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Bruton's Tyrosine Kinase is a Potential Therapeutic Target in Non-Small Cell Lung Cancer Cells

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An honors thesis presented to the Department of Biological Sciences, State University of New York at Albany in partial fulfillment for graduation with honors in biology and graduation from the Honors College

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Abstract

Acknowledgements

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Introduction

Lung and bronchus cancer are the leading causes of cancer-related deaths in the United States, representing 13.2% of all new cancer cases in the U.S. In 2010-2014, new cases of lung cancer was 55.8 per 100,000 men and women per year and the number of deaths was 44.7 per 100,000 men per year (SEER 2016). Based on data collected between 2007-2013 by the SEER program of the NIH Nation Cancer Institute, only 18.1% of people diagnosed with lung and bronchus cancer survive 5 or more years and in 2017, it is estimated that there will be 222,500 new cases of lung and bronchus cancer and 155,870 people will die from the diseases (SEER 2016).

The problem with common cancer treatments, surgery, chemotherapy, and radiation therapy, is that they are too invasive and cause damage to neighboring non-cancerous cells. Many new cancer treatments use targeted therapy for treating diverse types of cancer by taking advantage of specific proteins found in cancer cells but not in healthy tissues and block cancer cell survival signals.

Bruton's tyrosine kinase (BTK) is a Tec family kinase whose native form, BTK-A, is commonly found and directly involved in B lymphocyte development, signaling, and survival (Wang 2016). Mutation of the BTK coding gene, *Btk*, can cause X-linked agammaglobulinemia, a genetic disorder that results in low levels of mature B cells, resulting in severely compromised immune functionality in patients lacking a functional *Btk* gene (Honihberg 2010).

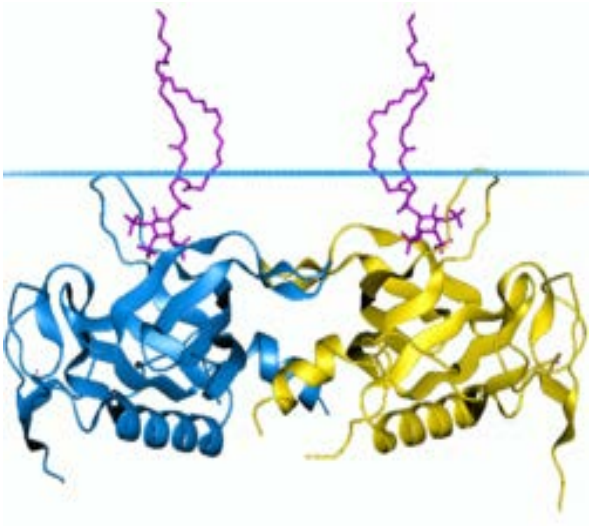


Figure 1. The structure of Bruton's Tyrosine Kinase

BTK-A is the native form of the tyrosine kinase found in B lymphocytes. BTK-C is an alternate isoform of BTK transcribed from an alternate promoter, resulting in an amino-terminus extension in the protein. This isoform of BTK has been found to be expressed at low levels in breast and prostate cancer cells as a survival factor (Eifert 2013; Kokabee 2015).

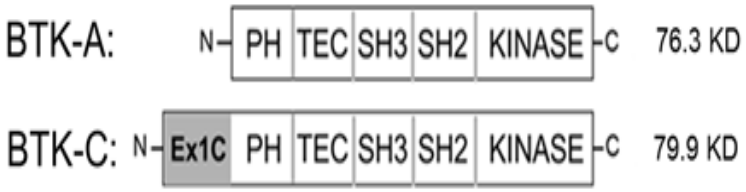


Figure 2. The BTK-C isoform contains an N-terminus extension.

In B-lymphocytes, BTK is a cytosolic kinase typically found in its inactive, unphosphorylated form, but upon activation, it affects other downstream proteins such as EGFR and AKT growth factors and innate immune response receptors. (Akinleye, Chen, Mukhi, Song, & Liu, 2013). This initiates a variety of cellular processes such as survival and proliferation, motility, cytokine production, and antigen presentation. Known BTK inhibitors, ibrutinib and AVL-292, have been shown to block the phosphorylation of BTK at the Tyr-223 residue and inhibit the downstream effects of the growth factors, reducing cell proliferation. Due to its safeness and bioavailability, Ibrutinib is currently being used in Phase III clinical trials for treating patients with B-cell neoplasms

(Akinleye, Chen, Mukhi, Song, & Liu, 2013) Dosages of 420 and 840 mg/day of ibrutinib are well tolerated.

