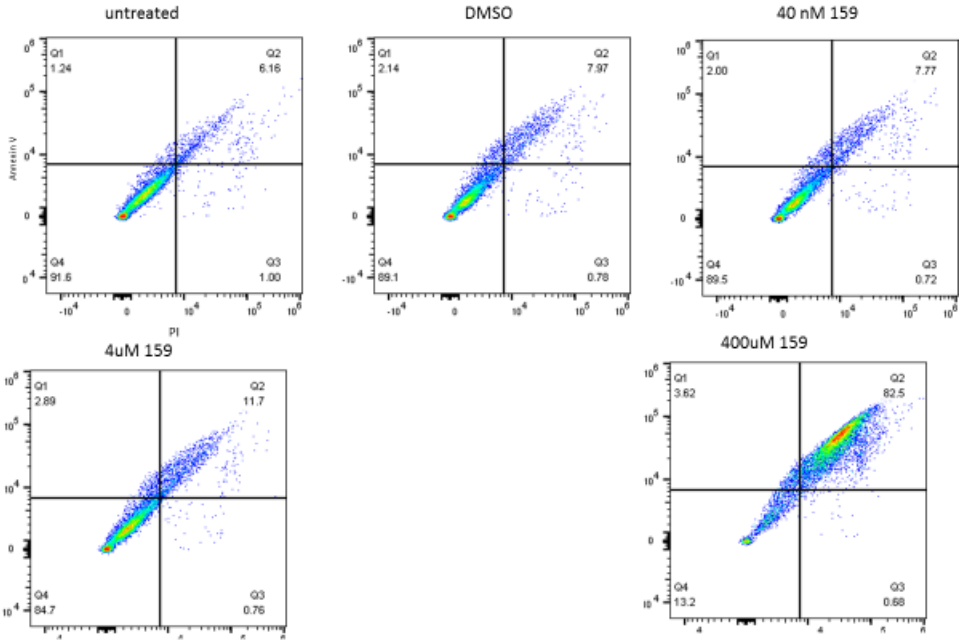

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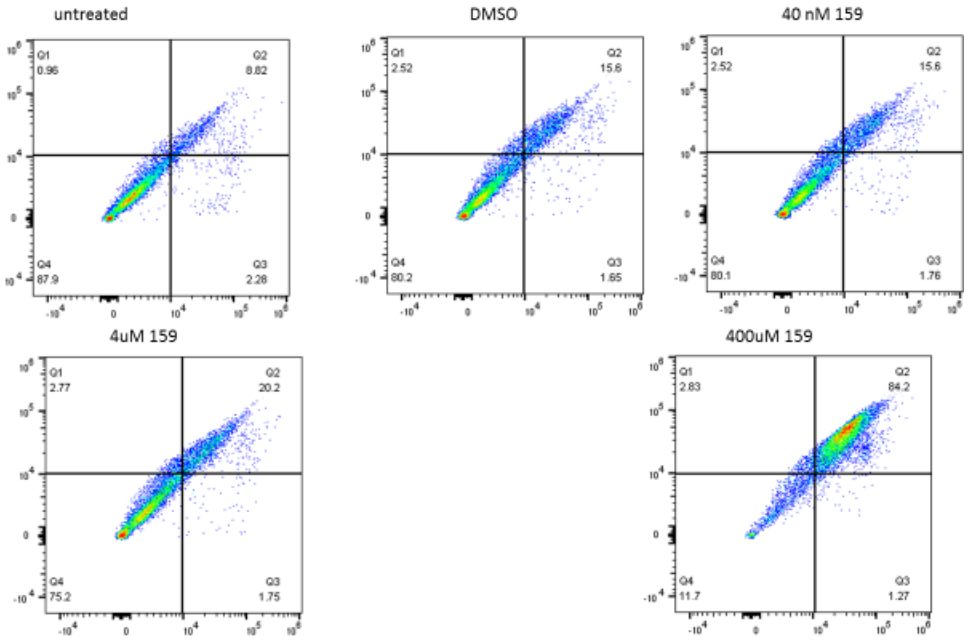
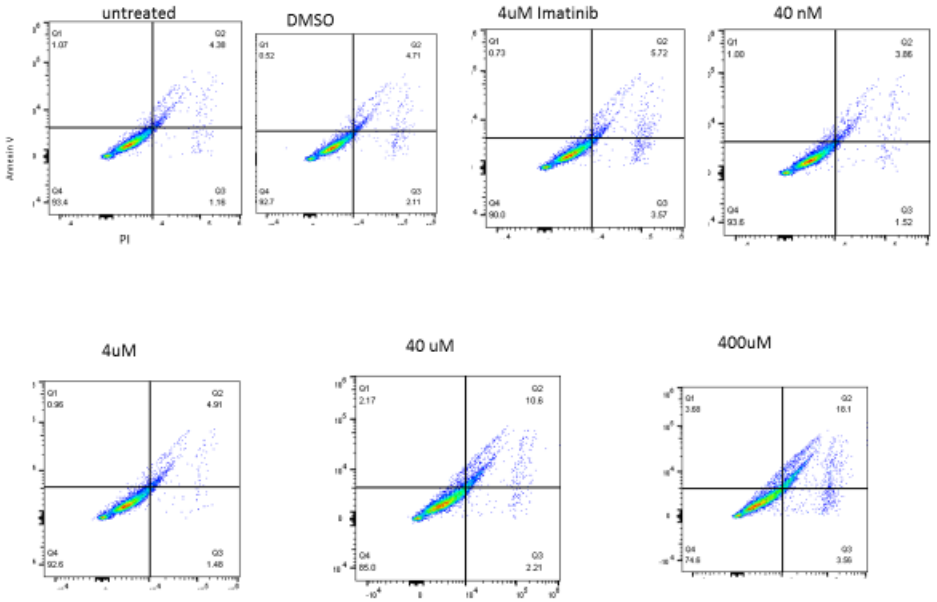
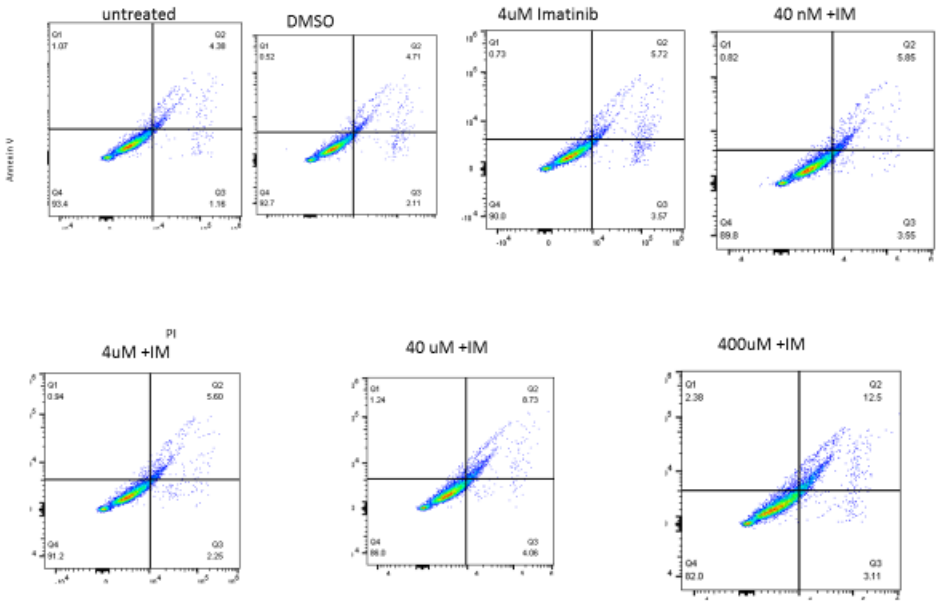


