Ibrutinib as a potential therapeutic option for HER2 overexpressing breast cancer – the role of STAT3 and p21

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By
Chandra Bose Prabaharan

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Room 116 Thorvaldson Building

110 Science Place

Saskatoon, Saskatchewan, S7N 5C9, Canada
Abstract

Treatment options for HER2 overexpressing breast cancer are limited, and the current anticancer therapies are associated with side effects. Tyrosine kinases perform crucial roles in cell proliferation, differentiation, and signal transduction process. Hence, tyrosine kinase inhibitors (TKIs) have been investigated as potential therapeutic options in the treatment of cancers. Ibrutinib (Imbruvica), an FDA approved tyrosine kinase inhibitor (TKI) for BTK, has shown to be effective in HER2 overexpressing breast cancer cell lines. However, the exact mechanism of action of ibrutinib in HER2 overexpressing breast cancer remains unknown. In this study, we have performed a kinome array analysis of ibrutinib treatment in two HER2 overexpressing breast cancer cell lines - BT474 and SKBR3. Our analysis shows that ibrutinib induces apoptosis through the activation of caspase-8, caspase-3, and PARP1 via the caspase-dependent extrinsic pathway. This result was further supported by changes in nuclear morphology, pSTAT3Y705 upregulation and p21T145 downregulation in both of the cell lines on treatment with ibrutinib. We for the first time show the involvement of the STAT3-p21 axis in bringing about apoptosis and propose that STAT3 activation is a passive response against DNA damage to promote cell cycle progression. These results have application in understanding the possible mechanism of action of ibrutinib in these cell lines and also suggest that inhibitors of pSTAT3 may be potential options for combination therapy with ibrutinib in HER2-overexpressing tumors.
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I dedicate this thesis to them.
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<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-Phosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton’s Tyrosine Kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic Lymphocytic Leukemia</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cells</td>
</tr>
<tr>
<td>DEP</td>
<td>Differentially Expressed Phosphoprotein</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast Growth Factor Receptors</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-Like Growth Factors 1 Receptor</td>
</tr>
<tr>
<td>ITK</td>
<td>Interleukin-2-Inducible T-Cell Kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinases</td>
</tr>
<tr>
<td>MTT</td>
<td>4,5-Dimethylthiazol-2-Yl-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>ORA</td>
<td>Over Representation Analysis</td>
</tr>
<tr>
<td>PDGFRs</td>
<td>Platelet-Derived Growth Factor Receptors</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-Dependent Kinase 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone Receptor</td>
</tr>
<tr>
<td>RGC</td>
<td>Receptor Guanylyl Cyclases</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer And Activator Of Transcription 3</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine Kinase Inhibitors</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XLA</td>
<td>X-Linked Agammaglobulinemia</td>
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</table>
CHAPTER 1: INTRODUCTION

Among women, breast cancer is the most common type of cancer contributing to mortality worldwide\(^1\). According to the data available, there are significant variations in incidence, mortality, and survival of patients among different countries and regions\(^1\). In Canada, one in eight women are affected by breast cancer and this contributes to 13% of all cancer-related deaths in women\(^2\). In 2017, approximately 26,300 women were diagnosed with breast cancer, which accounts for 25% of all new cancer cases\(^3\). On average, 72 Canadian women are diagnosed with breast cancer every day. It is found that metastatic breast cancer is one of the significant contributing factors for patient mortality\(^4\). Epidermal growth factor receptor 2 (HER2) is overexpressed in 30% of metastatic breast cancer patients\(^5\)\-\(^7\). HER2 overexpressing breast cancer is an aggressive type of cancer associated with poor prognosis and the current treatment options results in adverse drug reaction\(^5\)\-\(^7\). Currently, chemotherapeutic drugs such as doxorubicin, epirubicin, paclitaxel, cyclophosphamide and carboplatin are used for treating breast cancers\(^8\). However, the therapeutic index of many chemotherapeutic agents is narrow and they have unpredictable efficacy and toxicity issues.

1.1 Targeted Treatment options for HER2\(^+\) breast cancer

The current targeted treatment options include small molecule inhibitor and monoclonal antibodies. So far, FDA has approved 48 small molecule protein kinase inhibitors and nearly 1000 drugs are under clinical investigation in the hope for creating more options in targeted therapies\(^9\).

The current targeted treatment options for HER2\(^+\) breast cancer include trastuzumab, pertuzumab, and lapatinib\(^10\). Trastuzumab and pertuzumab are humanized monoclonal antibodies and lapatinib is a small molecule inhibitor designed explicitly for HER2\(^+\) breast cancer\(^11\). However, 20-50% of patients initially screened for treatment do not respond to the targeted treatment therapy, and 70% of initial responders acquire resistance to drugs such as
trastuzumab at later stages\textsuperscript{12}. Therefore, to avoid resistance and to explore novel effective treatment options, multi-target drugs and combination of drugs are being investigated.

1.2 Tyrosine Kinase Inhibitors (TKI)

Multi-target therapies are effective in treating breast cancer by modulating key processes of cell proliferation, migration, metabolism, anti-apoptotic signaling, and drug resistance\textsuperscript{13}. Tyrosine kinases are prime targets as these proteins play a crucial role in modulating various signaling processes\textsuperscript{14}. Small molecule inhibitors designed for various tyrosine kinases are named as tyrosine kinase inhibitors (TKI). These TKI generally inhibit multiple kinase targets with varied inhibitory potency\textsuperscript{15}. Several TKIs such as sunitinib, axitinib, neratinib, and pazopanib have been implicated in the treatment of HER2\textsuperscript{+} breast cancer either alone or in combination with trastuzumab and other chemotherapeutic agents such as Capecitabine\textsuperscript{16}. However each of these drugs has its limitations and advantages, since it has different targets, which might be present in different signaling pathways\textsuperscript{17}. Hence, there is a continued need to expand the array of TKI as treatment options for breast cancer.

1.3 Ibrutinib

Ibrutinib is an FDA approved irreversible tyrosine kinase inhibitor for Bruton’s Tyrosine Kinase (BTK) for the treatment of B-cell malignancies\textsuperscript{18}. BTK is involved in B-cell signaling and other important signaling pathways such as Fc receptor signaling, Chemokine receptor signaling and Toll-like receptors signaling\textsuperscript{19}. A novel isoform of BTK is overexpressed in breast cancer cells and protects them from apoptosis\textsuperscript{20}. Hendriks \textit{et al}. highlighted the importance of BTK in various signaling pathways in the context of its therapeutic inhibition\textsuperscript{21}. Ibrutinib is known to efficiently reduce the phosphorylation of HER1, HER2, and HER3 receptor tyrosine kinases\textsuperscript{22}. Anti-PD-L1 (programmed cell death ligand) and ibrutinib combination has been reported to have an anticancer effect in mice models of triple-negative breast cancer\textsuperscript{23}. PD-L1/PD-1 are immune checkpoints that include negative regulators
responsible for protection against autoimmunity. In tumor cells, PD-L1 are overexpressed and bind to PD-1 receptor on the activated T-cells leading to inhibition of cytotoxic T cell activity\textsuperscript{23}. Reports suggest that ibrutinib along with Anti-PD-L1 inhibits ITK (interleukin-2 inducible T-cell kinase) an essential enzyme of Th2 T cells and increase the activity of Th1 T cells which results in enhancing antitumor immune responses\textsuperscript{23}. Similarly, ibrutinib is reported for its therapeutic effect on HER2 overexpressing breast cancer by modulation of ITK and BTK\textsuperscript{24}. Apart from a few reports that suggest on the efficacy of ibrutinib in breast cancer cells, its molecular mechanism is still unknown.

We performed kinome array analysis to get information on key proteins involved in executing the effect of ibrutinib in HER2 overexpressing breast cancer cell lines BT474 and SKBR3. The data obtained from the kinome array was analysed and selected proteins (STAT3, p21, caspase 3, caspase 8 and PARP1) were validated using experimental procedure (western blot and RT-PCR). Our data suggest on the role of STAT3 and p21 in cell death brought about by treatment with ibrutinib.
CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

Cancer is a group of diseases characterized by uncontrolled proliferation and abnormal growth of cells. The growth of cancer often invades surrounding tissue and can metastasize to distant sites\textsuperscript{25}. According to WHO cancer statistics in the year 2015, about 90.5 million people had cancer, and about 14.1 million new cases occur each year\textsuperscript{26}. Usually, genes and proteins control cellular functions in our body, but when there is a change in these genes, the cellular function gets altered, resulting in uncontrolled proliferation. The key drivers of cancer that causes genetic changes are proto-oncogenes, tumor suppressor genes, and DNA repair genes\textsuperscript{27}. There are several types of cancer and are classified based on the type of cell the tumor cells resemble (Table 2.1).

Table 2.1 Categories of cancer based on cell type \textsuperscript{28}

<table>
<thead>
<tr>
<th>Tumor Types</th>
<th>Origin</th>
<th>Cancer</th>
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<tr>
<td>Carcinoma</td>
<td>Epithelial tissues, mucus membrane, breast, lung, colon and prostate</td>
<td>Adenocarcinoma, squamous cell carcinoma,</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>Connective tissues (bone, cartilage, fat, nerve)</td>
<td>Osteosarcoma, Chondrosarcoma, and liposarcoma</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>lymphatic system- the spleen, tonsils, and thymus</td>
<td>Hodgkin lymphoma, Non-Hodgkin lymphomas</td>
</tr>
<tr>
<td>Leukemia</td>
<td>Bone marrow, white blood cells and red blood cells</td>
<td>lymphoblastic leukemia, erythremia, granulocytic leukemia</td>
</tr>
<tr>
<td>Germ cell tumor</td>
<td>Pluripotent cells (testicle and ovary)</td>
<td>seminoma and dysgerminoma</td>
</tr>
<tr>
<td>Blastoma</td>
<td>Immature &quot;precursor&quot; cells or embryonic tissue</td>
<td>nephroblastoma, medulloblastoma, and retinoblastoma</td>
</tr>
</tbody>
</table>
2.2 Breast cancer

Breast cancer is a group of heterogeneous diseases indicated by uncontrolled growth of malignant cells in the epithelial tissue of the mammary glands. Breast cancer affects both the genders; however, it is found to be more frequent in women. It is reported that breast cancer accounts for 29% of all new cancer cases and 14% of all cancer-related deaths among women. According to the American Cancer Society reports of 2017 and 2018, 252,710 new cases of invasive breast cancer (the cancer invading surrounding breast tissues) are diagnosed among women, and 2,470 cases are diagnosed among men. Breast cancer typically develops in breast cells such as mammary glands, lobules, and connective tissues. Breast cancer development is primarily due to biological, genetic, and environmental factors such as smoking cigarette, inherited mutation of genes and harmful radiation exposure.

2.3 Types of breast cancer

2.3.1 Histological classification of breast cancer

Breast cancer is broadly categorized into in situ carcinoma and infiltrating (invasive) carcinoma. Further, in situ carcinoma is classified into two subtypes based on their growth pattern and cytological features as either ductal or lobular carcinoma. Through various research and examination of the breast tissues, pathologists have identified that ductal carcinoma is more common than lobular carcinoma.

The ductal carcinoma (DCIS) is a cancer that starts from milk ducts of mammary gland. It is also called as non-invasive carcinoma as it does not spread beyond milk duct into any surrounding breast tissues. It is further classified into five subtypes viz., comedo, cribiform, micropapillary, papillary, and solid (Fig 2.1). Comedo involves necrotic tissues with calcification, cribiform involves connective tissues of the breast, micropapillary and papillary carcinoma involves fibrovascular stalks lined by epithelial cells (finger like projections).
On the contrary, Invasive lobular carcinoma, invades surrounding breast tissues and spreads to the lymph nodes and other areas of the body. It is categorized into seven subtypes as infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary, and invasive papillary carcinoma. Tubular carcinoma involves axillary lymph nodes forming a tube-shaped structure, mucinous (colloid) involves mucus membrane for the spread of cancer. In infiltrating ductal carcinoma, the spread of cancer occurs in fatty tissues surrounding the ducts, whereas in lobular/ductal carcinoma the cancer spreads in lymph nodes surrounding the lobules of breast tissues.

