PDK-1/AKT PATHWAY AS TARGETS FOR CHEMOSENSITIZING EFFECTS

DISSERTATION

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By

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Cancer is the leading causes of human deaths all over the world. The growing understanding in cancer biology provides the researchers to develop molecularly targeted therapeutic strategies. However, the toxicity and resistance limit the applications for molecularly targeted agents. Here, we proposed that inhibiting PI3K/PDK-1/Akt signaling pathway, which is critical in controlling cell survival and proliferation, is able to enhance the therapeutic effects and overcome resistance of the current used molecularly targeted drugs.

Imatinib (STI571; Gleevec), a selective bcr-abl tyrosine kinase inhibitor, is approved to treat patients with chronic myelogenous leukemia (CML). However, patients in more advanced phases of CML frequently develop resistance to the treatment. Mutations within the kinase domain for the binding of imatinib attributes as one of the major imatinib-resistant mechanisms. The effects of imatinib each alone and in combination with OSU-03012, a novel celecoxib-derived PDK-1 inhibitor, was evaluated in a panel of Bcr-Abl positive Ba/F3 cell lines without or with mutations. The IC_{50} values for OSU-03012 alone were comparable, while the sensitivities to imatinib alone were differed. The combination treatment, however, caused a synergistic enhancement of apoptosis in these cells, including those that are resistant to imatinib.
HER2/neu is frequently over-expressed in breast cancers that are characterized by aggressive tumor progression and resistant to current therapies. The effects of trastuzumab (Herceptin), a monoclonal antibody targeting to HER2/neu, alone and in combination with OSU-03012 were evaluated in a panel of breast cancer cell lines. The IC\textsubscript{50} values for OSU-03012 alone in four cell lines were comparable, while the sensitivities to trastuzumab alone were mainly dependent on the HER2/neu expression level or resistant phenotypes. The combination treatments, however, caused a synergistic enhancement of anti-proliferation in HER2/neu positive cell lines, including SKBR3/IGF-IR, as a resistant phenotype, that responded poorly to trastuzumab.

These results demonstrate the potential clinical value of a therapeutic strategy to sensitize cancer cells to molecularly targeted drugs by co-targeting PDK-1/Akt signaling. More importantly, the co-treatments are able to overcome drug resistance.
Dedicated to my lovely parents
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<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>c-Cbl</td>
<td>Casitas b-lineage lymphoma</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myelogenous leukemia</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>ERCC1</td>
<td>Excision repair cross-complementing 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>Human epidermal growth factor receptor</td>
</tr>
<tr>
<td>FDA</td>
<td>The U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FKHR</td>
<td>Forkhead transcriptional factor</td>
</tr>
<tr>
<td>GIST</td>
<td>Gastrointestinal stromal tumor</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis protein</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor-I receptor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activating protein kinase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>p70(^{S6K})</td>
<td>Ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP-ribosyl) polymerase</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent Kinase-1</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PI(3,4)P(_2)</td>
<td>Phosphatidylinositol-3,4-bisphosphate</td>
</tr>
<tr>
<td>PI(4,5)P(_2)</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PI(3,4,5)P(_3)</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome ten</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
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<tr>
<td>SHIP</td>
<td>Src homology 2 domain-containing inositol phosphatase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>ILK-1</td>
<td>Integrin linked kinase 1</td>
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CHAPTER 1

INTRODUCTION

1.1 Molecular Targeting for Cancer Therapy

Cancer is a leading cause of human death all over the world. Disappointingly, there is no indication that the incidence of cancer will decrease in the future, due to improvements in medicine that result in greater life expectancies and reduce deaths from other diseases.

Traditional treatments for cancer, including surgery, radiotherapy and chemotherapy, are not specific and selective, and can lead to severe cytotoxic effects in normal cells. Therefore, the development of new strategies for cancer therapies are focused on targeting cancer cells specifically and selectively with less toxicity and side
effects. Our growing understanding of the cellular, molecular and genetic basis of how cancer cells arise, survive, grow, and metastasize provides researchers with the knowledge to design molecularly targeted therapeutic strategies that selectively block or interrupt specific pathways, proteins or genes that are critical to cancer cells, and minimize the effects to normal cells (1-3).