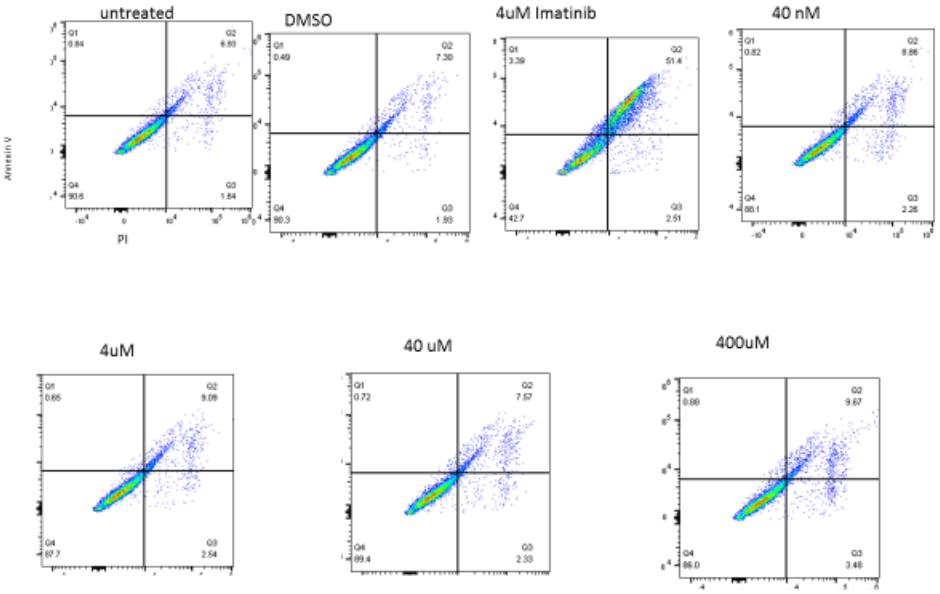
NIH3t3 Open Mat 1



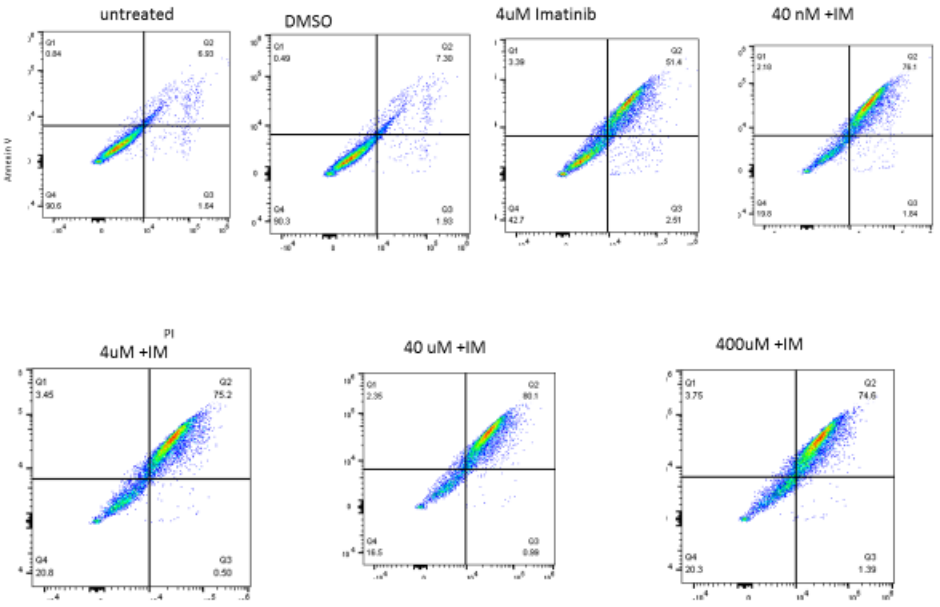
NIH3t3 Open Mat 1



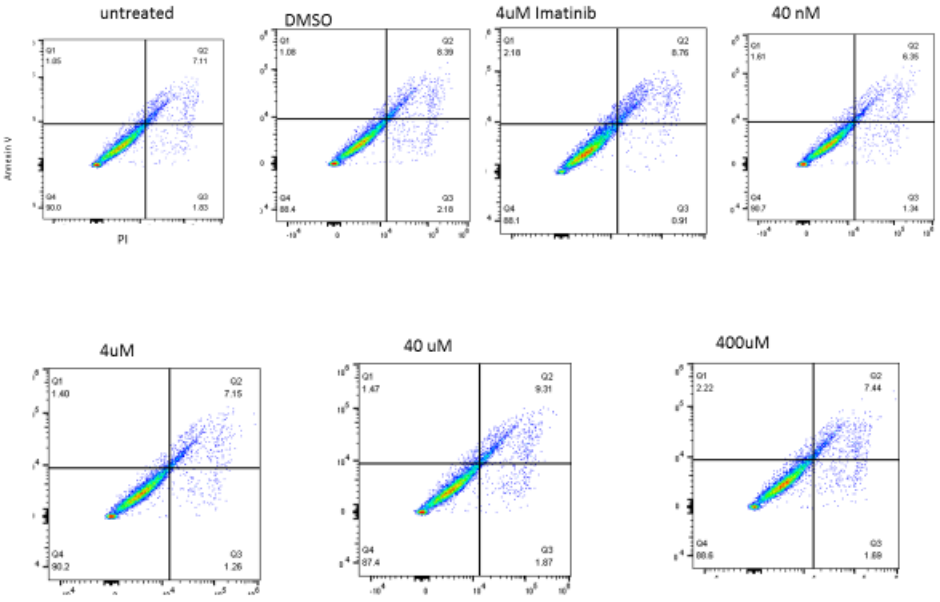
p210 Open Mat 1



p210 Open Mat 1



E255K Open Mat 1



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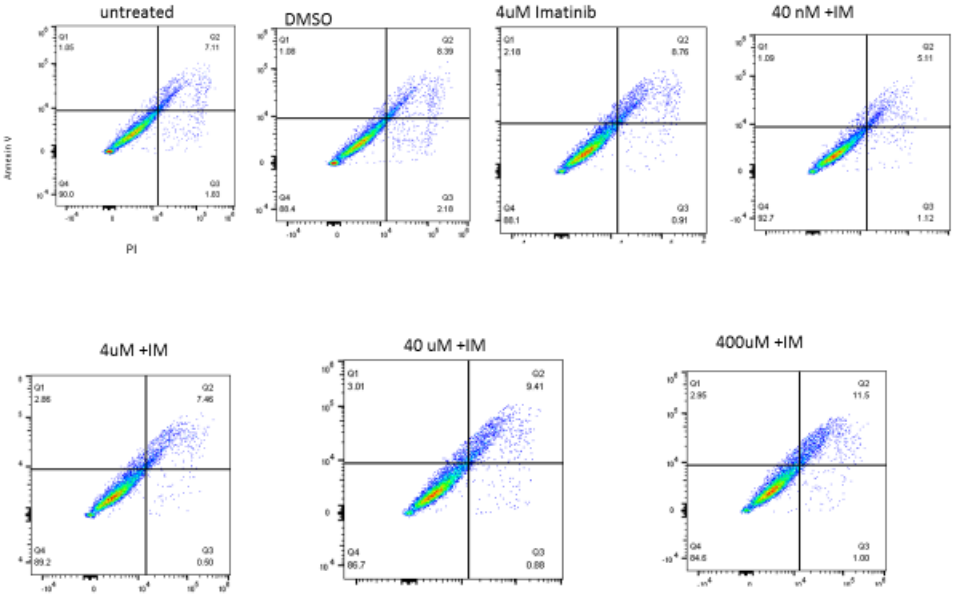
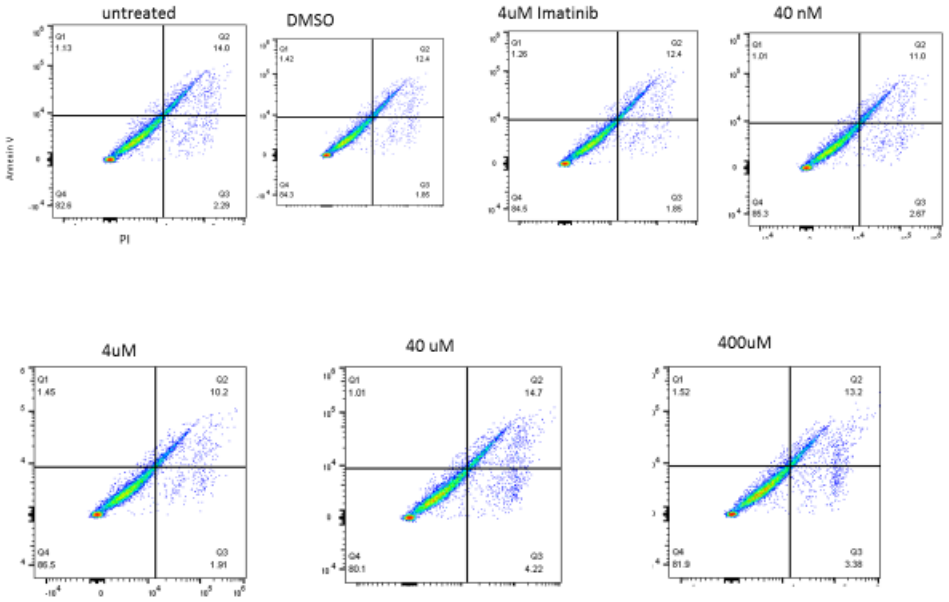
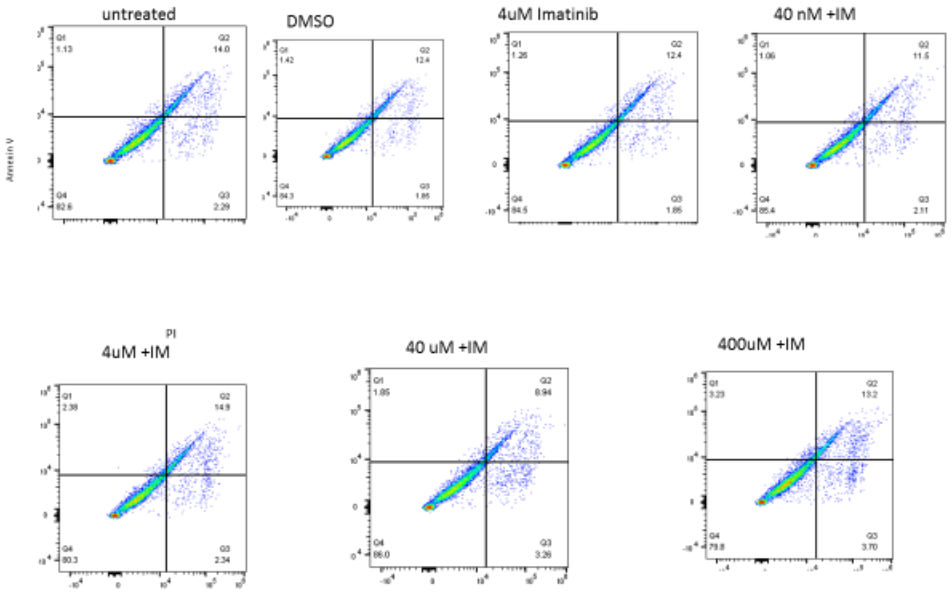