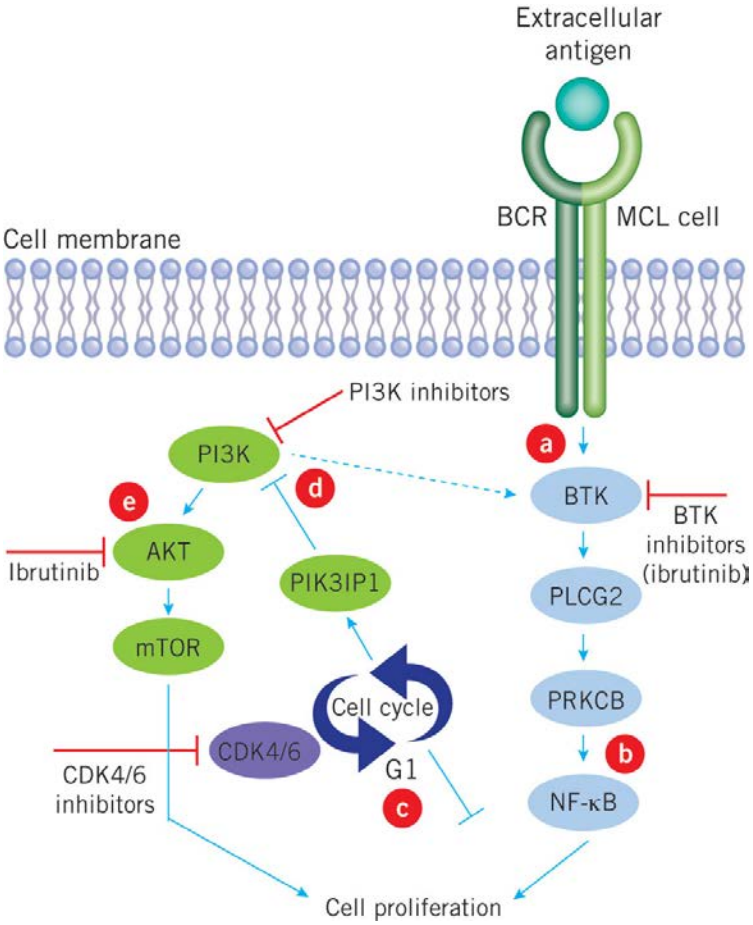
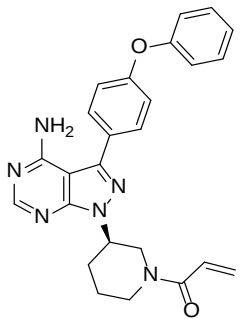


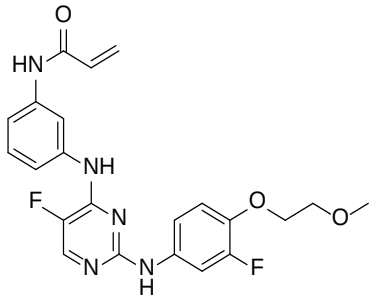
Figure 3. Inhibition of BTK affects downstream proteins that mediate cell survival.

Evidence shows BTK to be a target therapeutic in treating patients with non-Hodgkin B cell lymphoma (NHL), rheumatoid arthritis (RA), and breast cancer (Herman 2011). In HER2-positive breast cancer cells have been shown to be inhibited by not only its known inhibitor, lapatinib, but also by ibrutinib as well due to BTK inhibition and blockage of the AKT signaling pathway (Wang 2015). Recent evidence has shown BTK-C to be expressed in prostate cancer as well, and known inhibitors of BTK, such as

ibrutinib and AVL-292, have been shown to block BTK-C and prevent cancer cell proliferation (Kokabee 2015).



Ibrutinib



AVL-292

Figure 4. Molecular structure of BTK small molecule inhibitors

The goal of the research was to investigate whether BTK-C is expressed in lung cancer cells in the same manner as prostate or breast cancer cells. Through Next Generation RNA Sequencing of various lung cancer cell line transcriptomes, there has been shown high levels of BTK-C expressed in lung cancer cells (Figure 5). If BTK-C is expressed in lung cancer cells, then the tyrosine kinase can act as a potential therapeutic target for treating lung cancer. By blocking BTK-C activity with its known inhibitors, there may be a reduction in cell survival in BTK expressing lung cancer cells. Some non-small cell lung cancer cells (NSCLC) express high levels of EGFR and inhibition by erlotinib, a small molecule inhibitor, may also have an effect on cell proliferation. Other growth factors, such as HER2, may also play a role in cell survival and proliferation of lung cancer cells if expressed.