1.2 Phosphoinositide 3 Kinase (PI3K)/Phosphoinositide-dependent Kinase-1 (PDK-1)/Akt Signaling Pathway

Among the promising molecular targets for anti-cancer drug development, the PI3K/PDK-1/Akt signaling pathway has garnered substantial interest (4). As outlined in Fig. 1.1 (5), signaling through this pathway can start from the binding of growth factor with its receptor, such as insulin-like growth factor-I receptor (IGF-IR), human epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2/neu, or erb-B2), platelet-derived growth factor receptor (PDGF), vascular endothelial growth factor receptor (VEGFR) and etc.. The activated receptors undergo auto-phosphorylation and acquire tyrosine kinase activity. The activated receptor tyrosine kinase then activates PI3K. The main function of PI3K is to convert phosphatidylinositol-4,5-bisphosphate
[PI(4,5)P₂] into phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P₃]. PDK-1/2 and Akt are recruited to the plasma membrane by binding to PI(3,4,5)P₃ via their pleckstrin homology (PH) domains. After localizing to the membrane, PDK-1/2 is able to activate Akt by phosphorylation at Thr 308 and Ser 473. PDK-1 is first identified by Alessi et al. at 1997 to phosphorylate Akt at Thr 308. Meanwhile, the identity of the kinase that phosphorylates Akt at Ser 473, PDK-2, remains unknown. Several kinases have been proposed as PDK-2, including integrin linked kinase 1 (ILK-1), protein kinase Cα (PKCα), other AGC kinases or Akt itself through auto-phosphorylation (6-8). Activated Akt phosphorylates many downstream proteins which control cell proliferation and survival. The activity of Akt is negatively regulated by PI(3,4,5)P₃ phosphatases, phosphatase and tensin homologue deleted on chromosome ten (PTEN) and Src homology 2 domain-containing inositol phosphatase (SHIP), which are able to convert PI(3,4,5)P₃ back to PI(4,5)P₂ or phosphatidylinositol-3,4-disphosphate [PI(3,4)P₂]. In addition to Akt, PDK-1 also has been reported to activate PKC isoforms and ribosomal protein S6 kinase (p70S6K) (9, 10).

Among the many substrates of Akt are BAD and forkhead transcriptional factors (FKHR), which have pro-apoptotic functions. After phosphorylation by Akt at Ser 136,
BAD dissociates from Bcl-2 or Bcl-X_L, and loses the ability to induce apoptosis due to translocation from mitochondria to the cytoplasm (11). FKHR, a nuclear transcription factor that stimulates the transcription of apoptotic proteins, is bound by 14-3-3 protein after phosphorylation at Ser 256 and is then degraded in the cytoplasm (12). Akt also controls cell cycle progression by inactivating p27 and glycogen synthase kinase 3β (GSK-3β), or by activating mammalian target of rapamycin (mTOR) and murine double minute 2 (MDM2). p27, a cyclin-dependent kinase (CDK) inhibitor, and GSK-3β, which is involved in inhibition of metabolism and growth factor-stimulated protein synthesis, play roles in the induction of cell cycle arrest. Phosphorylated p27 is translocated from the nucleus, the site of its activity, to the cytoplasm where it is degraded. In the case of GSK-3β, phosphorylation causes the loss of its kinase activity, thereby facilitating the cellular growth response to growth factor stimulation. MDM2, an ubiquitin protein ligase that promotes the degradation of p53 degradation, is activated by Akt-catalyzed phosphorylation at Ser 186. mTOR, a kinase involved in cell cycle progression and DNA recombination, is activated by phosphorylation at Thr 2446 and Ser 2448. The transcription factor, NFκB, is another important downstream protein activated by Akt. The transcriptional activity of NFκB is up-regulated after
phosphorylation by Akt. However, controversy exists about the direct involvement of Akt in the phosphorylation of the NFκB inhibitory protein, IκB kinase, which results in the release of NFκB from IκB.

The involvement of PI3K in signal transduction is not limited to downstream signaling through PDK-1/Akt. Cross-talk between PI3K and other signaling pathways, such as the mitogen-activating protein kinase (MAPK)1/2, and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways extends the influence of PI3K in biological regulation.

1.3 PI3K/PDK-1/Akt Pathway in Tumorigenesis

A lot of evidences have implicated aberrations in the PI3K/PDK-1/Akt pathway in tumorigenesis (5, 13).

Receptor tyrosine kinases (RTKs), the initiated signaling elements of PI3K/PDK-1/Akt pathway, are found to be highly activated and dysregulated due to over-expression, truncation, or mutation in certain cancer types. Examples include HER2/neu in breast and ovarian cancer or EGFR in lung, breast, prostate, colon, ovarian, or head and neck cancer.
The central element of this pathway, PI3K, is also altered in various cancers. The $PIK3CA$ gene that encodes the p110α subunit of PI3K is amplified in ovarian and cervical cancer, and the p85 subunit of PI3K is mutational activated in colon and ovarian cancers.

In addition, although no modified or mutated Akt genes have been found in mammals, genes encode Akt have been found to be amplified in human cancers. There are three Akt isoforms: Akt1, Akt2, and Akt3. Amplification of $Akt1$ gene is found in gastric carcinoma (14), $Akt2$ is discovered to be amplified in ovarian, pancreatic, gastric and breast cancer (15, 16), and $Akt3$ mRNA over-expresses in hormone-independent breast and prostate cancer (17).