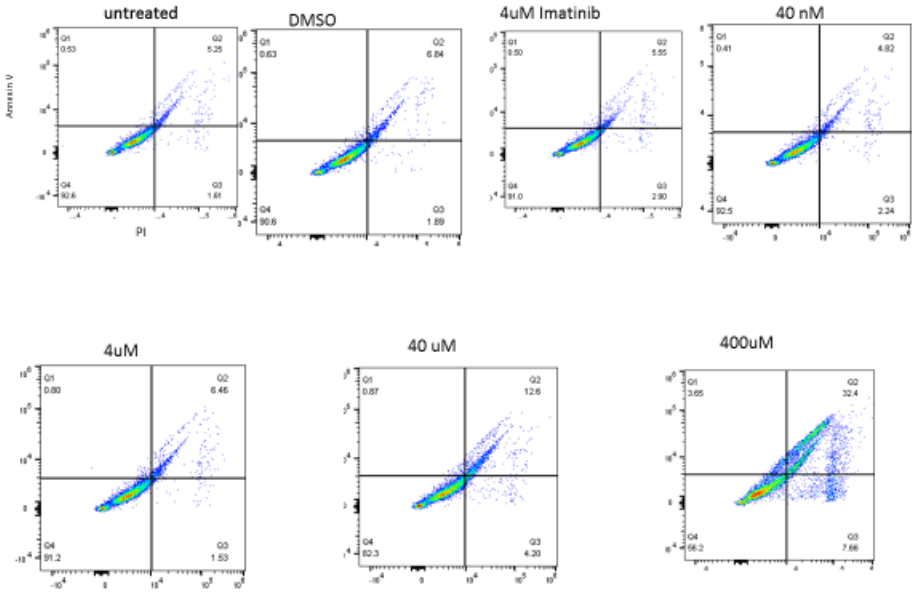
T315I Open Mat 1



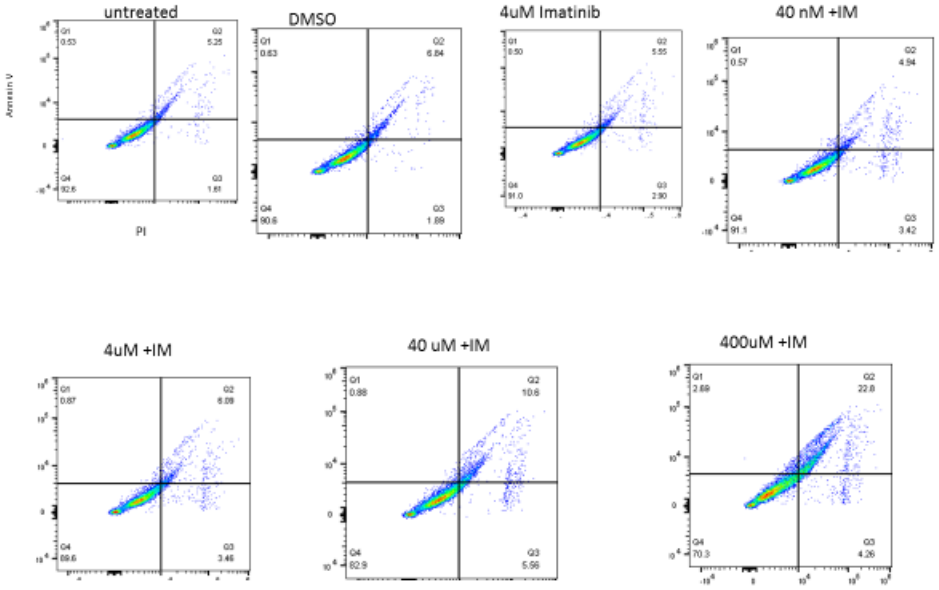
T315I Open Mat 1



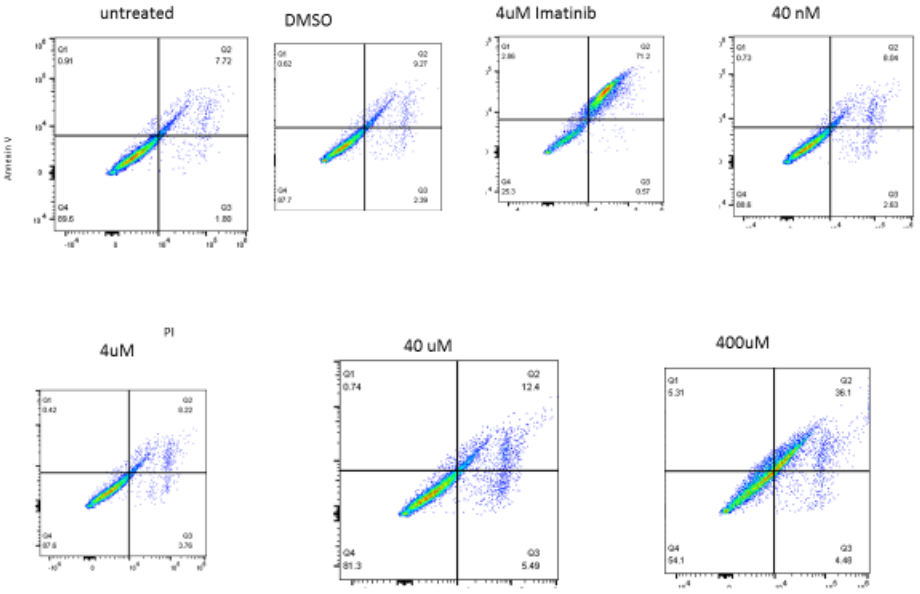
NIH 3T3 Open Mat 2



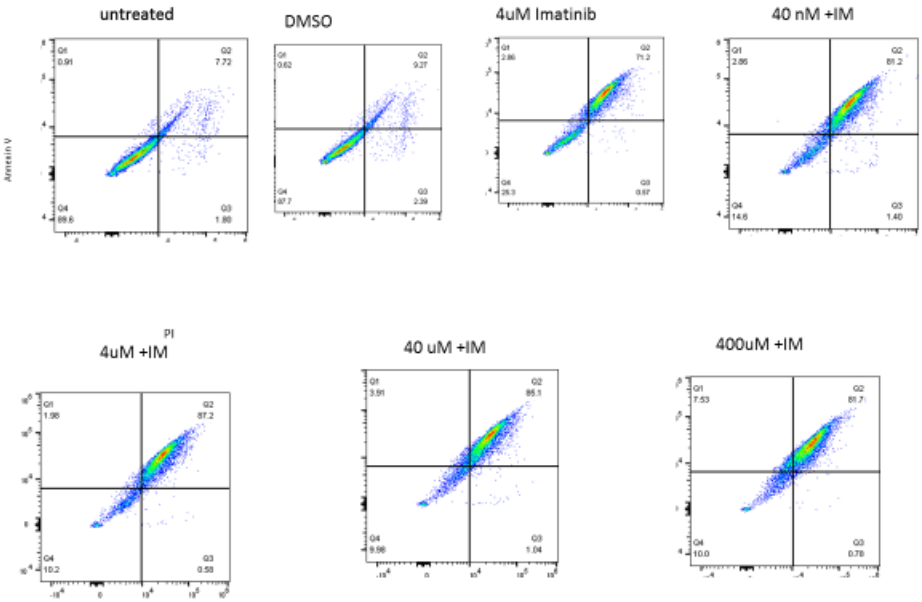
NIH 3T3 Open Mat 2



p210 Open Mat 2



p210 Open Mat 2



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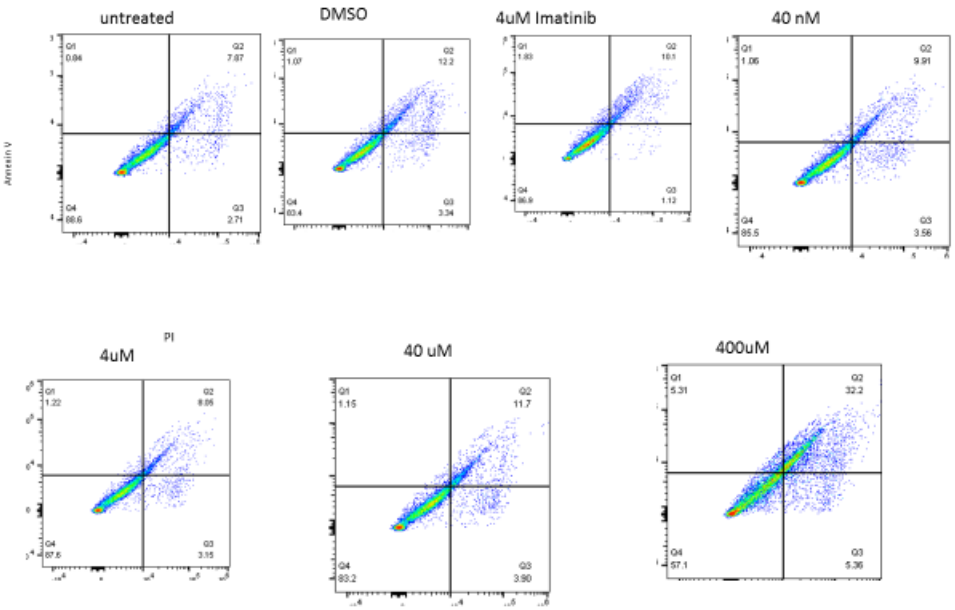

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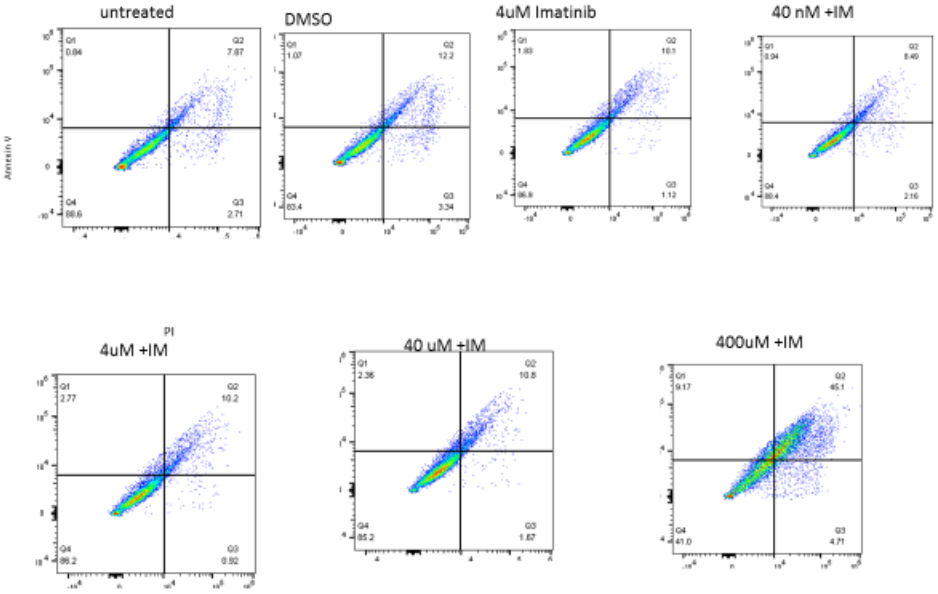
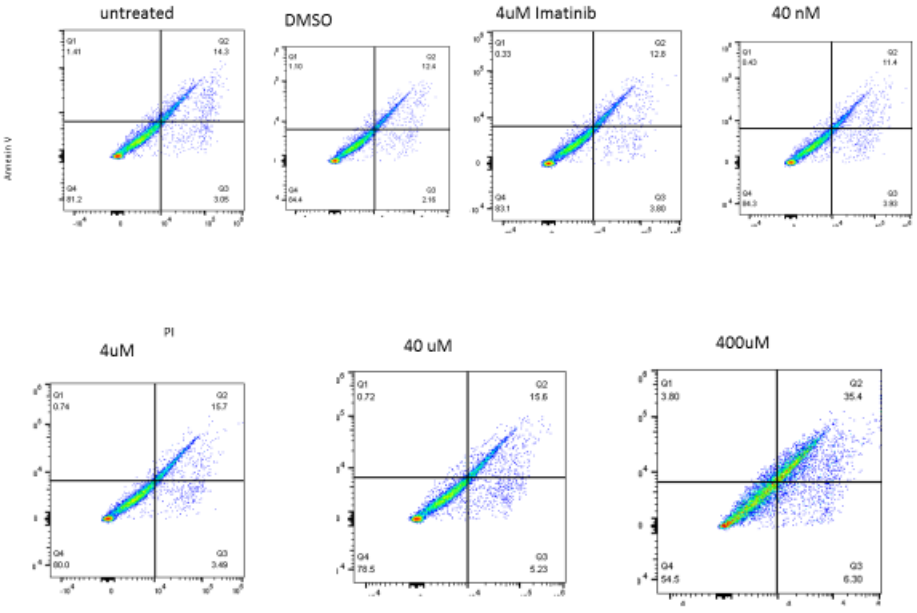
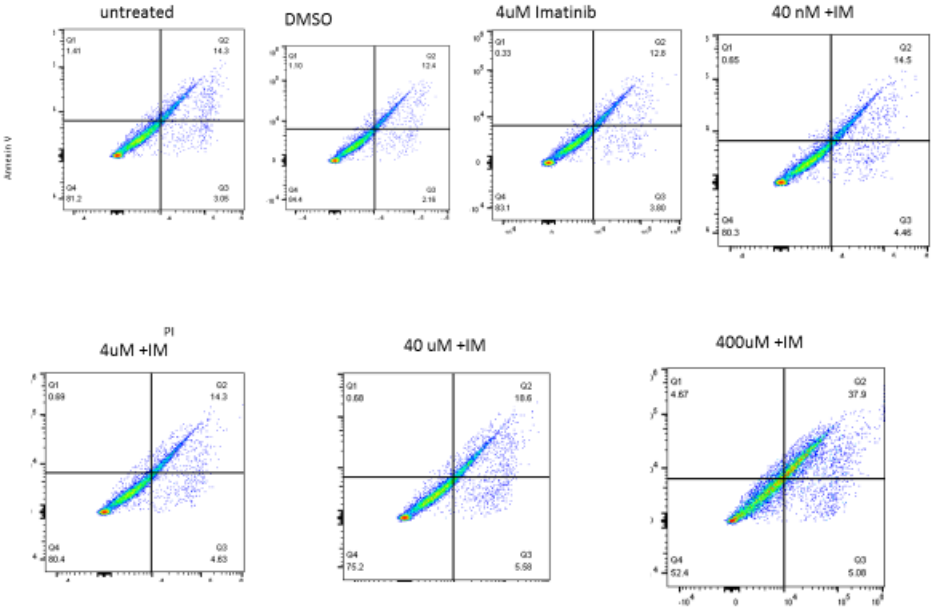


T315I Open Mat 2



T315I Open Mat 2



NIH 3T3

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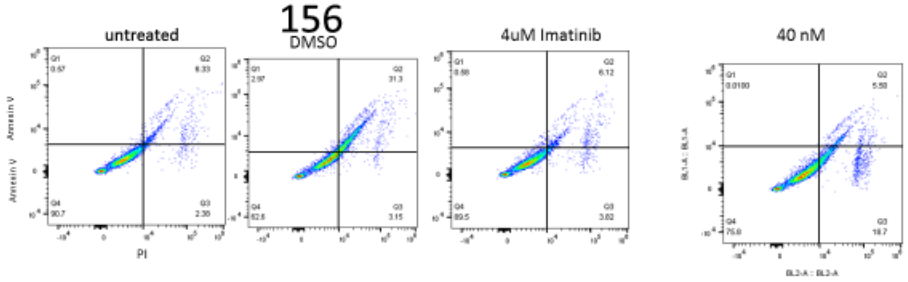