2.3.2 Molecular classification of breast cancer

The new technological advancements led to classify breast cancer based on molecular markers such as Estrogen receptor (ER), Progesterone receptor (PR), ErbB2 (HER2/neu), and p53. Research on molecular characteristics in breast cancer has led to identification of several intrinsic molecular subtypes such as basal-like, ErbB2+ (HER2+), normal breast-like, luminal subtype A, luminal subtype B, and Claudin-low (Fig 2.2). Microarray gene expression and unbiased hierarchical clustering were used to identify these molecular subtypes. In recent years, cost-effective new methods such as PAM50 (50-gene signature PCR array), is used to predict risk of relapse in ER+ and ER- breast cancer patients compared to the models utilizing only clinical variables such as tumor size and histologic grades.

2.3.3 Functional classification of breast cancer

Apart from the molecular and histological classification, breast cancer can be classified based on Cancer Stem Cells (CSC). Cancer stem cells were isolated and characterized as tumor-initiating cells in common malignancies. There are no conclusive biomarkers for CSC. However, several laboratories have identified that biomarkers in mammary stem cells can be used for Cancer Stem Cells (CSC), such as CD44, CD24, CD49f/α 6-integrin, CD29/β 1-integrin, and ALDH1.
Fig 2.1 Histological classification of breast cancer indicating several subtypes adapted from reference 34

Fig 2.2 Molecular classification of breast cancer with various subtypes adapted from reference 34
2.4 HER2 overexpressing breast cancer and signaling

HER2+ breast cancer is an aggressive type of breast cancer and about 15-20% of breast cancer patients worldwide are diagnosed with HER2 subtype, and about 50% of these patients express estrogen and/or progesterone receptors (ER/PR)\textsuperscript{43}. It is reported that in HER2+ breast cancers there is about 40-100 fold increased HER2 receptor protein expression on tumor cell surface\textsuperscript{44}. The diagnosis of HER2+ breast cancer in patients is carried out using immunohistochemistry, fluorescence in situ hybridization, and mRNA microarray. HER2 is a member of the transmembrane receptor tyrosine kinases family (HER1-4). The HER receptor has an ectodomain or ligand binding region and an intracellular cytoplasmic tyrosine kinase domain\textsuperscript{45,46}. These proteins are encoded by genes expressed on chromosomes 7, 17, 12, and 21 respectively.

There are specific soluble ligands identified for the ectodomains of HER1 (EGFR), HER3, and HER4. There is no specific ligand for HER2. HER2 exists in open conformation for dimerization and it is constitutively active\textsuperscript{45,46}. Once a ligand binds to the HER1, HER3 and HER4 receptor, a conformation change facilitates homo or heterodimerization with another HER family proteins\textsuperscript{44}. HER2 appears to be a preferred binding partner compared to other members of ERBB family for the formation of heterodimers, as the four members of ERBB family show a high degree of homology with significant sequence divergence in C- terminal residues\textsuperscript{47}.

The conformation changes in HER2 results in autophosphorylation of tyrosine residues within the intracellular domain which results in activation of downstream signaling pathways, including MAPK (mitogen-activated protein kinase pathway, PI3K (phosphatidylinositol 3'-kinase) and AKT pathway. As a result of these signaling pathways various nuclear factors are recruited to regulate the transcription of certain genes responsible for cell cycle progression,
proliferation and survival. Eventually, dysregulation of these pathways and transcription factors alters the expression of genes that contribute to the malignant phenotype \(^45,48\) (Fig 2.3).

The cells expressing HER2, HER3, and HER4 homodimers do not induce tumor growth. Whereas, cells expressing HER1 (EGFR) alone or co-expression HER 2/3 can influence tumor growth. However, aggressive tumor growth is found only in HER2 overexpression cells\(^44\). Apart from HER2 overexpression, there are other membrane receptors such as insulin-like growth factors 1 receptor (IGF-1R) and hepatocyte growth factor receptor (HGFR or MET) that can influence tumor growth. Similarly, alterations in tumor suppressor genes INPP4-B and PTEN can also contribute to malignant phenotypes \(^48,49\). Hence, finding a blocking agent for such a complex circuit is difficult.

Fig 2.3 HER2 dimerization and its downstream signaling adapted from reference\(^50,51\)
2.5 Breast cancer treatments

Breast cancer treatment normally includes surgery, radiation therapy, hormonal therapy, chemotherapy, and targeted therapy\textsuperscript{52}. The treatment options for breast cancer patients are generally based on age, tumor size, histological grade, metastatic stage, the status of hormone receptors, and HER2 expression\textsuperscript{52}.

Most patients with breast cancer normally undergo breast-conserving surgery. This type of surgery depends mainly on the stages of breast cancer\textsuperscript{53}. After surgery, to curtail the recurrence of breast cancer, patients undergoes radiation therapy or hormonal therapy. The radiation therapy include direct radiation through the skin to the tumor and tissues around it. This treatment also prevents metastasis in patients\textsuperscript{54}. Hormonal therapy on the other hand prevents breast cancer in patients by blocking hormone receptor ER/PR on the cell surface using competitive antagonists such as tamoxifen\textsuperscript{52, 55}.

2.5.1 Chemotherapy

Over a decade, cytotoxic chemotherapy in both early stages and advanced stages of breast cancer has made significant progress\textsuperscript{56}. Chemotherapy is given to cancer patients after they undergo other therapies such as surgery, radiation therapy\textsuperscript{57}. The route of administration of chemotherapy drugs to patients are normally through oral, intravenous, and intrathecal. The chemotherapeutic drugs are cytotoxic in nature and cause stress in cancer dividing cells leading to cell death\textsuperscript{57}. The commonly used chemotherapy drugs for breast cancer are taxanes such as paclitaxel, docetaxel, and albumin-bound paclitaxel and anthracyclines such as doxorubicin, epirubicin, and pegylated liposomal doxorubicin\textsuperscript{58, 59}. However, these first-line drugs tend to fail in many breast cancer patients\textsuperscript{60}. Hence, second line chemotherapeutic drugs such as capecitabine, gemcitabine, ixabepilone, and vinorelbine are given to these patients as single-agent chemotherapy or combination chemotherapy\textsuperscript{61}. Examples for combination chemotherapeutic drugs are paclitaxel with bevacizumab (triple-negative breast cancer), capecitabine with
docetaxel (Hormone receptor breast cancer) and capecitabine with trastuzumab (HER2+ breast cancer)\textsuperscript{61}.

2.5.2 Targeted therapy

Targeted therapies have fewer side effects compared to chemotherapy drugs. \textsuperscript{62} So far, several molecular targets have been identified in various types of cancers\textsuperscript{63}. Targeted therapy drugs against these targets include monoclonal antibodies, small molecule inhibitors, prodrug therapies and antibody-conjugated nanoparticles\textsuperscript{64}.

2.5.2.1 Monoclonal antibodies for HER2 inhibition

Trastuzumab (Herceptin\textsuperscript{®}) and pertuzumab (Perjeta\textsuperscript{®}) are recombinant humanized monoclonal antibodies approved by FDA for HER2\textsuperscript{+} breast cancer treatment. Trastuzumab is a IgG1 class monoclonal antibody that inhibits EGFR and prevents HER2 heterodimerization\textsuperscript{65}. However, its exact mechanism of action is unclear. Similarly, pertuzumab is a monoclonal antibody used to treat HER2 overexpressed patients. It binds with HER3/HER2 and prevents dimerization\textsuperscript{66}. Reports suggest that combination therapies have better response among the patient population\textsuperscript{47, 67}. Hence, Pertuzumab is combined with trastuzumab and docetaxel for the treatment of HER2\textsuperscript{+} metastatic breast cancer patient population\textsuperscript{68, 69}.

2.5.2.2 Small molecule kinase inhibitors

Synthesis of a small molecule inhibitor targeting a defined kinase target is challenging \textsuperscript{70}. This is because the ATP binding site is common among kinases and hence developing an inhibitor for a specific target is difficult. Also, the drug has to compete with a millimolar concentration of ATP inside the cell \textsuperscript{71}. Since FDA’s approval of a small molecule inhibitor imatinib in 2001 (Gleevec\textsuperscript{@}) for BCR-ABL, there was a steady increase in the development of small molecule kinase inhibitors as this drug served as a prototype for next-generation inhibitors\textsuperscript{72-74}.

In HER2 positive breast cancer, the small molecule drugs, tyrosine kinase inhibitors (TKIs) are found to be a promising anticancer agent. Unlike monoclonal antibodies, TKIs can penetrate
the Blood-brain barrier (BBB) because small molecule kinase inhibitors are usually 400Da to 500 Da in molecular weight and this facilitates easy diffusion across the membrane\textsuperscript{75}. TKIs has the ability to block multiple receptors of HER Family simultaneously. For example, lapatinib plus capecitabine has been shown to have a significant effect in BCBM (Breast cancer brain metastases)\textsuperscript{76}. Lapatinib (Tykeb/Tyverb\textsuperscript{®}) is a small molecule TKI developed as a dual tyrosine kinase inhibitor targeting EGFR/HER2\textsuperscript{77}. The drug lapatinib has been reported to induce apoptotic response towards breast cancer cell lines SKBR3 and BT474\textsuperscript{77}. Similarly, the drug AST-1306 is a weak irreversible EGFR and HER2 inhibitor with moderate drug efficacy and has been reported for its tumor suppression activity in ovarian cancer cell line SK-OV-3\textsuperscript{78}. On the other hand, AZD8931 is a reversible inhibitor of EGFR, HER2, and HER3 and it is more effective than lapatinib and gefitinib in treating NSCLC. This drug is highly selective towards HER family kinases \textsuperscript{47, 79}. There are other irreversible TKI drugs such as CI-1033 (Canertinib), CUDC-101, TAK-285, PF299804, PF299 (Dacomitinib) and EKB-569 (Perlitinib) designed to target multiple kinases\textsuperscript{47}. 
2.6 Challenges in breast cancer treatments

Since early 2000, the overall treatment for breast cancer has improved due to early detection, advanced treatment options, and increased awareness among people\textsuperscript{80}. However, new treatments and methods are still needed, as the treatment response rate is low among the patient population. There are many factors responsible for a low treatment response such as drug resistance, increased side effects, poor prognosis, cancer metastasis, etc. So far, chemotherapy drugs are dominated in breast cancer treatment, as these drugs are used as first-line therapies for breast cancer \textsuperscript{58, 81}. However, in recent years, patients tend to develop resistance to chemotherapies\textsuperscript{82}. Researchers have suggested various mechanisms for drug resistance. One such mechanism that has been reported is increased expression of ATP binding cassette the ABC transporters\textsuperscript{82}. ABC transporters along with multidrug resistance proteins (MRPs) efflux the drug molecules out of the cells\textsuperscript{83}. ABC transporters can be blocked using inhibitors for effective chemo-drug treatment, but their therapeutic results are not satisfactory\textsuperscript{84}. Hence, the challenge in finding an effective therapeutic option remains the same.

2.7 Ibrutinib

Ibrutinib is an irreversible tyrosine kinase inhibitor designed to target BTK (Bruton Tyrosine Kinases) as it covalently binds to cysteine residues Cys-481 in the ATP binding domain of BTK\textsuperscript{85}. Ibrutinib was initially designed by Celera genomics as a compound for studying BTK function. According to the Wall Street Journal, the global drug sale of ibrutinib was 1 billion US dollars and expected to reach $5 billion in 2020\textsuperscript{86} (Fig 2.4).