Moreover, PTEN, a negative regulator of Akt signaling, is mutated or absent in a number of human cancers, especially glioblastoma, lung and prostate cancer. The study of over-expressing PTEN in different cell lines suggests that PTEN functions as a tumor suppressor to inhibit cell growth and increase sensitivity to apoptosis. The pro-apoptotic effects mediated by PTEN may through Akt-dependent or –independent pathways (18). In Akt-dependent pathway, un-regulated PTEN are strongly associated with activation of Akt in cancer cells. Meanwhile, in Akt-independent pathway, recent studies have
demonstrated that the phosphatase activity of PTEN is able to regulate many other substrates which are also critical in controlling cell biology. Not surprisingly, another phosphatase that negatively regulates Akt signaling, SHIP, has been shown to be a crucial inhibitor of cell activation and proliferation (19, 20). However, the loss or mutation of SHIP has not been reported in humour tumors.

Alterations in the PI3K/PDK-1/Akt pathway can lead to abnormal cell functions, such as proliferation, differentiation, survival, and migration. The accumulation of uncontrolled cell signaling facilitates the acquisition of a cancerous phenotype.

1.4 Inhibitors of the PI3K/PDK-1/Akt Pathway

During the past couple of decades, substantial drug development efforts have been directed at targeting elements within the PI3K/PDK-1/Akt pathway. So far, RTKs are probably the most successful targets for drug development within this signaling pathway. The activity of RTKs can be blocked by small molecules that interact with the intracellular catalytic or allosteric domain of the receptor, or by antibodies that specifically bind to the extracellular domain and interfere with natural ligand binding. Gefitinib (Iressa) and erlotinib (Tarceva) are small molecules that target the ATP binding
domain of EGFR and disrupt this signaling pathway (21, 22). Both agents are approved by the U.S. Food and Drug Administration (FDA) for use in the treatment of locally advanced or metastatic non-small cell lung cancer. Indeed, many small molecules with the same mechanism of inhibition, but which target different RTKs, are in different phases of clinical trials. Trastuzumab (Herceptin) is the monoclonal antibody specific for HER2/neu, and has been approved by the FDA for the treatment of metastatic breast cancer with HER-2/neu over-expression (23). Other examples of therapeutic monoclonal antibodies are cetuximab (Erbitux) which targets EGFR and is approved for the treatment of colorectal cancer (24), and panitumumab (ABX-EGF) which also targets to EGFR and is in phase III clinical trial for the treatment of metastatic colorectal cancer (25).

Fig. 1.2 shows the chemical structures of some synthetic molecules used as inhibitors of PI3K/Akt pathway.

Compared to RTK inhibitors, few agents that inhibit the PI3K/PDK-1/Akt pathway have achieved clinical relevance. Wortmannin and LY294002 are two PI3K-specific inhibitors that are broadly used in biological research, but not in clinical study due to their toxicity and bioavailability. As for Akt inhibitors, a series of phosphoinositol lipid analogs have been designed to interrupt the interaction between PI(3,4,5)P₃ and the PH
domain of Akt and thus prevent Akt from being recruited to the plasma membrane and activated by PDK-1. Perifosine (KRX-0401), one of these phosphoinositol lipid analogs, is the only Akt inhibitor in clinical trials (Phase II) in which it is being evaluated for the treatment of advanced soft tissue sarcoma (26). The inhibitors of mTOR, a downstream effector of Akt, are derivatives of rapamycin. In spite of the original clinical application for rapamycin and its derivatives as immunosuppressors, CCI-779 and RAD001 (Everolimus) are two mTOR inhibitors currently in clinical trials as cancer therapeutic agents (27, 28). Additionally, UCN-01, a derivative of staurosporine, is a non-specific kinase inhibitor that targets different proteins in the PI3K/Akt pathway (29).

Recently, the cyclooxygenase-2 (COX-2) inhibitor, celecoxib (Celebrex), was shown to induce apoptosis in cancer cells via the COX-2-independent mechanism of PDK-1 inhibition (30-32). Moreover, celecoxib is shown to compete with ATP in binding to the binding pocket within PDK-1. Based on these findings, celecoxib was used as a scaffold to develop a novel class of PDK-1 inhibitors without COX-2 inhibitory activity (33, 34). A series of celecoxib derivatives were screened with PDK-1 kinase assay and anti-proliferation activity in prostate cancer cells, PC-3. OSU-03012 is an optimal PDK-1 inhibitor with tolerable toxicity (Fig. 1.3). OSU-03012 has been shown to possess anti-
tumor activity \textit{in vitro} and \textit{in vivo} (34, 35), and is currently undergoing preclinical testing under the Rapid Access to Intervention Development program at the National Cancer Institute.

1.5 Resistance to Chemotherapeutic Agents

Drug resistance, which exists in the original tumor or develops during the course of treatment, is a critical factor limiting the effectiveness of anti-cancer drugs and a major cause of the failure of chemotherapy. Moreover, not only conventional chemotherapeutic drugs, but also newly developed molecularly targeted agents face the challenge of drug resistance.

Fig. 1.4 is the cartoon summarizes the various mechanisms involved in drug resistance to anti-cancer drugs (36). The detail descriptions are outlined below (36, 37).