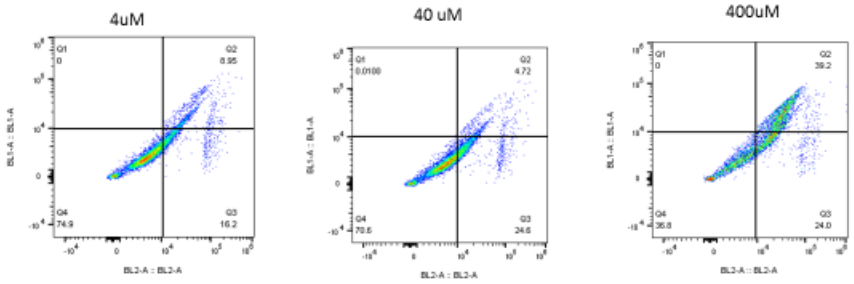

P210

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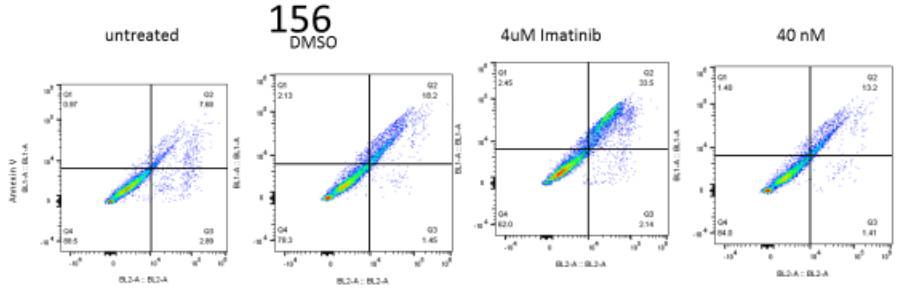

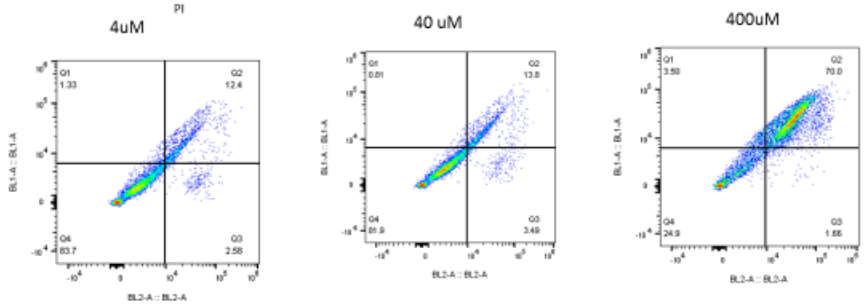

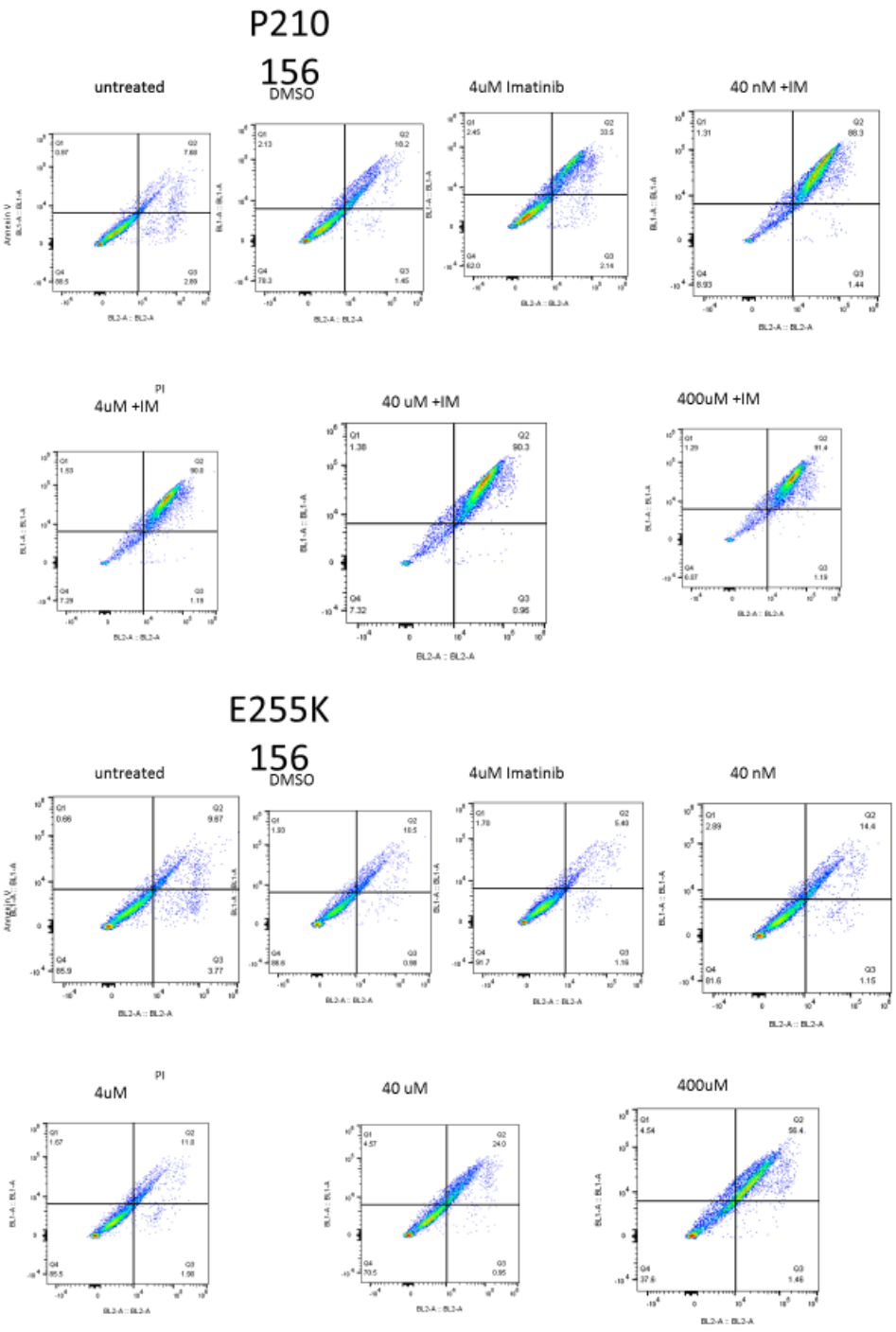

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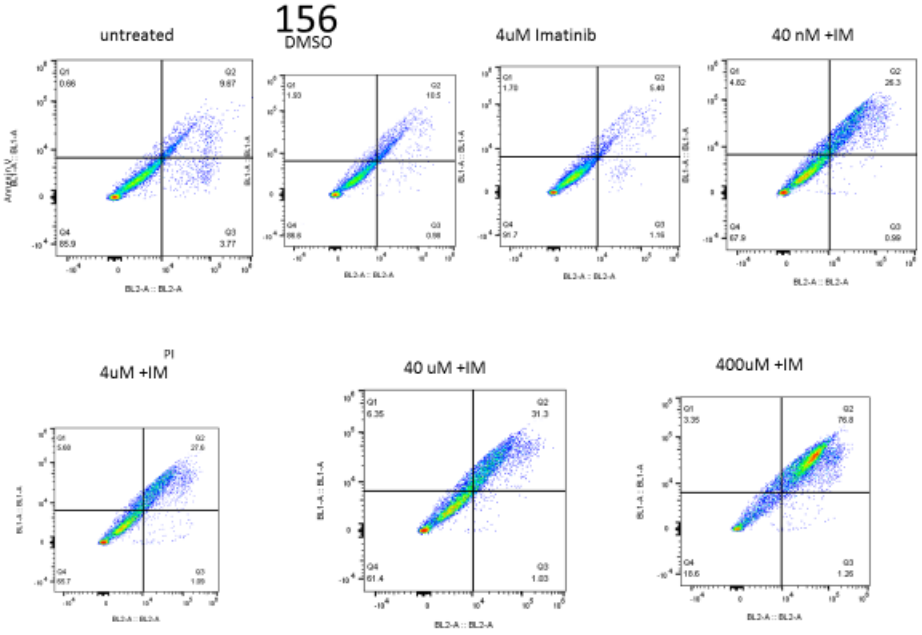

E255K

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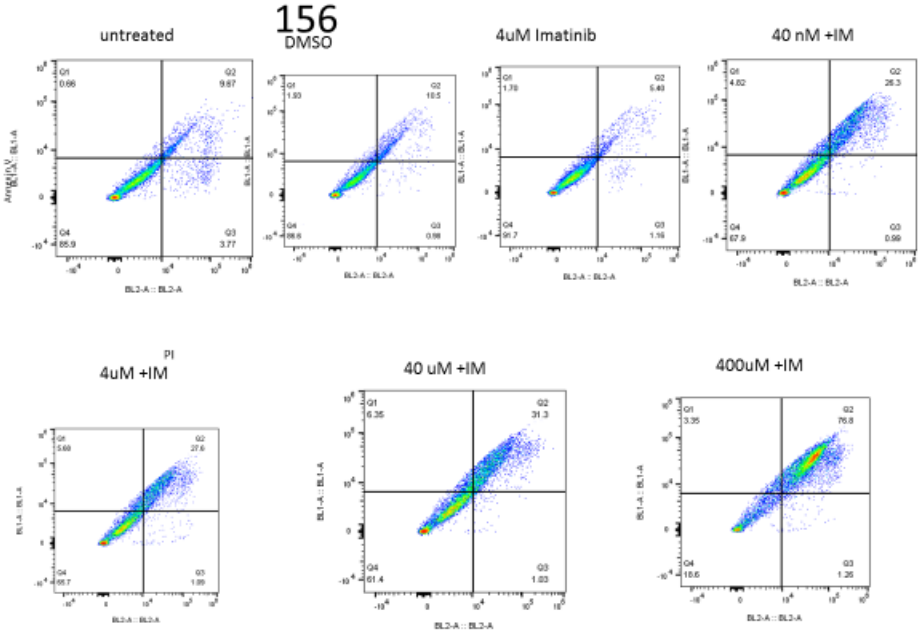

T315I

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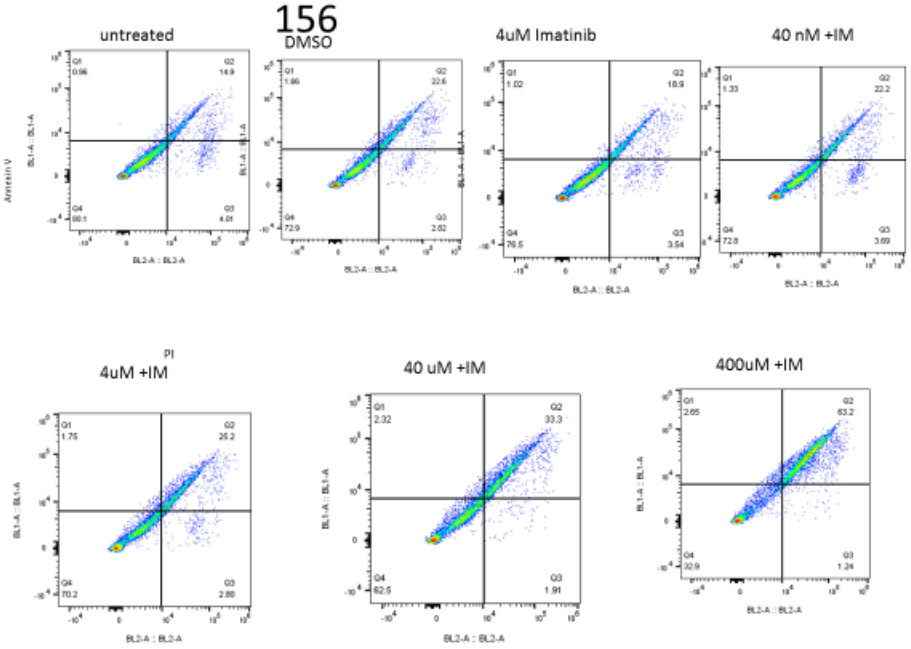