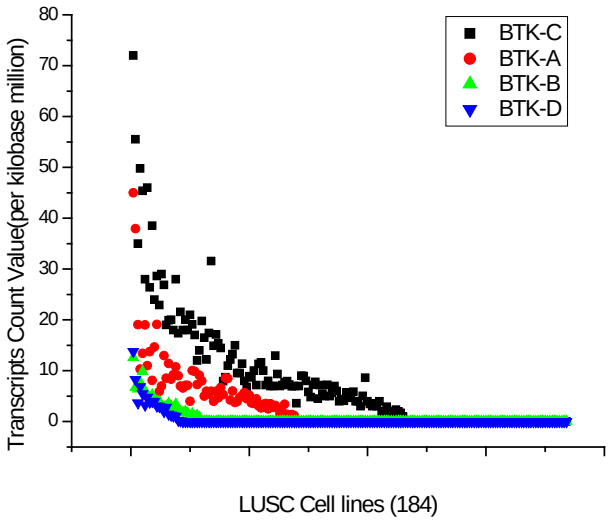


Figure 5. RNA-Seq of lung cancer cell lines shows expression of BTK-C.

Materials and Methods

Cell Culture

Cell line A549 (non-small cell lung cancer) was obtained from the American Type Culture Collection. These cells were found to express BTK (X. Wang unpublished). Lung cancer cells were cultured in Dulbecco's Modified Eagle Medium (gibco) (500 mL total stock) supplemented with 10% Fetal Bovine Serum and 1% of Penicillin-streptomycin (Cellgro).

Chemicals and Biologicals

BTK inhibitors were used in a cell viability assay to check for drug effectiveness in killing A549 cells. Ibrutinib was purchased from ChemieTek, AVL-292, also known as Spebrutinib, was purchased from MedKoo Biosciences, Erlotinib, an EGFR inhibitor, was purchased from Tarceva. Lapatinib, a HER2 inhibitor, was purchased from TYKERB. Each drug was dissolved and diluted in DMSO.

Immunoblot

To confirm the expression of BTK in A549 cells, an immunoblot analysis was necessary. Cell extracts were prepared using non-denaturing lysis buffer (20 μM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 μM EDTA), supplemented with complete cocktail of proteinase inhibitors (Roche). Protein extracts were separated by SDS-PAGE, transferred to Immobilon-FL (Millipore, Billerca, MA) membranes and immunoblotted according to standard protocols. Primary monoclonal anti-BTK antibodies (E-9) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-rabbit IgG-HRP antibodies (sc-2204, Santa Cruz Biotechnology) were

used for imaging the immunoblot, using the iBright^{TM} CL1000 Imaging System® (Thermofisher)

Cell Survival Assay

Approximately 5000 A549 cells per well were grown in a 96-well plate and treated with BTK-inhibitors and growth factor inhihibtors. Cells were treated with 10 nM, 1 µM, 10 1 µM. and 30 µM of each inhibitor diluted in DMSO. Control cells were treated with DMSO alone and cell proliferation continued without treatment. When the control wells reached ~80% confluency, the cells were fixed with 5% formaldehyde, washed with 1X PBS, and nuclei were stained with Hoechst 33342 (Molecular Probes® Invitrogen). Cell images were obtained using an IN Cell Analyzer 2200 (GE Healthcare) high content imaging system, with at least 9 fields imaged per single well of each experiment. Cell numbers were determined using IN Cell Investigator 3.4 high content image analysis software (GE Healthcare).

Results

Immunoblot Analysis

The purpose of the immunoblot was to assess for BTK-C expression in A549 cells, and identify its function in signaling pathways. Due to time constraints, results for the immunoblot could not be obtained in time before the completion of this thesis. The immunoblot procedure was attempted four times but BTK-C did not appear on the transfer membrane upon imaging. Despite not being able to obtain results from the immunoblot, from the RNA-seq analysis of lung cancer cell transcriptomes (Figure 5), there is reason to suspect expression of BTK-C in the A549 cell line.

Cell Survival Assay