Ibrutinib is clinically effective against B-cell lymphoproliferative diseases, especially chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), and Waldenstrom macroglobulinemia\textsuperscript{29, 87}. Ibrutinib was granted approval by FDA in November 2013 for the treatment of relapsed mantle cell lymphoma and relapsed refractory chronic lymphocytic leukemia in February 2014 \textsuperscript{88}.
Apart from BTK, ibrutinib is also reported to have inhibitory activity on several other kinases such as ITK, TEC, BLK, JAK3, EGFR HCK, and HER2. ibrutinib multi target binding activity is screened using kinativ-biochemical kinase profiling assay that measures the efficacy of small molecule inhibitors binding to various kinases. About 89 druggable targets with 50% inhibition were identified upon treatment with ibrutinib. However, among these targets, only a few genes are well studied for their mechanism.

2.7.1 Role of Ibrutinib in HER2+ breast cancer

Ibrutinib has a dual activity towards TEC family kinases (BTK, ITK/EMT/TSK, BMX and TXK/RLK) and HER kinases. Reports suggest that treating HER2+ breast cancer with ibrutinib leads to a reduction in the phosphorylation of the receptor tyrosine kinases HER 1-3. This leads to modulation of downstream target AKT, MAPK signaling pathways resulting in delaying of G1/S phase in the cell cycle and increase apoptosis. ibrutinib has been reported to have an IC50 of 0.03µM for SKBR3 and MCF-7 cell lines. A novel isoform of BTK (BTK-C) is found in most of the breast cancer cell lines and tissues. BTK-C is a downstream target of SRC that contributes to the NRG1 and EGF growth factor-mediated drug resistance in HER2+ breast cancer. A xenograft study in mice shows the ability of ibrutinib to target both HER2 and BTK-C and abolish the NRG1 or EGF mediated drug resistance in HER2+ breast cancer. Hence, BTK and BTK-C are considered as potential therapeutic targets of ibrutinib. Therefore, ibrutinib could be a possible drug for treating HER2 overexpressing breast cancer.

Apart from HER2 (ERBB2), ibrutinib is reported to have inhibitory activity on ERBB4 expressing breast cancer cells. At the protein level, ibrutinib dephosphorylates ERBB4 and other downstream targets such as MEK, ERK and AKT. Reports suggest that ERBB4 cell lines responds to ibrutinib in WNT pathway-dependent manner. Hence, ibrutinib along with
WNT inhibitors is reported to increase the possibility to treat ERBB4 expressing breast cancer cells\(^91\).

### 2.8 Bruton Tyrosine Kinase (BTK) - a targetibrutinib

Bruton’s tyrosine kinase (BTK) is a member of the non-receptor tyrosine kinases (TEC family kinases) and is a cytoplasmic protein predominantly expressed in hematopoietic cells\(^92\). BTK is involved in multiple signaling pathways regulating cell proliferation, differentiation, survival, and activation, of B-lineage lymphoid cells\(^93,94\).

BTK is expressed in hematopoietic cells and it also involved in B-lymphocytes production and maturation\(^95,96\). Initially, researchers had identified that BTK is localized in the cytosolic membrane. However, later with various supporting data, BTK was also reported to be localized in nucleus and plasma membrane\(^97\). B-cell receptor (BCR) activates downstream targets such as PI3K which phosphorylate PIP2 and generates phosphatidylinositol-3,4,5-triphosphate (PIP3). After sufficient accumulation of PIP3 at the inner surface of the cell, PIP3 interacts with BTK PH-domain and recruits BTK to the plasma membrane\(^98,99\). This is one of the reasons why BTK is also found in the plasma membrane. BTK is reported to activate anti-apoptotic signaling pathways. BTK act as an upstream regulator for the MAPK pathway, STAT5 pathway, and NFκB pathways\(^92\).

A defective BTK gene in human causes a phenotypic disease called X-linked agammaglobulinemia (XLA) and patients have failure of B-cell development resulting in markedly decreased production of all classes of immunoglobulins\(^98,100,101\). Rawlings et al. have shown that BTK is phosphorylated at site Y\(^551\) by Src family kinases Blk, Lyn, and Fyn. This phosphorylation further leads to the activation of the transcription factor NF-κB, transcription of BTK gene and inhibition of apoptosis\(^99\). Moreover, this is an evidence that BTK plays a role in apoptosis\(^102,103\). Likewise, BTK is responsible for phosphorylating PLCγ2 at positions Y\(^753\) and Y\(^759\), which is essential for the lipase activity of PLCγ2\(^104\). Reports also suggest that BTK
is accountable for recruiting phosphatidylinositol-4-phosphate 5-kinase (PIP5K), thereby stimulating a positive-feedback loop that generates phosphatidylinositol 4,5-bisphosphate (PIP2), which serves as a substrate for both PI3K and PLCγ2\textsuperscript{104,105} (Fig 2.4).

BTK is involved in multiple pathways, including chemokine receptors and TLR signaling\textsuperscript{104}. It is a key signaling molecule for chemokine receptors CXCR4 and CXCR5, that helps in lymphocyte migration\textsuperscript{104}. BTK has been shown to contribute to Toll-Like Receptor (TLR) signaling by interacting with five different molecules such as the intercellular domains of most TLRs, the downstream adaptors MYD88 and MYD88 adaptor-like protein (MAL), IL-1R-associated kinase 1 (IRAK1) and TIR domain-containing adaptor protein inducing interferon-β\textsuperscript{104}. \textsuperscript{104}
Fig 2.4 B-cell receptor pathway involving BTK and its downstream signaling adapted from reference 106, 107.
2.9 STAT3
STAT3 acts as a driving transcriptional regulator and modulates complex array of functions in
tumorigenesis. STAT3 is activated by phosphorylation of tyrosine residue site Y705. Normally STAT3 activation is tightly regulated which induces dimerization, nuclear translocation, and DNA binding. Reports says that STAT3 is constitutively active in cancer patients and plays an important role in tumor growth and metastasis. As STAT3 is a crucial transcription factor in regulating cellular proliferation, invasion and migration, it plays a major role in cell cycle progression.

2.10 p21
p21WAF1/CIP1 (referred as p21) is a protein that contributes to DNA repair mechanism by promoting cell proliferation, apoptosis and transcription when DNA damage occurs in a cell. p21 is known as an inhibitor of CDK complexes (CDK1, 2 and CDK 4/6). p21 regulate cell cycle by inhibiting the activity of CDK complexes. Also, p21 can regulate cell cycle progression without CDK involvement by binding with PCNA (proliferating cell nuclear antigen) complexes. p21 inhibit cyclin-dependent kinases either through p53-dependent or p53-independent mechanisms.

2.11 Apoptosis
Apoptosis act as a homeostatic mechanism in maintaining cell population in tissues. Apoptosis is generally characterized based on biochemical mechanism and morphology of the cell. Apoptosis also occurs as a defence mechanism during the immune response, cell damage due to diseases, stress and injury. There are various conditions that trigger apoptosis such as heat, radiation, hypoxia and cytotoxic drugs. However, alteration in apoptotic process and the inability to cause cell death, leads to cancer, autoimmune diseases, neurodegenerative disorders and other disorders. Apoptosis occurs through highly complex energy dependent mechanism involving cascades of signaling proteins.
There are two pathways involved in apoptosis - intrinsic and extrinsic pathway\textsuperscript{116} (Fig 2.5). In intrinsic pathway, mitochondria plays a crucial role. When the cell is about to undergo apoptotic cell death, the mitochondria releases cytochrome c through Bak and Bax (Bcl-2 protein family). Then, the released cytochrome c binds with apoptotic protease activating factor Apaf-1 and procaspase -9 to form a complex called Apoptosome. Further, Apoptosome complex activates effector caspase -3 and leads to cell death\textsuperscript{117}. However, in extrinsic pathway two mechanisms have been reported so far, TNF alpha induced model and Fas-Fas ligand mediated model. In TNF induced model the TNF alpha binds to TNFR1 receptor and activate caspase 8 via intermediate proteins such as TRADD and FADD\textsuperscript{113, 117}. Then, the caspase 8 activates effector caspase (caspase -3) leading to cell death. On the other hand, in Fas ligand mediated model, Fas ligand (FasL) binds to Apo1, a Fas receptor, forming a complex called DISC (death induced signaling complex). This complex contains the protein FADD (Fas associated death domain protein) which activates protein caspase 8. The caspase 8 activate effector caspase 3 leading to apoptotic cell death\textsuperscript{117}. 
Fig 2.5 Apoptosis mechanism-intrinsic and extrinsic pathway adapted from reference\textsuperscript{118}. 
2.12 Human kinome - a potential source for targets

The human kinome is an extensive network of protein kinases and lipid kinases. It contains 518 protein kinases and 20 lipid kinases which constitute 2% of the entire human genome\textsuperscript{119}. These protein kinases are responsible for phosphorylating one-third of all proteins\textsuperscript{71, 120}. Reversible phosphorylation of proteins and lipids are critical components of various signaling pathways regulating cell growth, differentiation, proliferation, angiogenesis, apoptosis, cytoskeletal rearrangement, and metabolism\textsuperscript{119}. Kinases mediate the phosphorylation of proteins or lipids through a transfer of phosphate from the higher energy precursor molecule ATP or GTP to proteins or lipids\textsuperscript{121, 122}. On the other hand, the dephosphorylation of proteins is mediated by phosphatases through the removal of a phosphate group from proteins or lipids\textsuperscript{122} (Fig 2.6). The phospho-acceptor sites in proteins are the hydroxyl group of serine, threonine, or tyrosine residue\textsuperscript{122}. These kinases, when altered due to mutations or random changes in expression, cause aberrant cellular signaling leading to diabetes, cancer, cardiovascular, neurodegenerative, developmental, immune, and behavioral disorders. Thus, human kinome is an important hotspot for drug target discovery\textsuperscript{119}.

2.12.1 Types of kinases

Human kinome is classified into eight group based on sequence similarity in kinase domain and they are described below.

2.12.1.1 Tyrosine kinases

Tyrosine kinases are a group of enzymes capable of phosphorylating the amino acid tyrosine and lead to conformational change and activation of other proteins. Tyrosine kinases constitute 90 enzymes out of 518 kinases and are classified as cell-surface receptor kinases and non-receptor kinases\textsuperscript{123}.
Fig 2.6 The post transitional modification of proteins
phosphorylation and dephosphorylation of proteins by kinases and phosphatases adapted from reference\textsuperscript{118}
The members of cell surface receptor kinases are human epidermal growth factor receptor (HER/EGFR) family, the insulin receptor (IR) and the closely related insulin-like growth factor 1 receptor (IGF1-R), the platelet-derived growth factor receptors (PDGFRs), and the fibroblast growth factor receptors (FGFRs). The members of non-receptor kinases include Src, Abl, and JAK kinases.

The JAK (JAK1 and JAK 2) protein has a recognisable kinase domain and a second domain with similarity to kinases. JAK is conjugated with STAT and is activated upon interaction with the C-terminal of receptor tyrosine kinases. Reports state there are about 26 tyrosine kinase inhibiting drugs approved by FDA as tyrosine kinases have a driving role in carcinogenesis. Reports also suggest that tyrosine kinases act as an ideal target for small molecule inhibitor production. This is because tyrosine kinase mediate various intracellular signals that activate cell survival, progression and migration. (Fig 2.7)

2.12.1.2 Tyrosine kinases like (TKL)

The Tyrosine kinases like (TKL) is a group of serine/threonine kinases. The TKL include interleukin-1 (IL-1) receptor-associated kinase (IRAK), the RAF kinases, the LIM domain kinase (LIMK), and the transforming growth factor-beta (TGFβ) receptors. These kinases are considered as potential targets and many acts as proto-oncogenes.