NIH 3T3

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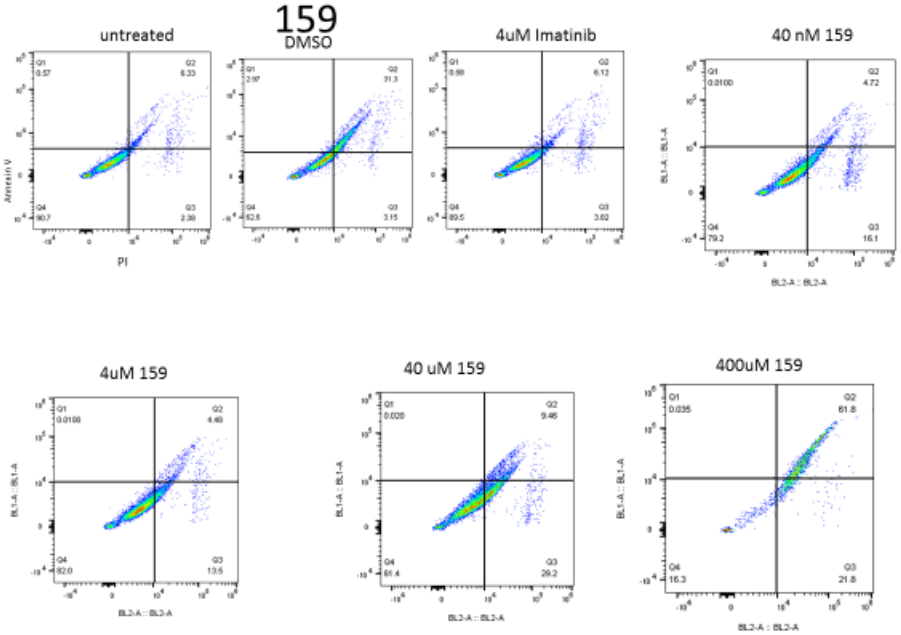

NIH 3T3

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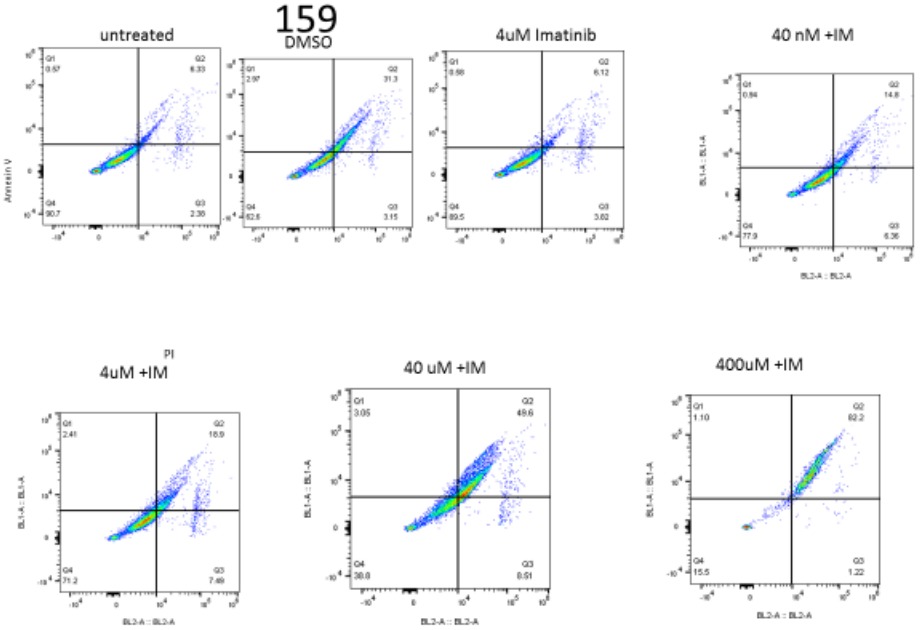

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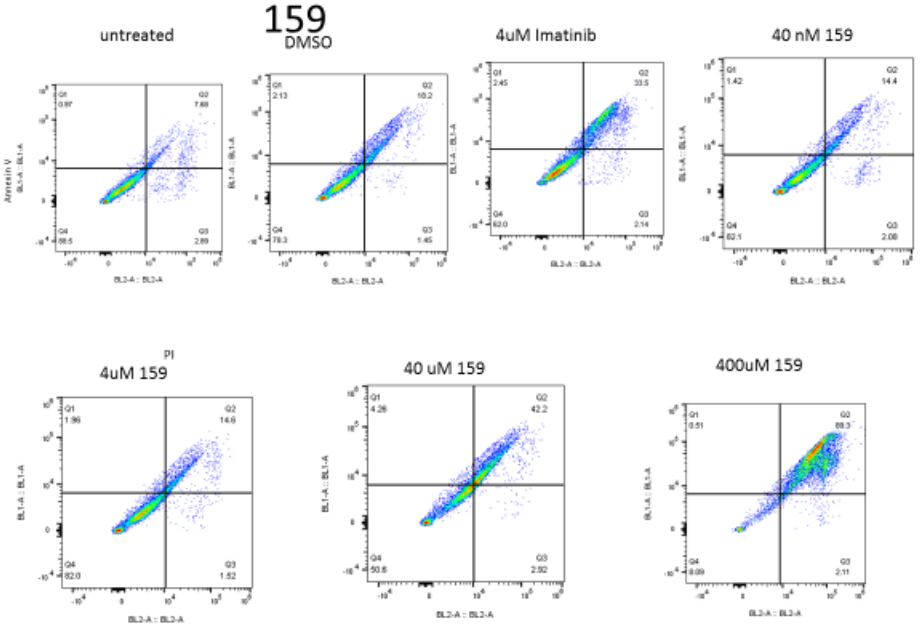

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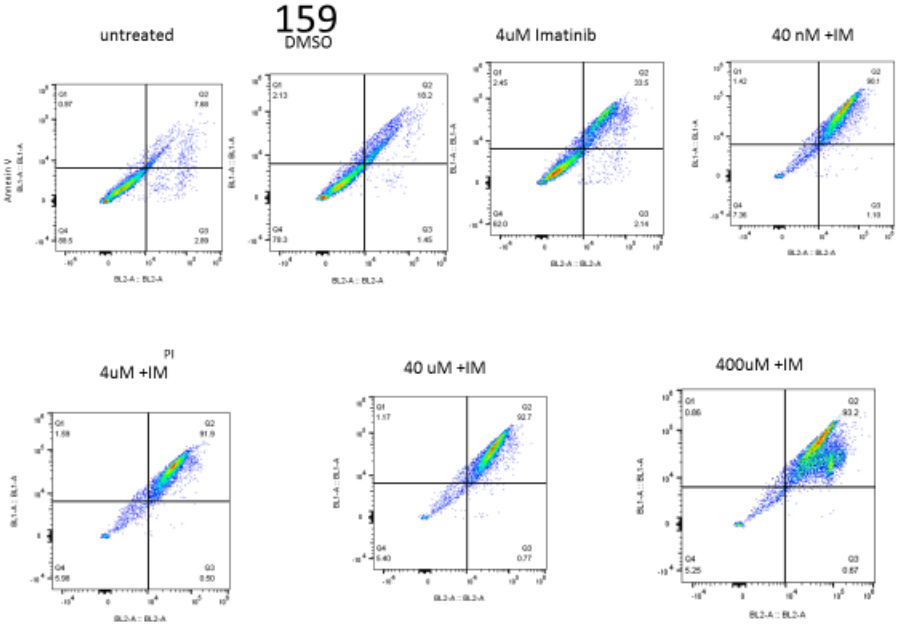

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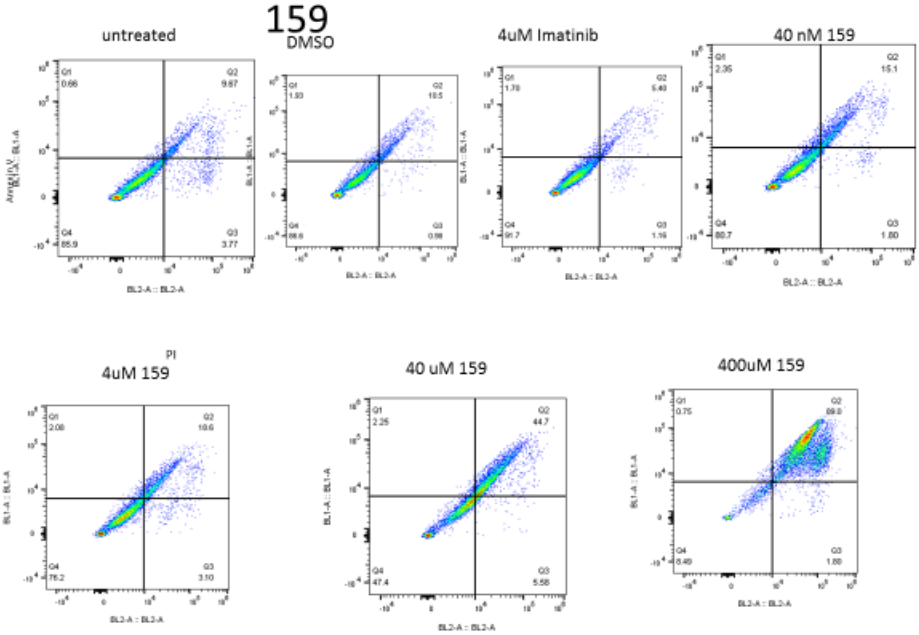

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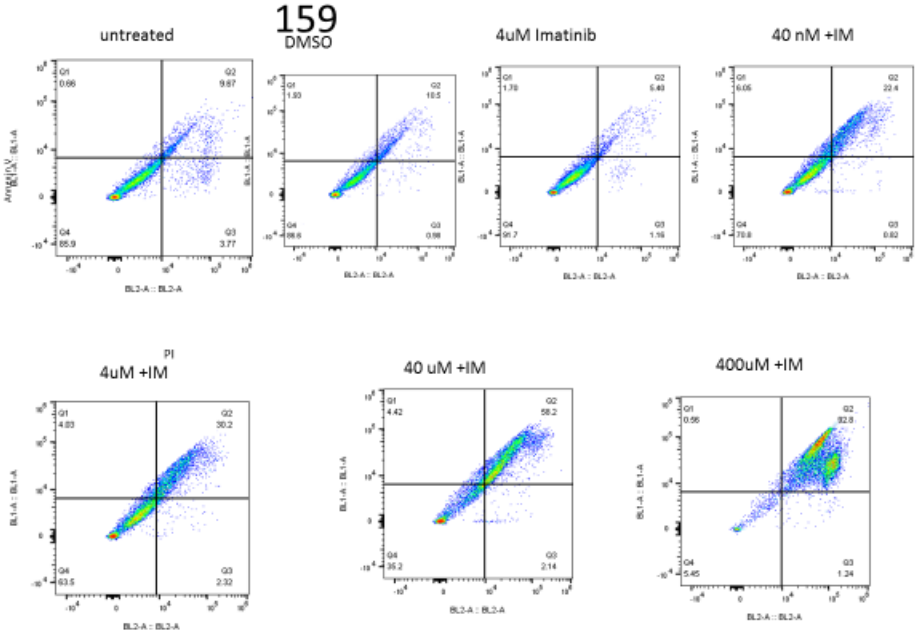