- Drug influx and efflux

The accumulation of drugs within cells results from the balance between influx and efflux. The mechanisms for uptake of chemotherapeutic drugs by cancer cells are varied and include diffusion, transport or endocytosis. The effectiveness of drugs clearly decreases as the cell’s ability to take-up drugs is diminished. Drug efflux is
associated with a group of energy-dependent transporters, called ATP-binding cassette (ABC) transporter proteins, such as P-glycoprotein (Pgp) and multi-drug resistance proteins (38, 39). The role of ABC transporters in drug resistance is well documented and can involve higher expression levels in tumor cells or increases in expression after chemotherapy (40).

- Drug inactivation

This mechanism of resistance decreases the amount of active drug available for binding to its targets after metabolic modification resulting in inactivation. Also, for agents administered as pro-drugs, the level of the enzymes that convert pro-drugs into their active form is associated with the drug effect.

- Drug targets.

Alteration in the drug target is another important mechanism of drug resistance. The expression level of drug target is a key factor dictating drug sensitivity. For instance, an increase in the levels of a drug’s target is likely to reduce cells’ responsiveness to the chemotherapeutic agent. Furthermore, mutation of the target that prevents access of the drug to its binding site or reduces the binding affinity will also result in drug resistance.
- DNA damage repair

For those chemotherapeutic agents that induce DNA damage directly or indirectly, the ability to repair DNA in cancer cells will lead to drug resistance. After DNA damage, cells undergo cell cycle arrest which provides an opportunity for DNA repair. Incomplete repair will trigger cells to undergo cell death; i.e. apoptosis or necrosis. There are three major DNA repair mechanisms: base excision, nucleotide excision and mismatch repair. Among these, nucleotide excision repair (NER) is a flexible repair pathway involving the removal of a variety of bulky DNA lesions. The excision repair cross-complementing 1 protein (ERCC1) is one of the rate-limiting factors in the NER system, and high expression of ERCC1 has been shown to be associated with cisplatin resistance in ovarian cancer (41). On the other hand, deficiency of DNA mismatch repair (MMR) genes has been implicated in the development of resistance (42). The explanation is that DNA damage recognized by MMR may trigger the cell death pathway.

- Induction of apoptosis

The capacity of tumor cells to undergo apoptosis induced by chemotherapeutic agents is a critical factor in determining drug resistance. Two main pathways are
involved in the activation of caspases, which are the key elements regulating apoptosis. These two pathways are regulated by Bcl-2 family proteins, and by the tumor necrosis factor (TNF) receptor superfamily (43).

The Bcl-2 family can be divided into three subfamilies: the Bcl-2 subfamily which contains anti-apoptotic proteins, and the Bax and BH3 subfamilies which contain pro-apoptotic proteins. Up-regulation of proteins in the Bcl-2 subfamily, such as Bcl-2, Bcl-X\textsubscript{L}, or Mcl-1, and down-regulation of pro-apoptotic proteins, such as Bax or Bad, are associated with poor responses to chemotherapy.

The TNF receptor superfamily mediates apoptosis through the death receptors, such as Fas, DR4 or DR5, which are bound with Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL). Apoptosis induced by this pathway can be inhibited by decoy receptors, which bind to FasL or TRAIL, but lack the intracellular domains required for transmitting death signals. Moreover, FADD-like interleukin-1b-converting enzyme-inhibitory protein (FLIP) also inhibits caspase activation from death receptors.

The Apoptosis mediated through Bcl-2 family proteins or TNF receptor superfamily needs caspases to transmit death signals. Thus, the inhibitors of
apoptosis protein (IAP) family is another important group of proteins that regulates apoptosis by inhibiting caspase activity (44). Over-expression of the IAP proteins has been shown to inhibit apoptosis induced by chemotherapeutic agents.

- Pro-survival signaling

Abnormal signaling pathways that regulate cell proliferation, differentiation or survival have an impact on drug resistance. As previously mentioned, the PI3K/Akt pathway regulates different anti-apoptotic signals. The JAK/STAT pathway regulates the expression of the anti-apoptotic Bcl-2 subfamily. Another key pro-survival pathway involves TNFα/NFκB signaling. NFκB activates the transcription of different anti-apoptotic proteins, such as IAPs, FLIP, and members of the Bcl-2 subfamily. Therefore, NFκB is an important factor in drug resistance by preventing cell death (45).
Fig. 1.1 PI3K/Akt signaling pathway (5). (under the permission of Nature Publishing Group Copyright)
Fig. 1.2 The chemical structures of inhibitors of PI3K/Akt pathway.
Fig. 1.3 The chemical structures of OSU-03012, an optimal PDK-1 inhibitor.
Fig. 1.4 The mechanisms of resistance to chemotherapeutic agents (36).
CHAPTER 2

SYNERGISTIC INTERACTIONS BETWEEN IMATINIB AND THE NOVEL PHOSPHOINOSITIDE-DEPENDENT KINASE-1 INHIBITOR OSU-03012 IN OVERCOMING IMATINIB RESISTANCE

2.1 Background

2.1.1 Chronic Myelogenous Leukemia (CML)

There are four major types of leukemia: acute myelogenous leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, and chronic lymphocytic leukemia. Among the four leukemia types, CML is a kind of slow progressing cancer with malignant clonal disorder in hematopoietic stem cells (46, 47). In CML, the bone marrow stem cells uncontrolled grow into white blood cells and some of them never
become mature. This leads to increase in more immature differentiating myeloid cells in blood, bone marrow and body tissues. About 5,000 people each year are diagnosed with CML in the United States.