2.12.1.3 Serine threonine kinases (STK)

The STK are kinase enzymes that phosphorylate the serine and threonine residues at their respective OH group. They constitute 125 enzymes out of 518 human kinases. The STK are classified into three families based on their homology to the yeast proteins STK20 (MAP4K), STK11 (MAP3K), and STK7 (MAP2K). STK includes the p21-activated kinases (PAKs). p21-activated kinases are critical regulators as these proteins act as tumor suppressors or an oncogenes (Fig 2.7)
2.12.1.4 Casein kinase 1 (CSNK1/CK1)

The Casein Kinase group are Ser/Thr kinases expressed constitutively as they phosphorylate a wide variety of substrates involved in transcriptional regulation and cytoskeleton function\textsuperscript{119}. 

Fig 2.7

2.12.1.5 Other kinases

The other kinases are AGC, CAMK, CMGC, RGC, CK2, and IKB Kinases (IKKs). The AGC is a set of enzymes involved in regulating cell growth, proliferation, protein synthesis, glucose metabolism, and survival. AKT is a member of AGC kinases and acts as a substrate for 3-phosphoinositide-dependent kinase 1 (PDK1), which contains a pleckstrin homology (PH) domain that facilitates its membrane recruitment \textsuperscript{131} Fig 2.7. CAMK kinases are prominent members involved in calcium signaling. CHK1 and CHK2 are members of the CAMK group, plays a vital role in cell cycle checkpoints by initiating phosphorylation cascade that leads to DNA repair and cell cycle arrest \textsuperscript{18, 131}.

The CMGC group is a diverse group of kinases. Cyclin-dependent kinases (CDKs) and Mitogen-Activated Protein Kinases (MAPKs) are members of CMGC kinases involving in cell cycle progression, cell proliferation, differentiation, and apoptosis. They act as cell cycle regulators \textsuperscript{121}. The (RGC) receptor guanylyl cyclases are a group of enzymes that convert GTP to cyclic GMP. They represent the smallest of the kinase groups and lack critical residue for phosphate transfer \textsuperscript{119, 121}. Fig 2.7

2.12.1.6 Atypical kinases

The atypical kinases have a different domain compared to normal eukaryotic kinase domain. However, they demonstrate kinase activity \textsuperscript{121, 132}. The prominent members of atypical kinases include pyruvate dehydrogenase kinase, bromodomain kinases, BCR, phosphatidylinositol-3-kinase-related kinases (PIKKs), phosphatidylinositol-3-kinase (PI3K) and the mammalian
target of rapamycin (mTOR). These Kinases are considered as downstream targets for HER pathway. 

Fig 2.7 

*Fig 2.7 A Schematic representation of Human kinome tree, with approved drug targets. Figure generated using KinMap, and reproduced courtesy of Cell Signaling Technology, Inc.*
2.13 Kinome analysis and challenges

The major obstacle for kinome analysis is the nature of the substrate we usually employ. For kinases, protein is the physiological substrate, and the protein gets phosphorylated and transfers signals to various other proteins through protein-protein interaction. Production of a high-throughput proteins in an array format is difficult because the stability and yield of proteins are low. In order to overcome these challenges, scientists have found that many protein kinases recognize the phospho-acceptor site determined by residues surrounding the phosphorylated amino acid, as opposed to higher-order secondary or tertiary structures. Synthetic peptides modelled on the phosphorylation site may be suitable substrates with appropriate $V_{\text{max}}$ and $K_m$ values to that of the intact protein. Thus, after these findings peptide array constructs were employed for kinome and phospho-proteome analysis.

2.14 Kinome profiling using peptide array

Peptide array is used for kinome analysis and involves the array design and physiology of gene microarray. In gene microarray, nucleotide strands complementary to the gene of interest are constructed in an array format. However, in the peptide array, each spot is coated with a population of identical peptides representing a particular phosphorylation site. The peptides in the array construct are unique with ~15 amino acid in length with centrally positioned phospho-acceptor sites. To avoid errors and facilitate the analysis, each peptide is printed as multiple spots on the array (three to nine replicates for every unique peptide sequence).

The process starts by incubating the peptide array chip with cell lysate, in which active kinases phosphorylate peptides whose sequence match the recognition motif of that particular kinase. The phosphorylation event of each peptide is quantified by the use of phosphorylation specific-antibodies or radiolabelled ATP, which indicates the activation status of these corresponding kinases.
Similarly, there is an alternative approach for kinase analysis known as PAM chip approach. In this method, the peptides are immobilized in aluminium oxide surface and represent as a 3D array with 144 spots. The cell lysate is pumped through the matrix to facilitate the kinases to recognize the respective peptides for phosphorylation. The lysate is pumped repeatedly to phosphorylate further the peptides which are detected using fluorescent labelled phosphospecific antibodies.

The other method to measure the phosphorylation of peptides is bead-based assay where peptides representing specific phosphorylation site are linked through acrylamide beads. This method is not preferred nowadays, as it is difficult to synthesize the beads.

2.15 Data statistics and analysis

The microarray analysis is dominated by genomics with high-throughput mathematical manipulation and validation. It may be assumed that the mathematical manipulations and statistical tools developed for nucleotide arrays would be directly applicable to these peptide array technologies.

2.15.1 PIIKA 2 platform

In kinome array, peptides act as phosphorylation targets. To measure the phosphorylation events in high-throughput manner researchers have developed a Platform for Intelligent, Integrated Kinome Analysis (PIIKA). Further, to meet the programming limitation of the PIIKA module, higher version such as PIIKA2 was created. PIIKA2, is an updated version which acts as a standalone web platform.

PIIKA2 module can be accessed via http://saphire.usask.ca. This module allows users to determine the statistical significance between the actual clustered data and hypothesized clustered data. It also allows users to assess the significance of hierarchically clustered nodes using bootstrapping.
Statistical significance between the treated samples and control samples can be obtained using PIIKA2. The software also enables the user to access the false positive rates, false negative rates, and negative predictive values\textsuperscript{138}. The improved platform PIIKA2 also contains some visualization tools such as Principle component analysis (PCA) 3D plot and volcano plot\textsuperscript{138}. The entire software was developed using the R programming environment. The PIIKA2 version uses (GUI) graphics user interface which facilitates the user to access the software without programming codes\textsuperscript{138}.

2.15.2 Pathway analysis

Pathway analysis is used to identify related proteins within a biological pathway or building a new biological pathway from protein of interest.

Pathway analysis software’s uses three methods to analyze the pathways such as Pathway-over representation analysis (ORA), Functional class scoring, and pathway topology.

Pathway ORA in a pathway is based on the probability value (p-value) of the differential expressed genes or proteins. InnateDB is a web-based platform used as a pathway analysis tool for microarray data\textsuperscript{139}. It perform pathway enrichment based on pathway Over Representation Analysis (ORA)\textsuperscript{139}. InnateDB scores the pathway based on gene/proteins significance, fold change and number of genes/proteins involved in the pathway\textsuperscript{139}.

The major limitation of pathway analysis is that the databases are always ending and updated on regular basis. So complete annotation of the molecular network is not possible. Hence, to attain a perfect classification of biological pathways is always difficult.
CHAPTER 3: HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

We hypothesize that the TKI inhibitor ibrutinib effectively targets HER2 overexpressing breast cancer cells through a STAT3 and p21 axis leading to cancer cell apoptosis.

3.2 Objectives

- To check the efficacy of ibrutinib in HER2 overexpressing breast cancer cell lines SKBR3 and BT474
- To investigate the mode of cell death in SKBR3 and BT474 cells when treated with ibrutinib
- To evaluate the multi-target response of ibrutinib using kinome peptide array and to identify the key proteins involved in bringing about cell death (signaling pathways).
CHAPTER 4: MATERIALS AND METHODS

4.1 Reagents and antibodies

Rosewell Park Memorial Institute (RPMI) 1640 medium with L- Glutamine, McCoy 5A (modified medium), Phosphatase buffered saline (1X), Trypsin 0.25% (1X) solution, Penicillin (10,000 units/ml)-Streptomycin (10,000 µg/ml) solution, Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS) purchased from HyClone, USA. Ibrutinib (PCI-32765) inhibitor (Selleckchem, Houston, Texas, USA). BCA protein assay kit (Thermofisher, Waltham, Massachusetts, USA). The BTK siRNA and transfection reagents (Santa Cruz, Dallas, Texas, USA). 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), Annexin V-FITC kit (Sigma Aldrich, St. Louis, Missouri, USA). RIPA lysis buffer chemical composition were listed (Table 4.1). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster city, California, USA). TRIzol reagent from (Ambion life technologies, Carlsbad, California USA). Antifade Mounting Medium with DAPI (Vector Labs, Burlingame, California USA). Phosphatase inhibitor (Roche, Belmont, California USA). Ultrapure distilled water (Invitrogen, Carlsbad, California USA). Clarity Western ECL substrate (Bio-Rad, Hercules, California, USA). Trypan blue stain (0.4%) from life technologies, USA. Antibodies purchased for western blot were listed in (Table 4.2).

Table 4.1 RIPA buffer composition

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Stock Concentration</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5M</td>
<td>150mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5M</td>
<td>5mM</td>
</tr>
<tr>
<td>Tris, pH8.0</td>
<td>1M</td>
<td>50mM</td>
</tr>
<tr>
<td>Triton-X100</td>
<td>100%</td>
<td>1%</td>
</tr>
<tr>
<td>Sodium-deoxycholate</td>
<td>10%</td>
<td>0.5%</td>
</tr>
<tr>
<td>SDS</td>
<td>10%</td>
<td>0.1%</td>
</tr>
<tr>
<td>dH2O</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
All reagents and kits used were handled according to the standard procedures provided by the manufacturers. DNA/RNA samples were eluted into DNase-free ddH$_2$O and stored at -20 °C for future use. Cancer cells working stock for experiments were kept in liquid nitrogen. The protein samples were extracted using the inhibitor cocktail and stored at -80°C for further use. Standard biosafety guidelines and aseptic practices were followed at every step of the research work.

Table 4.2 Antibodies list

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-STAT3$^{Y705}$</td>
<td>Abcam, Cambridge, Massachusetts, USA.</td>
</tr>
<tr>
<td>p-p21$^{T145}$</td>
<td>Abcam, Cambridge, Massachusetts, USA.</td>
</tr>
<tr>
<td>AKT</td>
<td>Cell signaling, Danvers, Massachusetts, USA.</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>Abcam, Cambridge, Massachusetts, USA.</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Abcam, Cambridge, Massachusetts, USA.</td>
</tr>
<tr>
<td>PARP1</td>
<td>Abcam, Cambridge, Massachusetts, USA.</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Cell signaling, Danvers, Massachusetts, USA.</td>
</tr>
</tbody>
</table>

4.2 Cell lines and culture conditions

The BT474 and SKBR3 breast cancer cells was used for the study were purchased from American Type Culture Collection (ATCC). SKBR3 cell line was maintained in the McCoy 5A medium containing 10 % FBS, 1 % penicillin/streptomycin. BT474 was maintained in RPMI 1640 containing 10 % FBS, 1 % penicillin/streptomycin. ATCC guidelines were followed during storing, seeding, growing and harvesting the cell lines. Standard incubation conditions at 37°C in 5 % CO$_2$ were followed for culturing the above-mentioned cell lines. The cell cultures were replenished with fresh medium every 2 to 3 days and passaged 1:3 when they reached about 70% confluence. The cells were regularly checked for *Mycoplasma* contamination. The cell growth was monitored using a light microscope and ZOE Imager. An
automated cell counter from Bio-Rad with cell counting slide was used to count the cells at different steps of the experiments.