T315i

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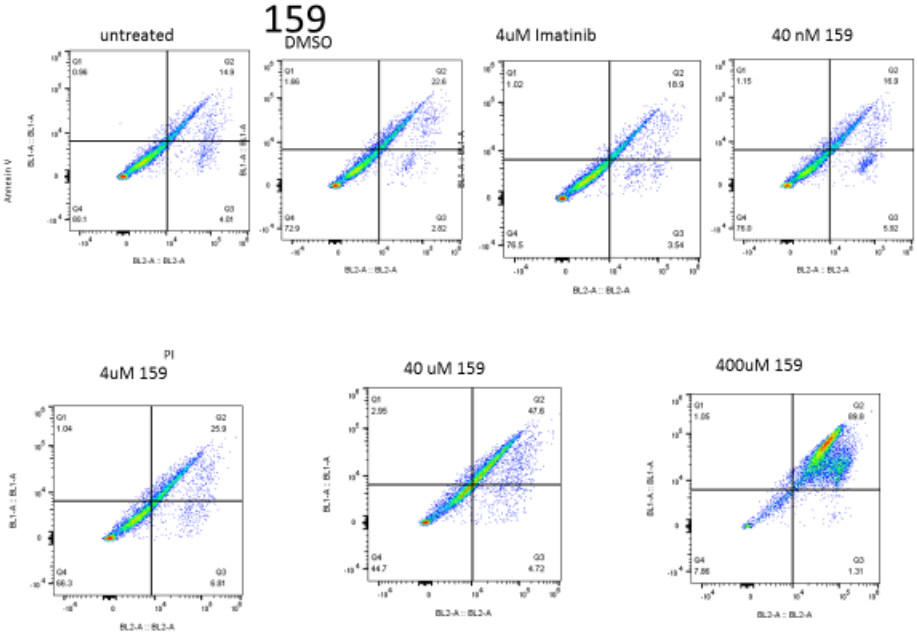

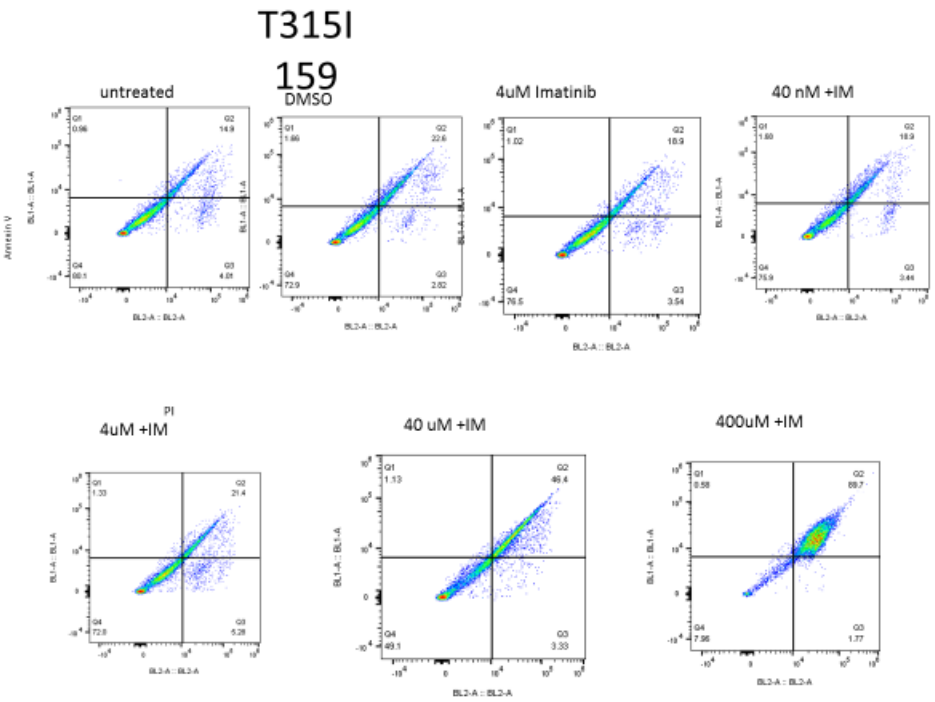

NIH 3T3

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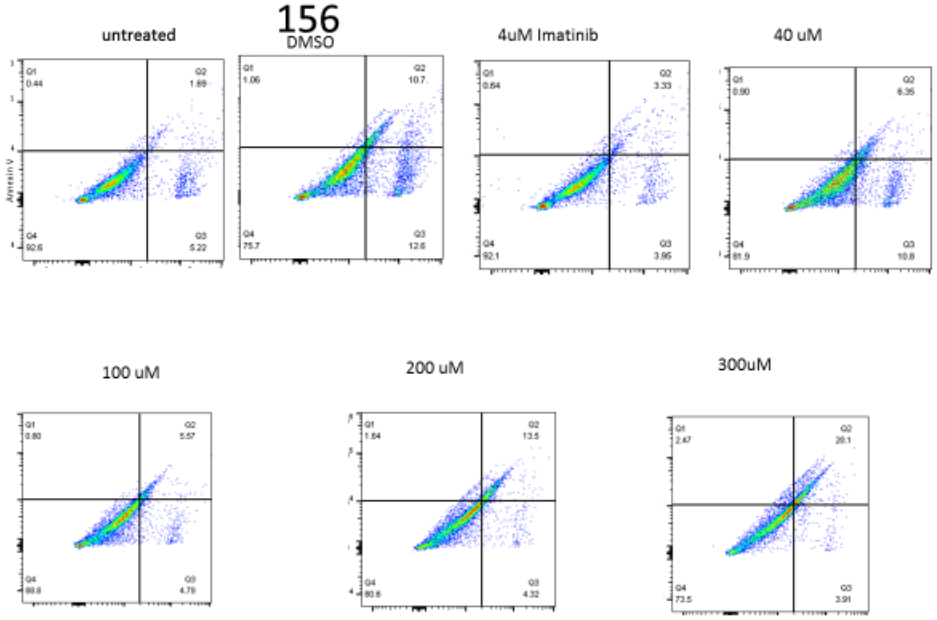

NIH 3T3

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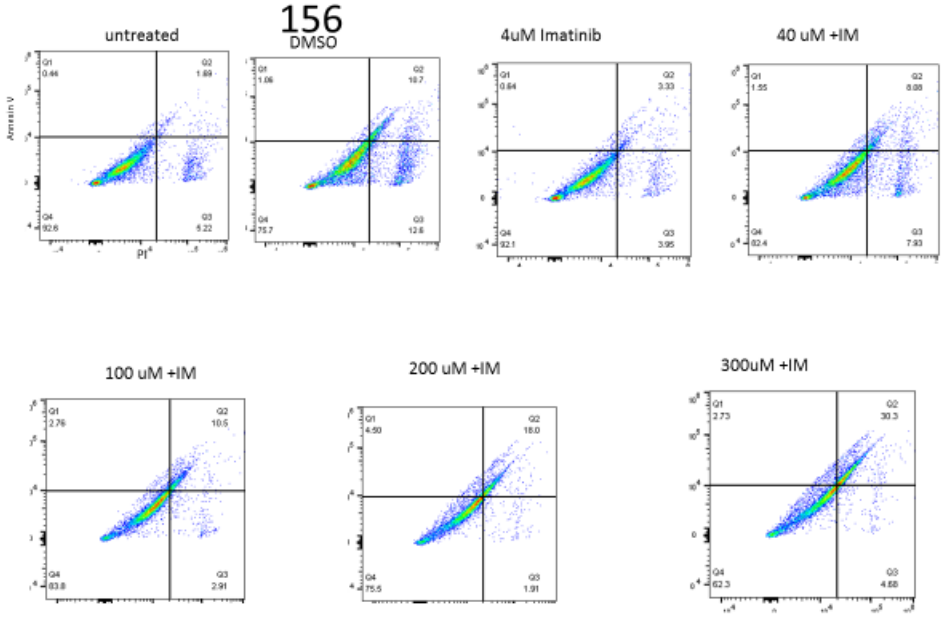