There are three clinical stages of CML: the chronic phase; the accelerated phase and blastic phase. The chronic phase can last from a few months to about 4 to 5 years and can be controlled with medications. After that, CML patients progress from chronic phase into accelerated phase. Only 3 to 6 month later, Accelerated phase rapidly advances to the final stage, blastic phase, which means the continual presence of greater than 30% myeloblasts in marrow or peripheral blood. Patients in blastic phase live an average of 3 to 6 months.

Nowell and Hungerford first described in 1960 that over 90% of the patients with CML can be observed to carry with the Philadelphia (Ph) chromosome in the bone marrow (48). This cytogenetic aberration is the translocation between the long arms of chromosomes 22 and 9; t(9;22), and results in a shortened chromosome 22, celled Ph chromosome (49). The translocation relocates abelson (abl) gene coded for a tyrosine kinase from chromosome 9 to the region of breakpoint cluster region (bcr) gene which is coded for a protein with serine/threonine kinase activity in chromosome 22. The bcr-abl
fusion gene encodes a fusion protein with abnormal tyrosine kinase activity which activates different downstream signaling pathways (Fig. 2.1). The diagnosis of CML is based on the detection of Ph chromosome in the bone marrow cells. However, how this genetic abnormal leads to CML is still not fully understood.

Stem cell transplantation is generally believed to be able to cure CML in selected and limited patients with the considerable risk of death. The treatment of stem cell transplantation is a procedure to try to restore the blood-producing stem cells after high-dose chemotherapy/radiotherapy has reduced them to dangerously low levels. Autologous stem cells collected the patient’s own stem cells or allogeneic stem cells collected from a related or unrelated donor are used in transplantation. The 5-year survival rate for patients to accept allogeneic stem cell transplant is decreased alone with the disease progression; approximately greater than 70% in chronic phase, 65% in accelerated phase and 25% in blastic phase. However, during the treatment, the stem cells reach critically low levels, and complications, such as anemia, infection and bleeding, can occur. Transplantation related mortality worldwide ranges from 5 to 40%.

Before the introduction of imatinib, hydroxyurea and interferon α are widely used in the conventional chemotherapy. Hydroxyurea is an oral chemotherapeutic agent that
controls the growth of white blood cells, but does not destroy bone marrow stem cells. The effects of hydroxyurea are to reduce the number of one particular type of nucleotide, adenine, which is important as DNA building blocks in the cells. Interferon $\alpha$ is a protein that occurs naturally in the body in very small amounts, and functions by stimulating the body's immune system to fight the cancer cells.

However, the development of imatinib provides the CML patients an alternative choice instead of interferon $\alpha$ or hydroxyurea now. From the phase 2 clinical study, almost 90% of the CML patients treated with imatinib show no further progression of their disease.

2.1.2 Bcr-Abl

Bcr-Abl protein is the product of the Ph Chromosome. $bcr-abl$ fusion gene with different breakpoints in $bcr$ gene is translated into three different Bcr-Abl isoforms: $p190^{Bcr-Abl}$, $p210^{Bcr-Abl}$ and $p230^{Bcr-Abl}$. While $p210^{Bcr-Abl}$ can be found in 90% of CML, $p190^{Bcr-Abl}$ exits in most Ph chromosome positive acute lymphoblastic leukemia (50), and $p230^{Bcr-Abl}$ might be associated with rare chronic neutrophilic leukemia (51).
The kinase activity in normal Abl protein is under careful control by the intramolecular binding of the N-terminal cap region to the Src-homology 1 (SH1) domain (52). However, Bcr-Abl fusion protein is not only lack of the cap region in Abl, but also that a dimerization domain in Bcr promotes the dimerization of two Bcr-Abl protein which leads to phosphorylate each other in kinase-activation loops. Therefore, the Bcr-Abl tyrosine kinase protein is constitutive activated and cause uncontrolled signaling transduction pathway (53). Besides, the normal Abl and Bcr-Abl proteins have different subcellular locations. The normal Abl is found in the nucleus and cytoplasm, and the nuclear Abl acts as pro-apoptotic protein in response to genotoxic stress (54). However, Bcr-Abl is only found in cytoplasm and anti-apoptotic.

Bcr-Abl has been shown to cause hematopoietic cell transformation in vitro and in vivo (55, 56). It is probably due to that Bcr-Abl protein uncontrolled phosphorylates many downstream substrates, including growth factor receptor-bound protein 2, CRK, Dok, paxillin et al. Consequently, multiple downstream signal pathways, such as PI3K, JNK, Myc, Ras are activated, and resulting to altered cellular adhesion, increased proliferation, reduced growth factor or cytokine dependence, inhibited apoptosis (57) (Fig. 2.2).
Although the essential components downstream Bcr-Abl to cause leukemogenesis are not yet well defined, the crucial role of Bcr-Abl in causing cellular transformation provides an attractive drug target.