4.3 Effect of ibrutinib on HER2 overexpressing breast cancer cell lines

4.3.1 Cell proliferation assay

The anti-proliferative effect of ibrutinib was assessed by MTT assay. MTT is a yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide that gets reduced by mitochondrial succinate in living cells forming insoluble dark purple colored formazan crystal. Normally organic solvents like DMSO solubilize the formazan product. Since the reduction of MTT can only occur in live cells, the level of reduction is a measure of the viability of the cells.

5 x 10³ cells/well (BT474 and SKBR3) were seeded in 96 well plates and incubated for 24hrs individually. After incubation, the cells were washed with fresh medium and treated with the desired concentration of ibrutinib dissolved in dimethyl sulfoxide (DMSO) for 72hrs. For vehicle control, the cells treated with DMSO were used. For negative control (blank) medium without cells was used. After treatment for 72hrs, the cells were incubated with 100 µl of medium containing 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) with 5mg/ml concentration at 37°C for 4hrs. Next, the MTT was removed from each well and replaced with 100 µl of DMSO to dissolve the formazan crystals, and the plates were read in synergy BioTek HT microplate reader at 570nm. The assay was performed as three independent experiments with each experiments containing technical triplicates. The mean value ± SD was calculated for each experiments. Cell viability was described as the ratio of absorbance (A570nm) of treated cells versus the ratio of absorbance(A570nm) of control cells. IC₅₀ was calculated as the concentration of ibrutinib needed to reduce 50% of absorbance relative to the vehicle (DMSO) and treatments using Graph-pad Prism6 software.
4.3.2 Flow cytometry – apoptosis validation

Flow cytometry analysis was carried out to assess the level of apoptosis in BT474 and SKBR3 cancer cells on treatment with ibrutinib. The assay was performed using Annexin V-propidium iodide kit. The cancer cells were seeded (1 x 10^6 cells/well) on 6 well plates (9 cm^2) individually to obtain 60–70% confluence in standard culture medium for 24 h. After treatment with IC_{50} concentration of ibrutinib for 72 hours, the cells were trypsinized, washed, and resuspended in 1x binding buffer. After resuspension, the samples were taken in small test tubes and incubated with 5µl Annexin V-FITC and 10µl of propidium iodide solution for 15 mins in dark. The whole experiment was conducted as per manufacturer's instruction (cat: APOAF - Annexin V-FITC Apoptosis Detection Kit, Sigma-Aldrich, St Louis, MO). Finally, the cells were analysed by flow cytometry (Cytoflex, Cytexpert, Beckman Coulter). The assay was performed as three independent experiments with each experiments containing technical triplicates. The best representative samples were used for analysis.

4.4 Effect of ibrutinib on BTK gene expression

4.4.1 BTK knockdown using siRNA

The siRNA BTK knockdown was carried out to suppress Bruton Tyrosine Kinase (BTK) gene expression. This assay was carried out to check ibrutinib's effect on HER2 overexpressing breast cancer after BTK gene knockdown., as ibrutinib were designed to target BTK specifically.

In our study, 2×10^5 cells/well were seeded in a 6 well plate with antibiotic free respective growth medium supplemented with FBS. The seeded cells were incubated at 37°C in a CO_2 incubator until they reach about 70% confluence, which usually takes 18-24hrs. 60 pmols of BTK siRNA were mixed with transfection medium and transfection reagent in 15ml Eppendorf tubes according to the manufacturer protocol. The mixture containing siRNA was incubated for 45 minutes at room temperature. In parallel, the cells seeded were washed with PBS and
made ready for transfection. After incubation of the siRNA mixture, the cells were transfected with medium containing the siRNA and incubated for 7 hr at 37°C in a CO₂ incubator. Finally, after transfection, the cells were resuspended with 2× normal growth medium. The whole transfection experiment was carried out based on Santa Cruz -siRNA transfection protocols. The transfected cells once replenished with the growth medium were treated with ibrutinib of desired concentration for 72hrs. The test was carried out in three different experiment with each containing technical triplicates to check the stable knockdown and effect of ibrutinib towards the cells. The validation of ibrutinib’s potential on multiple targets on both BT474 and SKBR3 cells were determined by comparing the results of quantitative RT-PCR and cell proliferation assay.

4.4.2 Reverse transcription and quantitative real time PCR

Quantitative real time PCR was carried out to determine the level of BTK gene expression based on Applied Systems cDNA protocol. First, the total RNA was isolated from the above experiment samples using TRIzol™ Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. After isolation purity of RNA was determined using NanoDrop™ and stored at 20°C for future use. Then, the total RNA from each sample was converted to cDNA using high capacity reverse transcriptase kit (Applied Biosystems). The Master mixture containing 0.8 µL of 25X dNTP (100 mM ), 2.0 µL of 10X RT Random Primers, 1 µL of MultiScribe™ Reverse Transcriptase, 2 µL of 10X RT Buffer, and 4.2 µL of DNase-free ddH₂O (total 10 µL) was used for cDNA conversion. 10µl of the above master mix and 10µl of total RNA(2µg) was added to obtain 20µl of reaction volume.

In order to perform this reaction, the temperature setup of the Thermo Cycler was set for 10 min at 25 °C, 120 min at 37 °C, 5 min at 85 °C. The resulting cDNA was stored at -20°C.

Finally, the real-time PCR reaction was performed using Bioline Sensifast SYBR green master mix. The BTK gene forward primer sequence 5’-GGTGGAGAGCGACGAGATAAA-3’ and
reverse primer sequence was 5'-CCGAGTCATGTGTTGGAATAC-3'. GAPDH was used as the control. The reaction was carried out as 50 cycles of amplification (5 sec at 95 °C, 10 sec at 60 °C, 15 sec at 72 °C). The StepOnePlus™ software (applied biosystem) was used to calculate the mean ct value for both GAPDH and BTK expression. The Δct method calculation was performed to find the relative gene expression (2-ΔΔct).

4.5 Nuclear morphology analysis / DAPI staining

DAPI (4',6-diamidino-2-phenylindole) is a blue-fluorescent DNA stain that targets the minor groove of double-stranded DNA. Upon binding to the AT region it exhibits 20 fold increased fluorescence. In our study, BT474 and SKBR3 cells were seeded at a cell density of 2x 10^5 cells on 35-mm cell culture plate and treated with IC50 of ibrutinib for 72hrs. Following treatment, cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS and mounted in 20 µl of Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) for 10 min and the nuclei were observed using ZOE Fluorescence Cell Imager (Bio-Rad).

4.6 Effect of multiple kinases

To check the effect of ibrutinib on cellular signaling patterns like phosphorylation events of multiple kinases and signaling proteins we employed kinome profiling (peptide array) a high-throughput analysis.

4.6.1 Peptide array

The peptide array chip used for our analysis was customized in Dr. Scott Napper's lab. The customization includes cancer-associated phosphorylated proteins that are differentially regulated in various types of cancers such as breast cancer, renal cell carcinoma, pancreatic cancer, and prostate cancer. The peptide array chip contains 1290 peptides specifically selected to identify the major signaling pathways involved in proliferation, metabolism, and apoptosis. DAPPLE 2 software was used for customization of peptide array. It identifies the
post-translational modification sites in human through homology-based prediction using mouse or fungi[146, 147]

**Fig 4.1 A schematic representation of peptide array process adapted from reference[137]**

Briefly, $1 \times 10^7$ cells of BT474 and SKBR3 both control and ibrutinib treated samples were collected and pelleted in 15ml Eppendorf tubes. The pellets were lysed using 100µl lysis buffer at 4°C for 10 min. The composition of lysis buffer is included in the Table 4.3. After lysis, the cells were centrifuged at 10,000 rpm for 10min at 4°C and the supernatant was collected. 70µl of the supernatant from each samples was mixed with 10µl activation mix containing 50% glycerol, 500µM ATP, 60mM MgCl$_2$, 0.05% (v/v) Brij 35, and 0.25mg/ml BSA. The mixture was then incubated in the array for 2hrs at 37°C and finally washed with 1% Triton X-100 and PBS. After, washing the peptide array slides were immersed in phosphospecific fluorescent ProQ Diamond phosphoprotein stain (Invitrogen, Canada) for 1 hr. After incubation, the slides were de-stained using 20% acetonitrile and 50mM sodium acetate at pH 4.0 for 10 minutes.
followed by rinsing the slides for 2 min with deionized water. Finally, the washed arrays were air dried for 20 min and then centrifuged at 300×g for 2 min to remove the remaining moisture. The arrays were analysed using GenePix Professional 4200A microarray scanner (MDS Analytical technologies, Canada.). Professional GenePix software (version 6.0) was used to collect spot intensity signal by comparing the differences between mean pixel intensity and the background intensity.\textsuperscript{138, 148} (Fig 4.1)

Table 4.3 Peptide array lysis buffer concentration

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 7.5</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>EGTA</td>
<td>1 mM EGTA</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1%</td>
</tr>
<tr>
<td>sodium pyrophosphate</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>sodium orthovanadate</td>
<td>1 mM</td>
</tr>
<tr>
<td>sodium orthovanadate</td>
<td>1 mM</td>
</tr>
<tr>
<td>sodium fluoride</td>
<td>1 mM</td>
</tr>
<tr>
<td>leupeptin</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>aprotinin</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>Phenylmethysulphonyl fluoride</td>
<td>1 mM</td>
</tr>
</tbody>
</table>
4.7 Data analysis

4.7.1 Differential expression in Phosphoproteins (DEPs)

After peptide array experiments the raw data obtained based on the spot intensities were processed using free web-based platform PIIKA 2 package. The software computes P-values and beta values for each phosphorylated protein by comparing the ratio of average phosphorylation expression between the control (untreated sample) and treatment (ibrutinib treated sample), based on Benjamin-Hochberg false discovery rate. This comparison gives us the differentially expressed proteins.

Finally, the differentially expression in phosphoproteins (DEPs) were plotted as volcano plots and heat map to identify the differences in expression of DEPs between two HER2 overexpressing cell lines. The heat maps are reported as one of the best ways to hierarchically cluster the proteins to acquire fold change differences among DEPs. The heatmap was plotted using bioinformatics tool -Heatmap.2 function in R/Bioconductor package “gplots” (http://cran.fhcrc.org/web/packages/gplots/gplots.pdf).

4.7.2 Functional gene annotation (Gene Ontology)

The data was submitted to the Funrich open access standalone tool for enrichment analysis. It is a bioinformatics platform designed for functional enrichment and interaction network analysis of gene and proteins. The tool allows us to depict Venn diagram and obtain information about gene ontology functions such as biological processes, molecular functions and cellular components of DEPs. GO terms with P≤0.05 were selected as significantly enriched and plotted using Microsoft excel.

4.7.3 Pathway analysis of significant DEP

The pathway analysis in both HER2 overexpressing cell lines was performed using InnateDB, a public platform which contains a database of genes, proteins and signaling pathways involved in humans, mice, and bovines to microbial infections. DEPs expression data obtained from
PIIKA software was used to identify enriched pathways. Over Representation Analysis (ORA) tool in InnateDB predicts biological pathways for DEPs. P-values were assigned to all the pathways based on the number of proteins and their fold change differences present in a particular pathway. The proteins in the significant pathways were mapped on to the pathway map using Pathview tool (KEGG pathway database platform) for the identification of the location of the protein in the pathway.