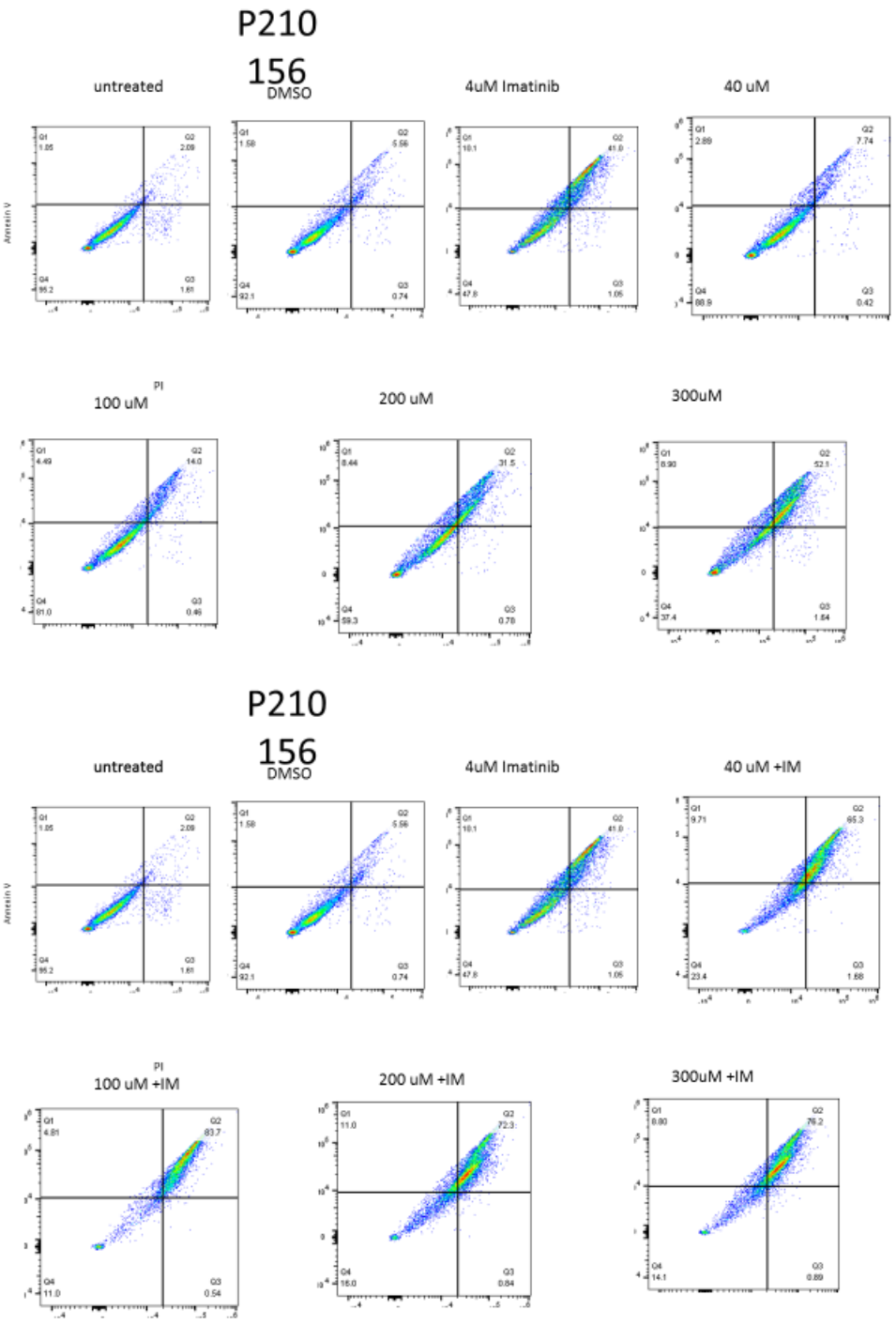

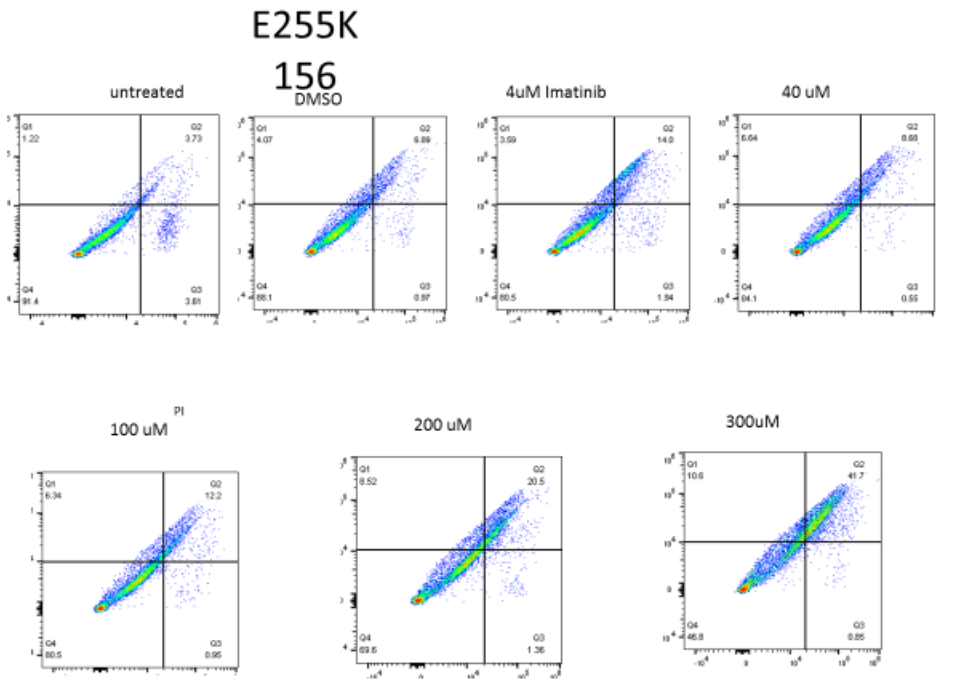

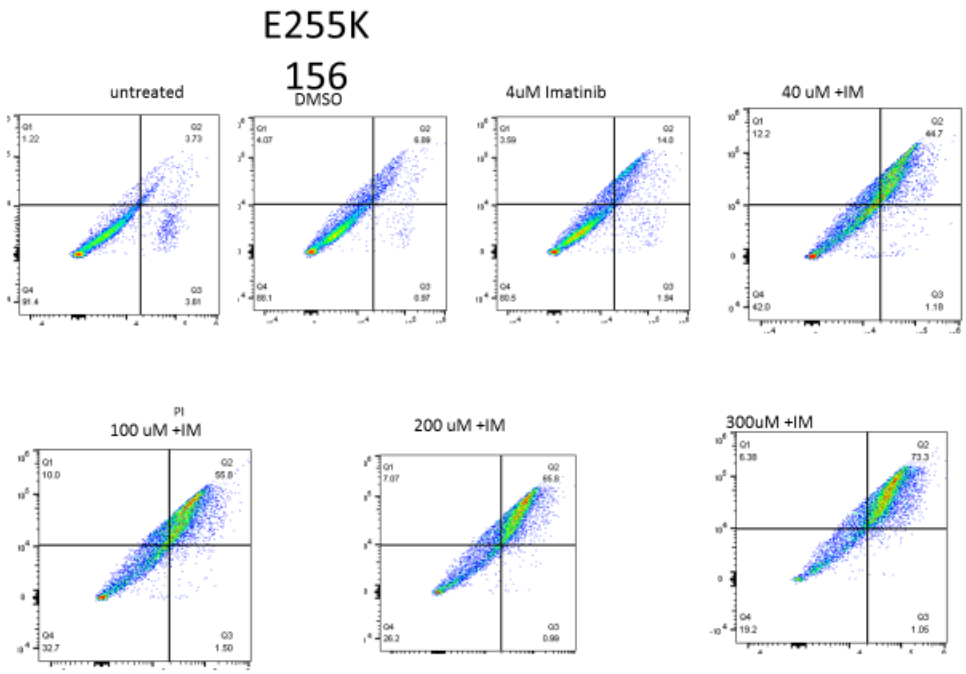

T315I

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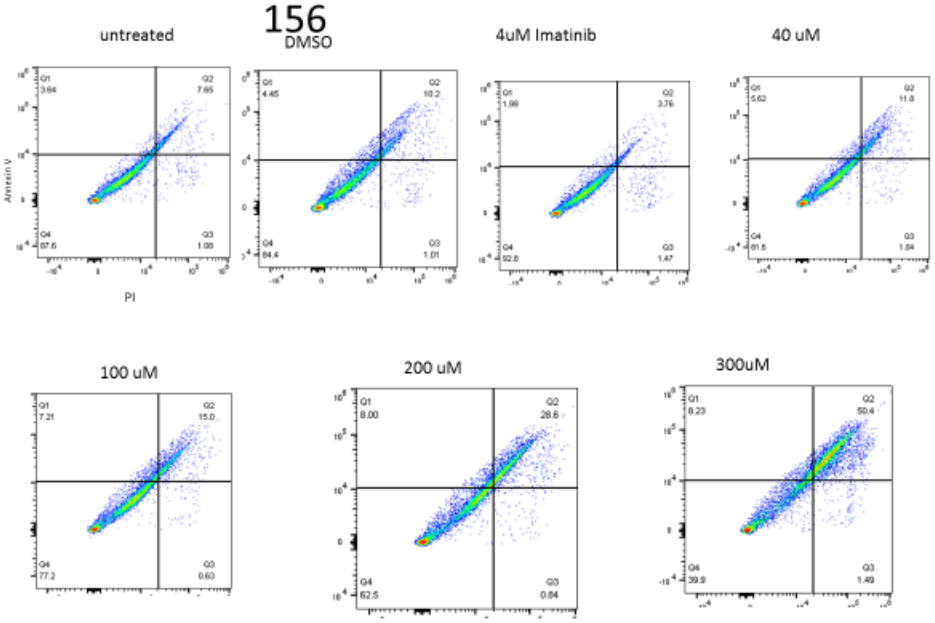

T315I

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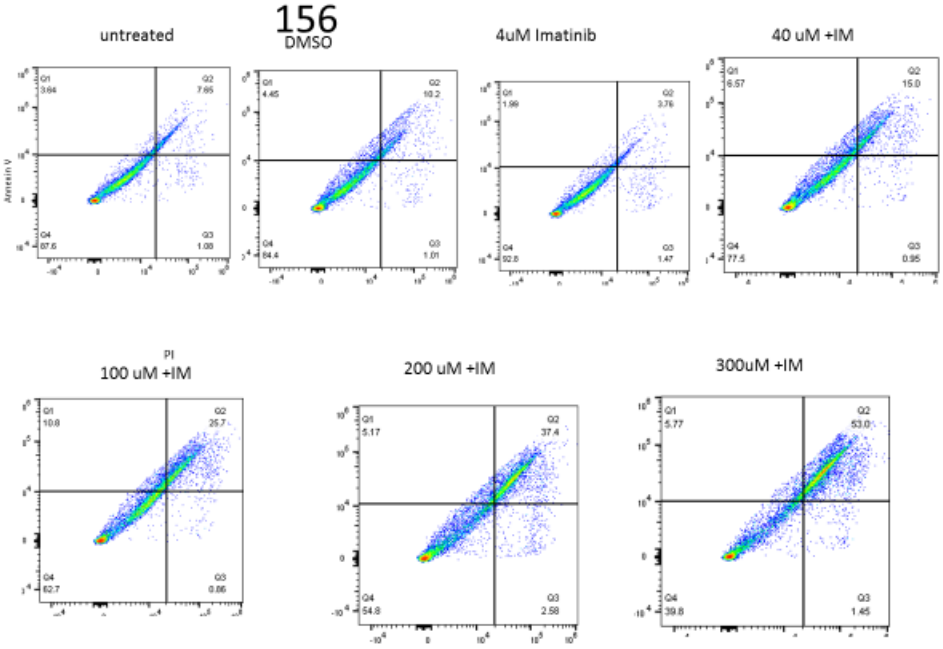