2.1.3 Imatinib

Imatinib, also known as STI571 or Gleevec, manufactured by Novartis Pharmaceuticals is approved by the FDA in 2001 to treat CML and gastrointestinal stromal tumor (GIST) which is a rare form of stomach cancer.

Imatinib is the first successful example that targets to the signaling transduction pathways which are associated with the cancer. Starting in the late 1980s, imatinib, a phenylamino-pyrimidine based molecule, is developed from a project initially focus on PKC inhibitors, but end up to discover the specific Abl tyrosine kinase inhibitors. Imatinib targets to the adenosine triphosphate (ATP) binding site of the Abl protein, and acts as a competitive inhibitor. All forms of the Abl tyrosine kinases, such as Abl, viral Abl and Bcr-Abl are able to be inhibited by imatinib (Fig. 2.3). From the X-ray crystal structure, imatinib binds to inactive form of Abl kinase. This indicated that imatinib can only inhibit the Bcr-Abl activity before it is activated by auto-phosphorylation.
In addition to Abl tyrosine kinases, imatinib also shows potent inhibition activity to against PDGFR, and c-KIT. The inhibition of c-KIT by imatinib is thought to be the reasons of the anti-tumor effects in the GIST.

2.1.4 Mechanisms of Imatinib Resistance

By treating with imatinib, the patients in chronic phase CML show good response, and over 50% of the patients occur a major cytogenetic response. However, patients in more advanced phases of CML, such as blast crisis, either fail to respond or quickly relapse, even show an initial response to imatinib (58, 59).

In clinical samples from imatinib-resistant patients, two major resistant mechanisms are observed: amplification and over-expression of the bcr-abl gene, and mutations of the Bcr-Abl in the kinase domain (60).

The amplification or over-expression of bcr-abl gene leads to the increase of Bcr-Abl protein expression, and are found in about 18% of imatinib-resistance patients. The increased Bcr-Abl level makes it possible to maintain the minimum amount of signaling transduction activity needed for survival (61, 62).
Mutations within the kinase domain of Abl protein represent 50 to 90% of patients, who are identified with secondary resistance which means the resistance is developed after an initial response. Among many different mutated amino acids have been detected, it can be distinguished as four clusters: ATP binding loop (P-loop), T315, M351, and activating loop (A-loop) (Fig. 2.4) (60, 63).

The P-loop includes amino acid 244 to 255 where the phosphate groups of ATP are bound with. Except Y253, the amino acids mutated in P-loop do not interact directly with imatinib. Thus, it is proposed that the mutations in P-loop may shift the conformation that is disfavor the binding of imatinib. T315 cluster contains two mutation sites, T315 and F317. T315 forms a hydrogen bond with imatinib, while F317 uses hydrophobic interaction to bind with imatinib. The M351 group that contains M351 and F486 are the amino acids far away from the imatinib binding site, which form hydrophobic patch to stabilize the C-terminal lobe. A-loop includes the amino acid from residues 381 to 402. Both M351 and A-loop clusters are associated with the conformational regulation of Abl kinase activity. The mutations within these areas may interrupt the inactive kinase conformations which are required for the imatinib binding.
Among these mutation sites, the residue E255 and T315 are two most frequently occurring mutations. E255K/V or T315I is largely insensitive to imatinib, and results to the IC$_{50}$ values from kinase activity assay or cell proliferation assay exceed the therapeutically achievable concentration.

Additionally, in the laboratory studies, it have been shown that over-expression of $MDR1$ gene, which encodes Pgp, decreases imatinib cellular accumulation and leads to imatinib resistance (64). Over-expression of LYN, an Src kinase, is also demonstrated in imatinib-resistant leukemia cell line (65).

Since the mutations in Bcr-Abl play a critical role for the resistant mechanism in the patients with relapsed CML, it is important to develop an alternative strategy to overcome this type of imatinib resistance.

2.2 Experimental Hypothesis

From a mechanistic viewpoint, Bcr-Abl protein activates multiple downstream signaling pathways which are critical for the cell proliferation, survival, and transformation (57). Recently, evidences indicates that the PI3K inhibitor, LY294002, but not the MAPK inhibitor, PD98059, is not only able to cause the growth inhibiting effects
in CML cells (66), but also to synergize with imatinib (67). These findings suggest that
PI3K/Akt pathway is important for Bcr-Abl mediated cell survival. Therefore, we
hypothesize that the apoptosis threshold for CML cells turned to be lower by inhibiting
PI3K/Akt pathway. Alone with the remaining inhibiting effects of imatinib, inhibitors
targeting to PI3K/Akt pathway can enhance the anti-proliferation effects and restore the
sensitivity to imatinib in imatinib-resistant cells.