4.8 Validation of protein expression on ibrutinib treatment using western blot

4.8.1 Sample preparation

1×10⁶ cells/well of (SKBR3 and BT474) cells were seeded in 6 well plates with normal growth medium for 24hrs to obtain 70% confluency. After, 24hrs, the cells were washed and replaced with fresh media containing 0.1µm ibrutinib. The experimental samples were collected on various time points until 72hrs. The cells were trypsinized, lysed with RIPA buffer containing phosphatase inhibitors and centrifuged at 12000 rpm for 20 min. The supernatant containing proteins were quantified using a standard BCA protein estimation kit (Pierce™ BCA Protein Assay Kit). Protein estimation assay was carried out based on the manufacturer's protocol, 10µl of standards and proteins were aliquoted in 96 well plate containing working reagent of 190µl. The samples were incubated for 30min at 37°C with 5% CO₂. The absorbance was measured in BioTek spectrophotometer at 562nm. The standard graph was plotted and the unknown concentration of proteins was calculated. The proteins were mixed with Lammeli buffer required for the gel electrophoresis. Finally, the protein samples were stored in -80°C for further use.
4.8.2 Western blot technique

SDS-gel electrophoresis was performed to separate proteins based on the molecular weight. 12-15% gel was prepared to get the precise location of the protein bands. Around 30µg of protein was loaded in each well, once loaded the samples were separated with 1X running buffer containing SDS, at 100V for 2hrs using Bio-Rad electrophoresis unit. After 2hrs the separated proteins in the gel were transferred to PVDF membrane using Bio-Rad western blot unit. The transfer materials include, filter paper, membrane, protein gel, sponges. These materials were soaked in 1X transfer buffer for 15 min. However, before soaking the PVDF membrane in transfer buffer, was activated using methanol for 30 sec. The transfer sandwich prepared was placed in the western blot unit with 1X transfer buffer and kept for overnight transfer with 35V at 4°C.

After the transfer of protein to the membrane, the membrane was blocked with 5% skim milk dissolved in TBST buffer for 1hr. This step is crucial to avoid non-specific binding of the desired antibodies. After blocking, the membrane was washed with 1X TBST buffer three times for 15 min. The membrane was incubated for 12hrs with primary antibody (prepared in 2% BSA dissolved in 1x TBST) at 4°C. After incubation with the primary antibody, the membrane was washed three times with 1x TBST for 15 min and incubated with HRP-conjugated secondary antibody for 1hr at room temperature. Finally, after incubation, the membrane was washed again with 1x TBST for 15min. The membrane was finally developed using Bio-Rad ECL-clarity™ substrate in Bio-Rad chemiluminescence imager. Primary antibodies used were anti p-STAT3 Y705 (1:2000; Abcam), anti p-p21 T145 (1:1000, Abcam), anti-AKT (1:1000; Cell Signaling Technologies), anti-Caspase 8 (1:1000; Abcam), anti-Caspase3 (1:1000; Abcam), anti-PARP-1 (1:1000; Abcam) and anti-GAPDH (1:5000; Cell Signaling Technologies), respectively and GAPDH served as control.
CHAPTER 5: RESULTS

5.1 Growth inhibition of HER2 overexpressing breast cancer cell lines by ibrutinib

To investigate the cytotoxic activity of ibrutinib on breast cancer cell lines - SKBR3, BT474 and MDA MB 231. Cell viability of the three cell lines was assessed using MTT cell proliferation assay, as described in the section (4.3.1).

BT474 (ER+, PR+, HER2+), SKBR3 (ER+,PR+,HER2+), and MDA MB 231 (ER-,PR-,HER2-) cells were exposed to ibrutinib for 24, 48 and 72hrs at concentrations of 2, 5, 10, 30, and 50 nM, respectively. The relative IC₅₀ values for BT474 and SKBR3 were found to be 9.94 nM and 8.89 nM at 72hrs. However, for MDA MB 231, IC₅₀ value could not be determined as there were no significant difference in cell viability. So we preferred to proceed with the BT474 and SKBR3 cell lines. (Fig 5.1). The inhibitory concentration was calculated using GraphPad prism 6 statistical software.

![Fig 5.1 Ibrutinib inhibiting breast cancer cell lines BT474, SKBR3 and MDA MB 231. The log concentration of ibrutinib was plotted in X-axis and cell viability % in Y-axis. The experiment was carried out in biological triplicates using 96 well plates. GraphPad prism 6 software was used to calculate statistical mean, error bar, standard deviation (SD) and relative IC₅₀ value.](image-url)
5.2 Ibrutinib inhibits cell proliferation through apoptosis

Cell proliferation assay indicated that ibrutinib would be a viable therapeutic option for HER2 overexpressing breast cancer lines SKBR3 and BT474. However, to investigate the mechanism of cell death, Annexin V/PI cell death assay using flowcytometry was performed. The Annexin V is a fluorescent compound that stains phosphatidylserine molecules on the surface of the cell membrane. When the cell initiates apoptosis the phosphatidylserine from the inner surface of the plasma membrane translocate itself to the outer surface of the plasma membrane which makes Annexin V bind and distinguishes the early stage of apoptosis. On the other hand, necrotic cells are permeable to propidium iodide. The propidium iodide can also conjugate with Annexin V and differentiate the final stage of apoptotic cells. This dual staining of fluorescence-activated cell sorter (FACS) analysis allows us to distinguish between live cells (stained negative for both annexin V-FITC and PI), early apoptotic cells (stained positive for annexin V-FITC and negative for PI), and late apoptotic-- or dead cells (stained positive for both and annexin V-FITC and PI). The protocol of this experiment has been outlined in section 4.3.2.

Our analysis has revealed that, in ibrutinib-treated BT474 cells, 43.09% of the cells were viable, 37.76% were in early apoptosis and 17.61% were in the late or final stages of apoptosis. Whereas in untreated BT474 78.04% of cells were viable, 7.51% were in early apoptosis, 11.33% cells were in late or final stages of apoptosis (Fig 5.2). Similarly, in ibrutinib-treated SKBR3 cells 32.23% of the cells were viable, 40.74% were in early apoptosis and 23.92% were in the late or final stages of apoptosis. Whereas in untreated SKBR3 cells 58.50% of the cells were viable, 20.06% of cells were in early apoptosis and 15.67% of the cells were in late or final stages of apoptosis (Fig 5.2). These results suggest that ibrutinib is effective in inhibiting cell proliferation of both cell lines by apoptosis.
Fig 5.2 FACS analysis for BT474 and SKBR3 cells treated with ibrutinib.
Scatterplot showing the percentage of ibrutinib treated BT474 and SKBR3 cells undergoing apoptotic change compared to the untreated cells. Staurosporine was used as a positive control.
5.3 BTK is not the only target for ibrutinib

As ibrutinib is a tyrosine kinase inhibitor, it has multi targets apart from BTK. To confirm this, we performed siRNA knockdown of BTK and then treated with ibrutinib.

5.3.1 siRNA knockdown BTK

Both SKBR3 and BT474 cell lines were transfected with BTK siRNA. The whole experiment was carried out as described in section 4.3.1 and 4.3.2. A stable knockdown was achieved after siRNA treatment, which was further confirmed using quantitative real time-PCR (Fig 5.3 A). On the other hand, the proliferation of both cell lines was also detected using the MTT assay. From our analysis, it was inferred that by quantitative real time-PCR, there is a reduction in the expression of BTK on treatment with ibrutinib. This could be attributed to the silencing of BTK gene by ibrutinib. The quantitative real time-PCR revealed that in BT474 and SKBR3 cell lines the siBTK treated cells, ibrutinib treated cells, siBTK+ibrutinib treated cells showed significant reduction in BTK gene expression in comparison with control samples. (Fig 5.3A). However, only in SKBR3 cells alone the combination (siBTK +ibrutinib) showed significant reduction in expression of BTK in comparison with siBTK and ibrutinib samples. These results are complemented by MTT assay for each of the treatments (Fig 5.3 B). The cell proliferation assay (MTT) revealed that in both the cells lines ibrutinib treated cells and siBTK and ibrutinib combination treated cells had significant reduction in cell population in comparison with siBTK and control samples. However, in both the cell lines there is no significant change in cell viability between ibrutinib and siBTK+ibrutinib treated cells. These findings suggest in absence of BTK cell death occurs which proves the multi target ability of ibrutinib in the two cell lines under investigation.
Fig 5.3 The siRNA knockdown was supported by quantitative RT-PCR gene expression analysis and cell proliferation assay. A. The bar diagram represent BTK relative gene expression, plotted in Y-axis and the data was normalized with GAPDH in both SKBR3 and BT474 cell lines. The figure compares, the control cells, siBTK transfected cells, the ibrutinib treated cells and siBTK transfection + ibrutinib treated cells. Data represent the means ±S.D. of three independent experiments. **P < 0.01, ***P < 0.001, compared with control, ## P< 0.001 compared with siBTK and $$$ P< 0.001 compared with ibrutinib. Statistical analysis include 2-way ANOVA with Turkey’s multiple comparison test using GraphPad prism.

B. The bar diagram represents MTT cell proliferation assay at 72 hours, comparing the normal breast cancer cells, siBTK transfected cells, the ibrutinib treated cells and siBTK transfection + ibrutinib. The experiment were carried out in 6 well plates with 0.1µM ibrutinib and the siRNA concentration (60 pmols) were based on the manufacturer's protocol. **P < 0.001, ***P < 0.001, compared with control and ## P<0.001 compared with the siBTK, Statistical analysis include 2-way ANOVA with Turkey’s multiple comparison test using GraphPad prism.
5.3.2 Expression of BTK in various cancer

The Cancer Genome Atlas (TCGA), is a database for cancer genomics. The database was created by joint effort between National Cancer Institute and National Human Genome Research Institute began in 2006. TCGA is a data portal that contains 20,000 primary cancer samples spanning 33 types of cancer. These samples are molecularly characterised by comparing with matched control samples. According to the expression data from The Cancer Genome Atlas (TCGA) BTK expressed in 33 types of cancer. The graph obtained from TCGA shows increased expression of BTK in diffused large B-cell lymphoma (DLBC) and acute myeloid lymphoma (LAML) compare to other type of cancer (Fig 5.4). This indicates that BTK is not a key gene for breast cancer.

**Fig 5.4** BTK gene expression in 33 types of cancer obtained from TCGA database. The boxplot is plotted with TCGA samples on X-axis and log2 fold change on Y-axis. Transcripts per million (TPM). Data extracted using http://ualcan.path.uab.edu/
5.4 Ibrutinib causes changes in nuclear morphology

Further, we assessed the effect of ibrutinib on induction of DNA fragmentation of tumor cells. Treatment of BT474 and SKBR3 cell lines with IC$_{50}$ concentration of ibrutinib for 48hrs and 72hrs induced DNA fragmentation. Apoptotic cells with condensed and fragmented nuclei were visualized by staining with 4′,6-diamidino-2-phenylindole (DAPI) in ZOE florescent cell imager (Fig 5.5)

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**Fig 5.5 Ibrutinib treatment induces DNA fragmentation.**

DAPI-stained micrographs for control, 48hrs and 72hrs drug exposure in BT474 and SKBR3 cells using a ZOE fluorescent cell imager. The control cells showed intact nuclei of uniform shape and size with smooth edges, whereas the ibrutinib treated cells showed nuclei with chromatin condensation and cracking (white arrow).
5.5 Peptide array

To further investigate the biological role of ibrutinib action towards HER2+ breast cancer cells, we performed peptide array analysis and 1290 peptides for each cell lines were considered. Out of 1290 phosphoproteins in the array we identified 407 phosphoproteins were differentially expressed in SKBR3 and BT474 with P-value <0.05 (t-test comparing treatment and control) when treated with ibrutinib. These phosphoproteins were considered to be differentially expressed.