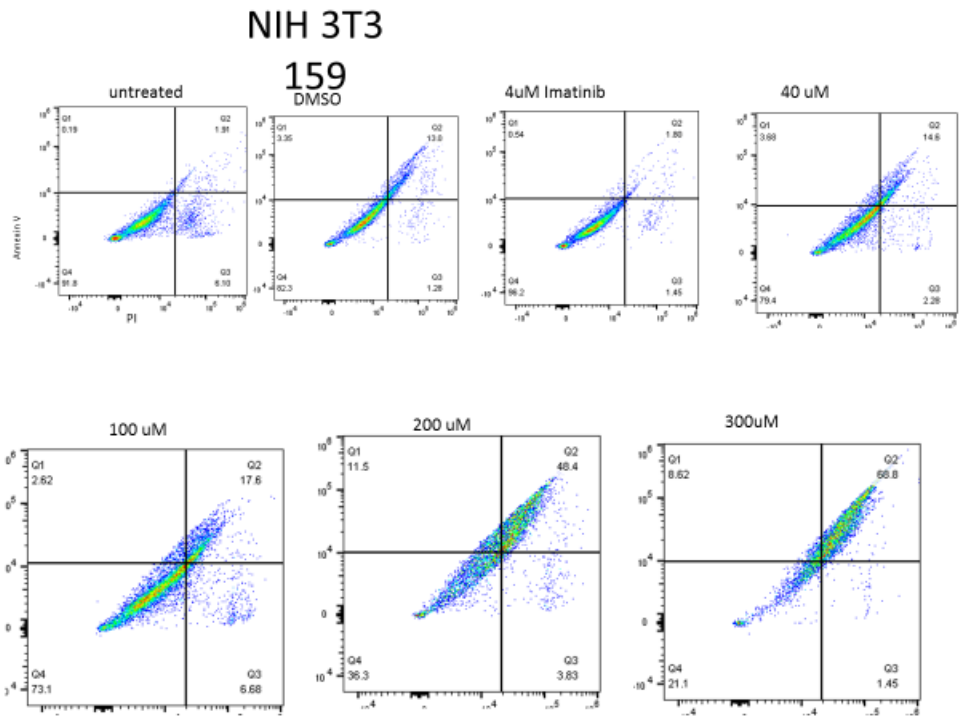

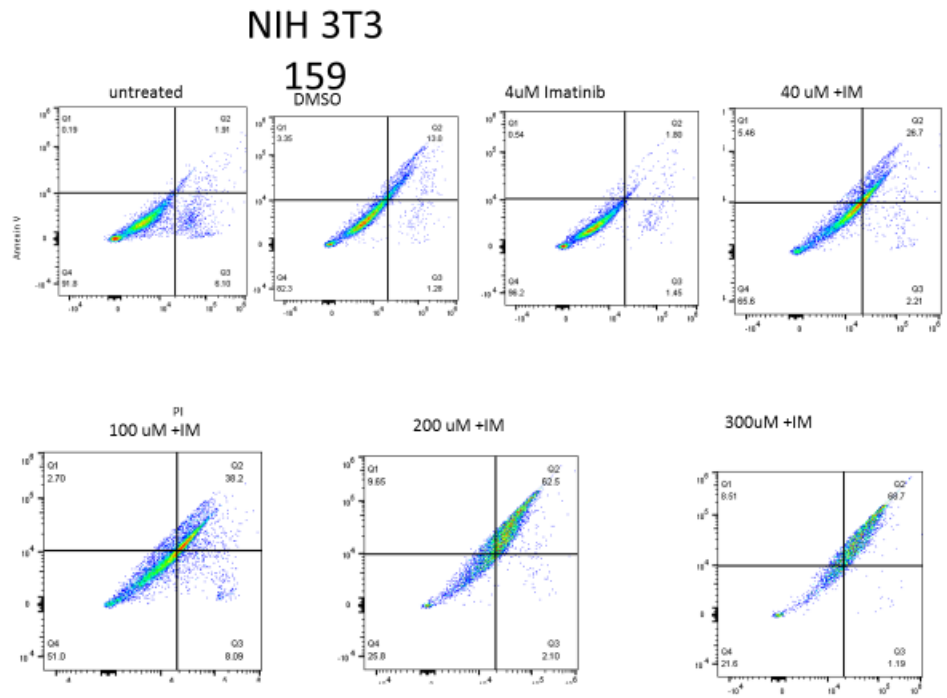

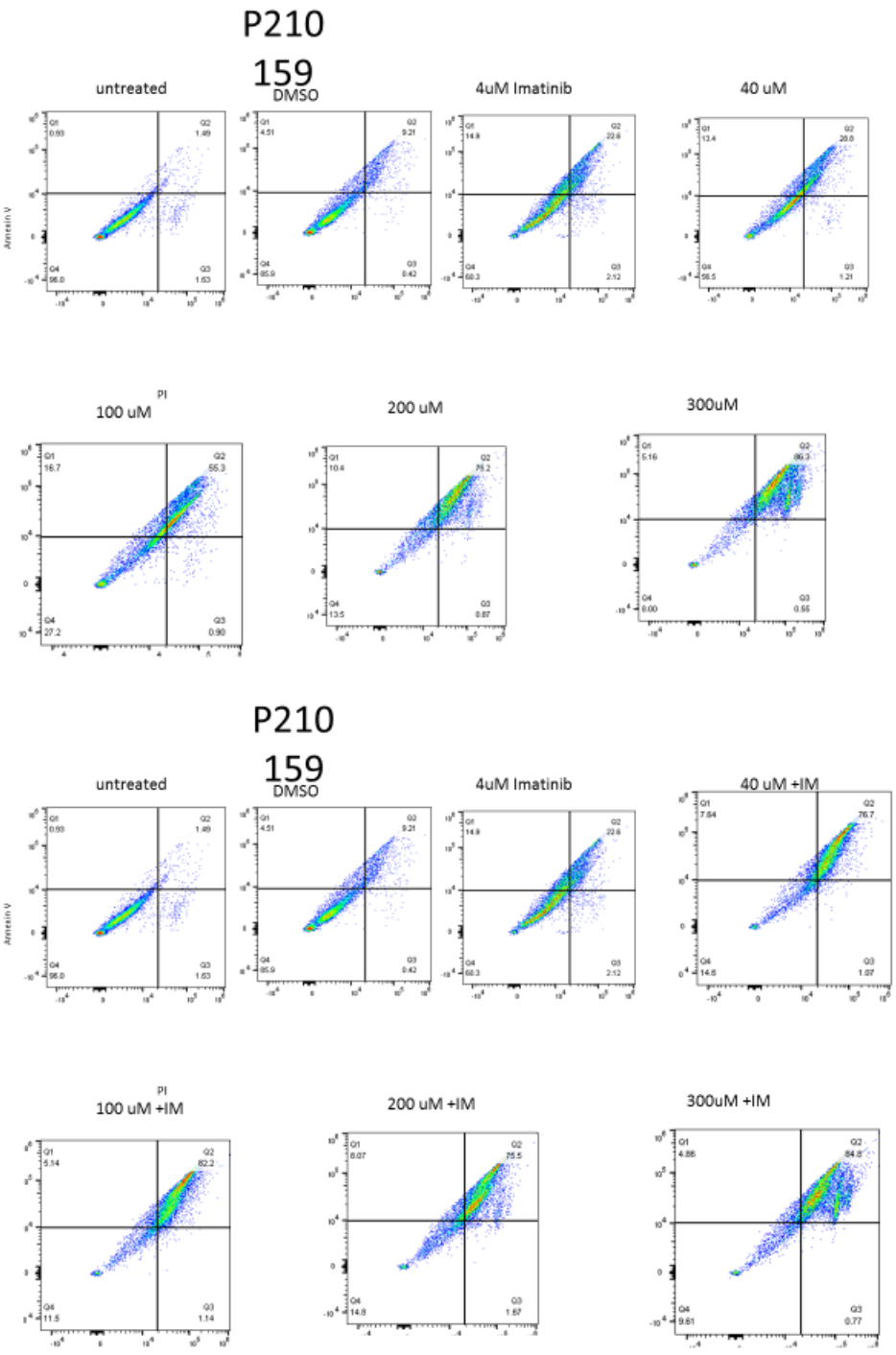

E255K

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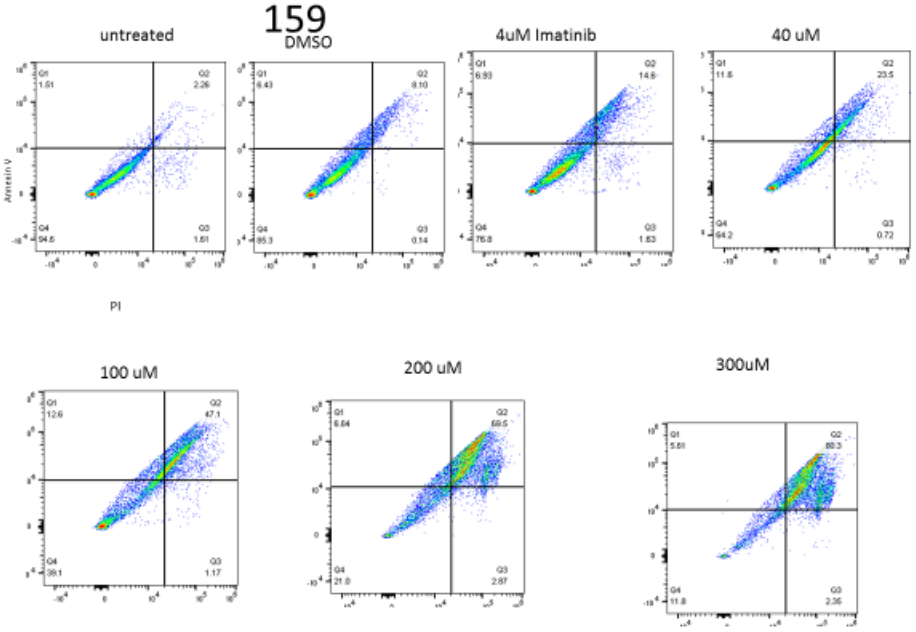

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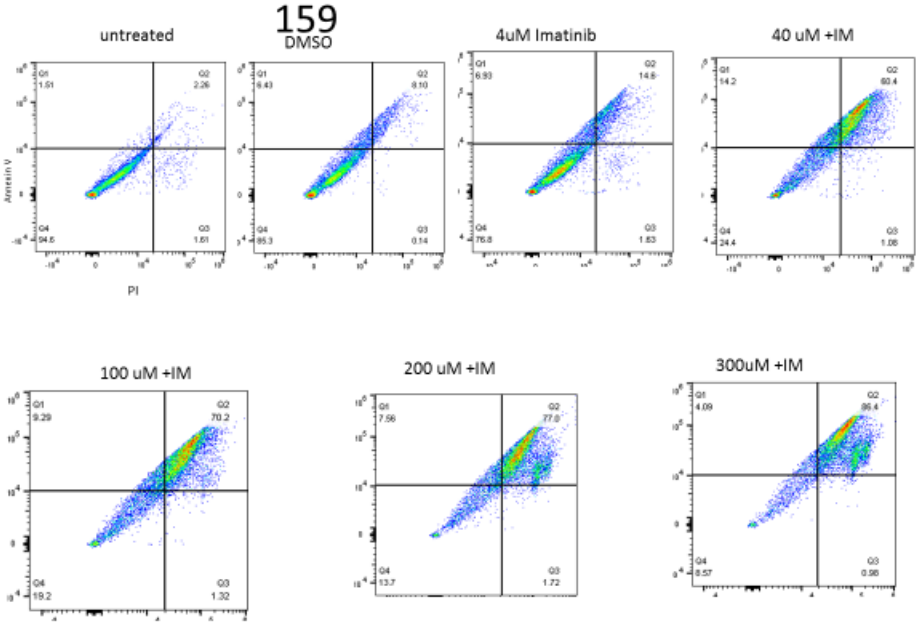

T315i

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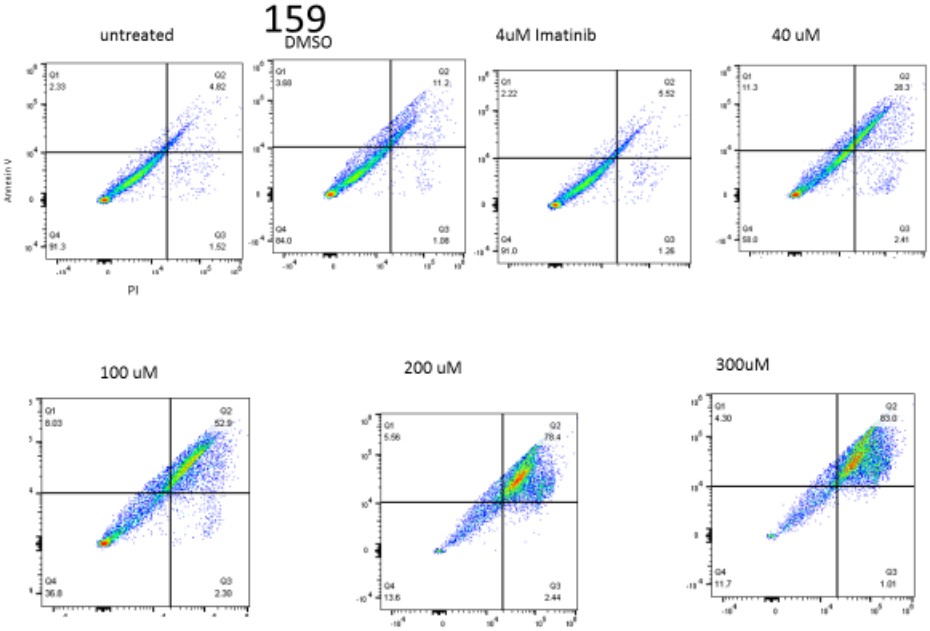

T315I

159