Moreover, since LY294002 is no more in clinical use, we examined the effects with
an optimal celecoxib-derived PDK-1 inhibitor, OSU-03012, alone or in combination with
imatinib.

2.3 Ba/F3 Cell Models

A parental murine myeloid hematopoietic cell line, 32D, was used as Bcr-Abl
negative cell line. The Ba/F3 cell line is a murine lymphoid cell line that is dependent on
interleukin-3 (IL-3) for growth. Ectopic expression of constitutive tyrosine kinases, such
as Bcr-Abl, make the Ba/F3 cells IL-3 independent (68). The stable clones expressing
Bcr-Abl in Ba/F3 were used as Bcr-Abl positive cell lines to mimic CML. There are three
different Bcr-Abl positive Ba/F3 cell lines: Ba/F3p210Bcr-Abl with wild type Bcr-Abl as
the sensitive cell line; Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} are cells with mutant Bcr-Abl in E255 or T315, respectively, to represent as imatinib-resistant cell lines. Besides, untransfected Ba/F3 cells lack of Bcr-Abl expression were used as control.

Fig. 2.5 was the western blot analysis that showed the protein expression level of Bcr-Abl, p-Thr\textsuperscript{308}-Akt, Akt and β-Actin. The Bcr-Abl protein was shown to over-express only in Ba/F3p210\textsuperscript{Bcr-Abl}, Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells, and no Bcr-Abl can be detected in 32D and Ba/F3 cell lines. Additionally, over-expression of Bcr-Abl was able to increase the levels of Akt phosphorylation without changing the total Akt level, and the increase was irrespective of the mutations in Bcr-Abl. The amount of β-Actin was used as protein loading control.

2.4 Sensitivity of Different Bcr-Abl Positive Cell Lines to Imatinib

2.4.1 Anti-proliferation Effects

The cell viability after treating cells with imatinib for 48 hours in medium with 10% fetal bovine serum (FBS) was tested by MTS assays (Fig. 2.6). The IC\textsubscript{50} values from the median dose-responses curves for Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} were 14 ± 4 and 30 ± 2 µM, respectively, while the IC\textsubscript{50} in wild-type Bcr-Abl cell line, Ba/F3p210\textsuperscript{Bcr-Abl},
was 0.13 ± 0.01 µM. The data indicated two orders of magnitude difference in sensitivity between resistant and wild-type cell lines. Our data was in line with the data from published report (69, 70). For the Bcr-Abl negative cell lines, 32D and untransfected Ba/F3, the IC$_{50}$ values were over 50 µM which indicated that Bcr-Abl protein was necessary for imatinib to perform its effects. The decreased cell viability in 32D and Ba/F3 cells with high imatinib concentration might due to the non-specific inhibition to other kinases.

2.4.2 Flow Cytometric Analysis with Annexin V/PI staining

The percentage of apoptotic cells induced by imatinib was determined by flow cytometric analysis with annexin V and PI staining (Fig. 2.7). Annexin V binds with phosphatidylserine externalized from inner plasma membrane after cells undergo apoptosis. Thus, annexin V signals indicated the apoptotic cells. Meanwhile, PI is able to stain the nucleotide fragments. Only in late stage of apoptosis that nucleus membrane disappears or in necrosis that the nucleus membrane is broken, the PI signals can be detected. In the pictures output from the flow cytometry, B1 quadrant represent annexin V$^-$/PI$^+$ which usually means necrotic cells, B2 is annexin V$^+/PI^+$ as apoptotic cells in late
stage, B3 is annexin V+/PI− as apoptotic cells in early stage, and B4 is annexin V−/PI− as vital cells. The percentage of apoptotic cells was defined as the sum of B2 and B3 quadrants.

Flow cytometric analysis indicated that at 1 µM imatinib, 97% cells underwent apoptosis in Ba/F3p210Bcr-Abl cells. However, even with 10 µM imatinib, the apoptotic cells were only 35% and 6% in Ba/F3p210E255K, and Ba/F3p210T315I cells, respectively. The data suggested that Ba/F3p210E255K, and Ba/F3p210T315I cells are resistant to the imatinib-induced apoptosis.

2.4.3 Effects of Imatinib Treatment on Akt and Cytochrome c

According to our hypothesis, the significant role of PI3K/Akt pathway in imatinib sensitivity was highlighted. Thus, Akt status after imatinib treatment was examined by western blotting.

The complete dephosphorylation of Akt in Ba/F3p210Bcr-Abl cells was found with the concentration of imatinib below 1 µM. On the other hand, imatinib was not able to cause decrease of phosphor-Akt in Ba/F3p210E255K and Ba/F3p210T315I cells until the concentration higher than 5 µM (Fig. 2.8 A).
The mitochondria mediated apoptosis would trigger the cytochrome c release. Thus, detecting the change of cytochrome c level in the cytoplasm provided another evidence for apoptosis, in addition to the phosphatidylserine externalization detected by annexin V (Fig. 2.8 B). The experiment of cytochrome c release indicated that only \( \text{Ba/F3p210}^{\text{Bcr-Abl}} \) was sensitive to imatinib-induced apoptosis at sub-\( \mu \text{M} \) range, but not \( \text{Ba/F3p210}^{\text{E255K}} \) and \( \text{Ba/F3p210}^{\text{T315I}} \) cells.