5.5.1 Differential phosphoproteins of SKBR3 and BT474

The normalization of array read count (RAW data) and statistical analysis was based on standardised algorithm that discovers quantitative changes in expression levels between experimental group is called differential expression. From our analysis on BT474 and SKBR3 cell lines, we found that among differential expression in phosphoproteins (DEPs) 209 phosphoproteins are common between BT474 and SKBR3. Apart, from this BT474 had 192 distinct phosphoproteins and SKBR3 had 72 distinct phosphoproteins (Fig 5.6 A). The fold change expression was measured based on the differential expression in phosphoproteins (DEPs). It was designated as upregulated or downregulated by comparing the relative mean signal of the corresponding peptide from untreated cells (control cells). To depict this data volcano plots were plotted (Fig 5.6 B) and hierarchical clustering (Heatmap) of all phosphoproteins (Fig 5.7.) was performed. The volcano plot and venn diagram were plotted using FunRich tool, a bioinformatics web-based platform exclusive for functional enrichment analysis. On the other hand, the Heatmap was also generated using R-platform (g plots package) programming platform to assess DEPs data. Thus, these plots give data on differences in expression of proteins between these two cell lines on treatment with ibrutinib. Thus, the DEPs could be our potential targets.
Fig 5.6: Venn diagram and Volcano plot showing HER2 overexpressing breast cancer cells SKBR3 and BT474.

(A) The Venn diagram represents the DEPs that are common and unique in the two cell lines.

(B) Volcano plots showing the distribution of the fold changes in DEPs. Phosphoproteins with fold change $\geq 1.00$ and $\leq 1.5$ are shown in red, $\geq 1.5$ as purple, $\geq -1.00$ and $\leq -1.5$ as blue and $\leq -1.5$ as green.
**Fig 5.7 Heatmap comparing HER2 overexpressing breast cancer cell lines.**

Heat map representing hierarchical clustering of the DEPs between BT474 and SKBR3. The plot shows the differences in expression pattern of phosphoproteins (upregulation and downregulation) among these cell lines when treated with ibrutinib. The left panel shows the level of phosphorylation of DEPs in BT474 and the right panel shows the level of phosphorylation in SKBR3. The data is displayed in a grid where each row represents a protein on the array. The intensity of the colour in the panel represents changes (not absolute values) of phosphorylation.
5.5.2 Gene ontology and pathway analysis

The gene ontology (GO) is a major bioinformatics initiative to assess the representation of properties of gene and gene products. Gene ontology is categorized in three domains namely cellular component, molecular function and biological process. The cellular component represents parts of the cell and its extracellular environment. The molecular component represents the elemental activity of a gene product at the molecular level such as protein binding, phosphorylation etc. The biological process represents operations or sets of molecular events with a defined beginning and end, applicable to the functioning of integrated living units such as cells, tissues, organs, and organisms. The subcategories in each domain of gene ontology are represented as GO-Term.

Based on p-value (Hypergeometric test) we categorised the molecular functional association and biological role of the phosphoproteins, by performing functional enrichment analysis using Funrich tool.

From our DEPs data, we analysed top 12 enriched Go-Ter ms in all three domains of gene ontology in both the cell lines (Fig 5.8). The analysis revealed that in SKBR3, protein phosphorylation, signal transduction, and negative regulation of apoptotic process are the most common GO categories \( (P \leq 0.05) \) represented in biological processes; Protein binding, protein kinase activity, and metal ion binding are the most common GO categories in molecular function and plasma membrane, cytoplasm, and nucleus are the most common categories in cellular location. In BT474, protein phosphorylation, signal transduction, and positive regulation of transcription from RNA polymerase II promoter are the most common GO categories \( (P \leq 0.05) \) represented in biological processes; ATP binding, protein kinase binding, and metal ion binding are the most common GO categories in molecular function and cytoplasm, plasma membrane, and nucleus are the most common categories in cellular location.
However, gene ontology does not provide exact information on function of the proteins unless it is coupled with pathway enrichment analysis data. Pathway enrichment analysis identifies significant biological pathways that are enriched in protein data sets. The study was performed using InnateDB a public online database with DEPs obtained from SKBR3 and BT474 cells. Based on p-value top 10 biological pathways were identified using this tool (Fig 5.9) The top four most represented biological pathways are EPO signaling pathway, IL-7 signaling pathway, VEGF signaling pathway, and JAK-STAT. We further mapped the pathway proteins in the respective pathways using path view tool (Fig 5.10). Since JAK-STAT pathway was of our interest, the genes in the JAK-STAT pathway are presented in a circos plot (R Package). The circos plot revealed JAK-STAT pathway relation with associated Go-terms such as MAPK cascade, apoptosis, protein phosphorylation, etc. The circos plot of biological processes among both cell lines revealed that some JAK STAT proteins are involved in apoptotic process. (Fig 5.11, 5.12, 5.13, 5.14, 5.15, 5.16)
Fig 5.8 Gene ontology- BT474 and SKBR3 Representation of top 12 enriched Go-Term in both cell line, Go terms (biological process, cellular components, Molecular function) on X-axis and respective gene count on Y-axis
Fig 5.9 Pathway enrichment using InnateDB BT474 and SKBR3
Representation of top 10 enriched pathways between HER2 overexpressing cell lines. **A, B:** Significant pathways on X-axis and respective protein count on Y-axis.
Fig 5.10 JAK-STAT pathway shared between BT474 and SKBR3.
The pathway mapping was done using Path view KEGG tool (R-platform). Both SKBR3 and BT474 phosphoproteins were mapped together in JAK-STAT pathway. Red color represents upregulation and Green color represents downregulation.
Fig 5.11 Circos plot representing gene ontology terms enriched in the JAK-STAT pathway for biological process and the associated genes in SKBR3 cell lines. The width of the chord represent the number of proteins linked with the respective biological process.
Fig 5.12 Circos plot representing gene ontology terms enriched in the JAK-STAT pathway for biological process and the associated genes in BT474 cell lines. The width of the chord represent the number of proteins linked with the respective biological process.
Fig 5.13 Circos plot representing gene ontology terms enriched in the JAK-STAT pathway for molecular process and the associated genes in SKBR3 cell lines. The width of the chord represent the number of proteins linked with the respective molecular process.
Fig 5.14 Circos plot representing gene ontology terms enriched in the JAK-STAT pathway for molecular process and the associated genes in BT474 cell lines. The width of the chord represent the number of proteins linked with the respective molecular process.
Fig 5.15 Circos plot representing gene ontology terms enriched in the JAK-STAT pathway for cellular components and the associated genes in SKBR3 cell lines. The width of the chord represent the number of proteins linked with the respective cellular components.
Fig 5.16 Circos plot representing gene ontology terms enriched in the JAK-STAT pathway for cellular components and the associated genes in BT474 cell lines. The width of the chord represent the number of proteins linked with the respective cellular components.
5.6 Western blots

Following the bioinformatic approach of identifying the pathways and the key regulating proteins responsible for ibrutinib action towards HER2 overexpressing cell lines, targets and pathways were validated by Western blot.

5.6.1 Effect of ibrutinib on STAT3-p21 axis and the apoptotic pathway

The HER 2 overexpressing breast cancer cells, SKBR3 and BT474 were treated with ibrutinib (0.1µmol/L) at various time points to determine the effect of ibrutinib on expression of phosphoproteins using western blots. Western blot analysis showed that ibrutinib has increased the phosphorylation of p-STAT3Y705, whereas ibrutinib has decreased the phosphorylation of p-P21T145. However, AKT showed no significant change after treatment with ibrutinib. This is in consistent with previous reports.24 It was observed that the level of expression of pro-caspase-8, pro-caspase-3, and PARP-1 decreased on treatment with ibrutinib. PARP1 antibody had significant differences where, it undergoes cleavage indicating apoptosis is caspase dependent. The western blot results are presented in Fig 5.17.
Fig 5.17 Changes in expression of p-STAT3, p-p21, AKT, pro-caspase 3, pro-caspase 8, PARP1 proteins in BT474 and SKBR3 cells - Cells were treated with 0.1 µM Ibrutinib for 6hrs, 12hrs, 24hrs, 48hrs and 72hrs respectively. GAPDH is the used as loading control. The images are representative gels from different experimental replicates.
CHAPTER 6: DISCUSSION

The overall treatment response rates for HER2+ breast cancer are unfortunately low and have undesirable side effects\textsuperscript{152}. Hence, efficient treatment options are required to enhance the survival of patients with breast cancer. Targeted therapy is one such treatment option which uses small molecule drugs and/or monoclonal antibodies to target cancer cells. Ibrutinib, a TKI is known for its anticancer effect on B-cell malignancies. Recent reports suggest that ibrutinib shows inhibitory effect on HER2+ breast cancer cell lines. Kinome analysis using peptide array platform can be used for identification of biomarkers in cancer and several diseases. It is a high throughput technology, similar in design of gene microarray chip. Here we show for the first time using kinome array platform for screening phosphoproteins involving kinases in HER2 overexpressing breast cancer cell lines after treatment with ibrutinib. In our study, we have extended the previously reported inhibitory action of ibrutinib towards HER2 overexpressing breast cancer by identifying its molecular mechanism of action.

6.1 Ibrutinib, BTK and HER2 in breast cancer.

Our investigation revealed that BTK is not the only target of ibrutinib. The siRNA knockdown of BTK does not affect cell proliferation significantly. On the contrary, ibrutinib on its own and ibrutinib combination with siBTK showed significant reduction in cell viability of both the cell lines (Fig 5.3). This states that in absence of BTK the cell death occurred. Hence it proves the multi target ability of ibrutinib.

According to The Cancer Genome Atlas (TCGA) database (UALCAN) analysis reveals that BTK is expressed in 33 types of cancers\textsuperscript{20}. However, BTK is significantly overexpressed only in B-cell malignancies in comparison to other types of cancer (Fig 5.4). This suggest that BTK is not a key gene in breast cancer.

Ibrutinib has been reported to reduce the phosphorylation of the receptor tyrosine kinases HER1, HER2, and HER3 along with its downstream targets such as BTK, AKT, ERK. in HER2
positive breast cancer cell lines \(^{22,85}\). It has also been reported that ibrutinib causes reduction of phosphorylation in histone H3 and it increases cleaved caspase 3 signals resulting in inhibition of growth in HER2+ breast cancer xenograft model \(^{85}\).

Chen et al also performed a high-throughput screening of two panels of tumor cell lines 230-cancer cell panel, the NCI-60 panel and other breast cancer cell lines (MDA-MB-453, SK-BR-3, and UACC-893) with ibrutinib treatment. The experiment was carried out to check growth inhibitory activity against these cell lines. It was reported that ibrutinib (<100 nmol/L) inhibits the growth of these cell lines with IC\(_{50}\) lower than that of lapatinib and dacomitinib \(^{24}\). They have also reported that ibrutinib shows antiproliferative activity towards several breast cancer cell lines expressing HER2 protein except for MDA-MB-361. In parallel, Wang et al reported that sequence alignment results of 10 kinases (Blk, Btk, Bmx, EGFR, HER2, ERBB4, Itk, JAK3, Tec, and Txk) in the human kinome could be potential targets of ibrutinib as they have a cysteine residue at an analogous position to the cysteine residue (Cys-481) near the ATP-binding pocket of BTK \(^{85}\). So far ibrutinib is marketed only for B-cell malignancies. However, many reports suggest ibrutinib has multi target potential. From our study, ibrutinib is also effective in HER2 overexpressing cell lines. Hence, we suggest that BTK is not the only target of ibrutinib in HER2 overexpressing breast cancer cell lines.

6.2 Effect of ibrutinib on signaling pathways

Pathway over-representation analysis (ORA) from our kinome data, shows that proteins in EPO signaling superfamily, IL-7 immune system superfamily, VEGF signaling, and JAK-STAT signaling superfamily are predominately represented in the ibrutinib treated cell lines data. Interestingly, we observed that for both EPO and IL7 receptor proteins, JAK-STAT pathway is the downstream target Fig 6.1. Furthermore, the JAK-STAT signaling contains a crucial transcription factor STAT3, which controls the expression of about 103 genes in the our dataset. Also, the JAK-STAT pathway is not reported as of today as a possible target for
ibrutinib. Apart from JAK-STAT pathway, erythropoietin (EPO) binding with erythropoietin signaling receptor (EPOR) plays a crucial role in erythropoiesis. It has been reported that over expression of EPOR in gastric cancer tissues and cells promotes tumor proliferation and survival. However, downregulation of EPOR by siRNA in gastric cancer does not affect cell proliferation significantly. Similarly, interleukin 7 (IL7) is a cytokine and plays an important role in T-cell and B-cell development. It has been reported that IL-7 shows antitumor effects in tumors such as leukemia, melanoma, lymphoma, prostate cancer, glioma, and glioblastoma. On the other hand, the Vascular Endothelial Growth Factor receptor signaling (VEGF) plays an important role in induction of angiogenesis. Monoclonal antibodies and small molecule inhibitors are used to block VEGF receptor and curtail angiogenic activities resulting in vascular regression in tumors. However, the lack of efficacy and side effects are a potential challenge in targeting VEGF signaling pathway proteins. Hence, we chose, JAK-STAT pathway as a potential pathway for action of ibrutinib on HER2 overexpressing breast cancer cell lines BT474 and SKBR3.
Fig 6.1 EPO and IL-7 signaling with common JAK-STAT downstream targets adapted from reference 156, 157
6.3 Ibrutinib causes apoptosis in HER2 overexpressing breast cancer

6.3.1 Caspase 8 and Caspase 3 role in HER2 overexpressing cell lines

Flow cytometry analysis showed apoptosis as a mechanism of cell death in both the HER2 overexpressing cell lines. Apoptosis initiation is caused by various pathological and physiological stimuli involving series of proteins. The results we obtained from western blot showed that ibrutinib induced apoptosis via caspase-dependent apoptosis pathway showing changes in expression of pro-caspases-8, pro-caspase-3, and PARP1 (Fig 5.17). Wang et al have also reported that ibrutinib cleaves procaspase 3 leading to caspase dependent apoptosis. As per apoptotic mechanism caspase 8 and caspase 3 cleavage indicate extrinsic mode of apoptotic cell death.

6.3.2 Ibrutinib affects DNA repair mechanism -PARP1

PARP1 poly (ADP-ribose) polymerase-1 is a nuclear protein. Its interaction with DNA is necessary for the catalytic centre organization. PARP1 is a the key enzyme associated with the repair of damaged DNA. The repair mechanism involves adding poly (ADP) polymers in response to cellular stress. PARP-1 cleavage acts as a signature biomarker for indicating DNA damage. Recent reports suggest that almost all caspases alter PARP-1 and hence PARP cleavage is considered to be a hallmark of apoptosis.

When ibrutinib was treated with HER2 overexpressing cell lines, we observed changes in nuclear morphology on staining DAPI. The changes in nuclear morphology is usually due to DNA damage. Hence, PARP-1 cleavage was analysed in these cell lines and our data showed PARP1 cleavage. This indicates DNA damage and caspase dependent apoptosis. From these results we suggest that ibrutinib possesses the ability to activate the effector caspases through an extrinsic pathway and affects DNA repair mechanism which may be one of the factors leading to apoptosis.
6.4 STAT3 role in HER2 overexpressing breast cancer

In our study p-STAT3<sup>Y705</sup> was constitutively active in the control cells. However, when cells were treated with ibrutinib there was an increase in the expression of p-STAT3<sup>Y705</sup> (Fig 6.2). Furthermore, the peptide array results show increased expression of CyclinD1 on treatment with ibrutinib. Chen et al. have reported that ibrutinib treatment in HER2 overexpressing breast cancer cell lines causes growth inhibition by arresting the cells in G1 phase of cell cycle and halt its progression <sup>24</sup>. According to literature reports cyclin D1 and c-Myc are required for regulation of G1 phase in cell cycle<sup>161</sup>. The constitutively activated STAT3 has been reported to be associated with activation of cyclinD1 and c-Myc expression<sup>162</sup>. This indicate that STAT3 acts as a driving a promotor for cell cycle progression. Reports suggest that STAT3 suppresses apoptosis in cancerous cells. On the other hand, several studies also indicate that STAT3 activation exerts tumor suppressor effects under specific conditions<sup>163-167</sup>. Studies have shown that when cells undergo DNA damage there is increase in expression of various proteins such as IL-6, p-JAK1 and p-STAT3. It is reported that, increase in expression is due to a response against the DNA damage to promote growth recovery process <sup>168</sup>. These results and reports coupled together suggest that STAT3 activation is a passive response against DNA damage to promote cell cycle.
6.5 The role of p21 in HER2 overexpressing breast cancer cell lines

Our investigations show that p21\textsuperscript{T145} is downregulated upon ibrutinib treatment in HER2 overexpressing cell lines. The western blot data shows a decrease in phosphorylation of p21 on treatment with ibrutinib, indicating that there is a decrease in expression of p21 resulting in cell cycle arrest and apoptosis. p21 act as a mediator of cell cycle arrest. In response to DNA damage in the cell p21 inactivates G1 phase cyclin/CDK complex of the cell cycle and inhibit DNA replication process\textsuperscript{111}. This process helps the cell to activate DNA repair mechanism as the cell cycle is halted or delayed by p21. Thus, p21 plays a mediator role in DNA repair mechanism.

Furthermore, reports also suggest that loss of p21 in DNA damaged cell undergo cell cycle arrest leading to apoptosis. This indicates that p21 inhibits apoptosis\textsuperscript{169}. However, other reports suggest that overexpression of p21 promotes apoptosis in chemical induced cancer cells or mutant or non-functional p53 cells\textsuperscript{170}. The dual (pro and anti-apoptotic) role of p21 is reported to depend on the nature of apoptotic stimulus and subcellular location of p21 protein\textsuperscript{171}.

p21 can block activation of procaspase 3 and other stress induced kinase which leads to inhibiting apoptosis when p21 is localised in cytoplasm. Reports also state that T145 phosphorylation of p21 can induce the relocalization of p21 from nucleus to cytosol inducing antiapoptotic activity\textsuperscript{171}. The growth inhibition or tumor suppression activity of p21 is associated with nuclear localization. However, the exact mechanism needs further elucidation \textsuperscript{169, 172-174 (Fig 6.2)}. Thus, we suggest that p21 induces cell cycle arrest due to DNA damage and promote apoptosis mediated by caspase-3, caspase-8, and PARP1 cleavage in HER2 overexpressing breast cancer cell lines.
Fig 6.2 Proposed mechanism of ibrutinib on HER2 overexpressing cell lines. The red arrows indicate upregulation and downregulation of differentially expressed phosphoproteins the question mark indicate the unknown proteins links yet to be identified. From our study we propose that STAT3 upregulation is a passive response as a result of an induction of DNA damage on treatment of ibrutinib and downregulation of phosphorylated p21 promotes cell cycle arrest and apoptosis in the two HER2 overexpressing cell lines.
CHAPTER 7: CONCLUSION

In this study, we showed that ibrutinib exhibits cytotoxic effect on HER2 overexpressing breast cancer cell lines BT474 and SKBR3. Flow cytometry and nuclear morphology analysis has suggested that ibrutinib causes apoptosis and nuclear degradation. Furthermore, the multi-target ability of ibrutinib was validated using siRNA knockdown experiment. To seek additional information about the molecular mechanisms of action of ibrutinib in HER2 overexpressing breast cancer cell lines, we performed kinome analysis and identified the significant pathways that lead to cell death by apoptosis. The JAK-STAT pathway along with its downstream p21 pathway was chosen as the pathways of choice for further investigation of the mode of action of ibrutinib. The results of changes in protein expression of STAT3, p21, caspase 8, caspase 3 and PARP1 on treatment with ibrutinib suggest the possible mechanism of action of ibrutinib in HER2 overexpressing breast cancer cell lines is through STAT3-p21 axis via extrinsic pathway (caspase 8, caspase3 and PARP1) leading to apoptotic cell death. We further, suggest that upregulation of STAT3Y705 acts as a passive response against DNA damage that occurs in the cells to promote cell cycle progression. In parallel, downregulation of p21T145 induce cell cycle arrest to promote apoptosis.

STAT3 inhibitors have shown to exhibit growth suppressive effect in pancreatic and breast cancer cells. Also reports have stated that pSTAT3 is related to trastuzumab resistance in HER2-positive breast cancers. STAT3 has been reported to bind to regulatory regions of proapoptotic genes and prevent gene expression leading to inhibition of apoptosis in prostate tumor cells. Thus, we propose that STAT3 inhibitors along with ibrutinib may be potential option for a combination therapy in HER2 overexpressing cell lines. This may help us to increase the efficacy of ibrutinib against HER2-overexpressing tumors.
7.1 Limitation and challenges

The first limitation in our study is the challenge associated with gene annotation in genome databases. After sequencing the genome, the process of identifying the coding sequence for the gene, exons and introns in the gene, the protein(s) encoded by the gene, regulatory regions and related information on protein function and class(es) is called gene annotation. These annotations are done by various computational programs which are developed by various groups, hence there is lack of consistency and variations are often seen. To overcome this limitation CCDS (consensus coding sequence) and RefSeq (reference sequences) are developed. However, the limitation persists, specifically with respect to protein annotation and function. We have taken precaution while assigning proteins to specific groups by using two software GenAlaCart and FunRich and in our opinion this has helped in correction of annotation to a significant degree.

The second limitation is that the Kinome array used in our analyses does not have complete set of kinases. However, it has 1290 proteins that have phosphorylation sites. These additional proteins do provide for extra information on pathways. The remaining gaps in deciphering the complete pathway can be filled by performing microarray analyses or RNASeq analyses for genome data for treated and untreated HER2 overexpressing cell lines (as elaborated in the future work).

Due to limitations in funds, the entire pathway of action of ibrutinib involving the STAT3-P21 axis could not be determined. This needs further investigation and may provide complete insights to modulation of this pathway by ibrutinib.
CHAPTER 8: FUTURE WORK

The first step would be to identify the complete repertoire of proteins in the STAT3-p21 axis that are modulated by treatment with ibrutinib. This will help fill the gaps in the proposed pathway of action for ibrutinib. This can be done by microarray and/or RNA seq analyses and mapping this data to information in literature. It would be interested to see what other transcription factors/co-transcription factors besides STAT3 are involved in modulating genes that bring about cell death by apoptosis. The entire set of proteins in the cell cycle could be investigated to see what stage the cell cycle is halted.

The data derived can be validated by creating cell line derived xenograft mouse models of cancer and treatment with ibrutinib. This will confirm our findings in vivo.

PK/PD studies (pharmacokinetics and pharmacodynamics) are available for ibrutinib but not for breast cancer (as ibrutinib is approved for B-cell malignancies). PK/PD studies will provide information on dose-concentration-response for ibrutinib specifically with reference to the absorbed, distributed, metabolized, and excreted (ADME) for the drug and the drug concentration and its biologic effect. Once the entire mechanism of action is known for the drug, phase 1 clinical trials can be tested. Ibrutinib is an approved TKI. Most of approved TKIs are effective in various types of cancers, as they act on pleiotropically by targeting multiple kinases. Hence, it would be interesting to see the effect of ibrutinib in other breast cancer cell line types and other cancer cell lines. This study can be expanded to find mechanism of action of ibrutinib other cancers, where it was found effective. Also, ibrutinib along with STAT3 inhibitor as a combination treatment (as discussed earlier) can be tested in HER2 overexpressing cell lines as several reports suggest that STAT3 is constitutively active in breast cancer cells and is involved in promoting cell cycle progression and perform anti-apoptotic
effect. Thus, this work is a promising start towards investigating ibrutinib’s potential in HER2 overexpressing breast cancer.
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