2.5 Sensitivity of Different Bcr-Abl Positive Cell Lines to OSU-03012

2.5.1 Anti-proliferation Effects

As shown in Fig. 2.9, the MTS assays indicated that 32D, Ba/F3, \( \text{Ba/F3p210}^{\text{Bcr-Abl}} \), \( \text{Ba/F3p210}^{\text{E255K}} \), and \( \text{Ba/F3p210}^{\text{T315I}} \) were equally susceptible to the OSU-03012 mediated anti-proliferation effects. The IC\(_{50}\) values were 4.4 ± 0.1 \( \mu \text{M} \), 4.8 ± 0.1 \( \mu \text{M} \), 4.9 ± 1.0 \( \mu \text{M} \), 4.8 ± 0.1 \( \mu \text{M} \), and 4.5 ± 0.3 \( \mu \text{M} \), respectively. The data here indicated that the sensitivity to OSU-03012 was irrespective of Bcr-Abl expression level and there was no cross-resistance between imatinib and OSU-03012 in the imatinib-resistant cells. In 32D and Ba/F3 cells, although the phosphor-Akt levels were relative low comparing with Bcr-Abl positive cell lines, the sensitivity to OSU-03012 did not show obvious different. The
lack of correlation between activated Akt level and drug sensitivity could be explained with that OSU-03012, a PDK-1 inhibitor, not only inhibits Akt, but also inhibits other downstream signaling that are also important for cell growth and survival, such as PKC and p70^S6K_, and the overall inhibition effects from all the downstream effectors of PDK-1 are similar in all the cell lines.

### 2.5.2 Flow Cytometric Analysis with Annexin V/PI Staining

Flow analysis indicated that with OSU-03012 treatment, the Bcr-Abl positive cell lines showed comparable dose-dependent increase in apoptotic cell death (Fig. 2.10). At 5 µM OSU-03012, the percentage of apoptotic cells were 41%, 38%, and 40% in Ba/F3p210^{Bcr-Abl}, Ba/F3p210^{E255K}, and Ba/F3p210^{T315I}, respectively. Meanwhile, at 10 µM OSU-03012, the percentage of apoptotic cells increased to around 90%.

### 2.5.3 Immunoblotting

From western blot analysis (Fig. 2.11 A), OSU-03012 was able to decrease phospho-Akt level with dose-dependent manner disregarding Bcr-Abl mutations.
Analyses of cytochrome c release, Poly(ADP-ribosyl) polymerase (PARP) cleavage alone with the flow cytometric analysis of annexin V staining, Bcr-Abl positive cells demonstrated that the OSU-03012-mediated cell death was mainly contributed from apoptosis (Fig. 2.10 and Fig. 2.11 B).

2.6 OSU-03012 Sensitizes Imatinib-resistant Cells to Imatinib-induced Apoptosis

2.6.1 Anti-proliferation effects

Since Ba/F3p210^Bcr-Abl cells is very sensitive to the imatinib treatment with IC_{50} less than 0.5 µM, we would like to focus on evaluating the combination effects of OSU-03012 and imatinib in imatinib-resistant cells only.

Ba/F3p210^{E255K}, and Ba/F3p210^{T315I} cells were exposed to various concentrations of imatinib in the present of 5 µM OSU-03012, or to different concentrations of OSU-03012 with 5 µM imatinib. In Fig. 2.12, the dose-response curves showed that OSU-03012 sensitized Ba/F3p210^{E255K} and Ba/F3p210^{T315I} cells to imatinib-induced anti-proliferation effects. IC_{50} for imatinib was reduced from over 10 µM to about 1 µM in the present of 5 µM OSU-03012. Also, 5 µM imatinib sensitized Ba/F3p210^{E255K} and Ba/F3p210^{T315I} cells to OSU-03012-mediated anti-proliferation effects with the reduction of IC_{50} from 5
µM to less than 3 µM. However, 5 µM OSU-03012 was not able to sensitize 32D and Ba/F3 cells, which are lack of Bcr-Abl expression, to the treatment of various concentrations of imatinib.

2.6.2 Flow Cytometric Analysis with Annexin V/PI Staining

Annexin V analysis demonstrated that the combination of OSU-03012 with imatinib lead to enhanced apoptosis (Fig. 2.13). In combination with 5 µM OSU-03012, treating Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells with imatinib at 1, 2.5 and 5 µM caused 50%, 70% and over 95% apoptotic cell death, respectively. Considering that 5 µM OSU-03012 alone lead to about 40% of apoptotic cells, and 10 µM imatinib alone caused 35% and less than 10% apoptotic death in Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I}, respectively, the apoptotic effects induced by combination treatment was more than additive.

2.6.3 Combination Index

The combination index (CI) proposed by Chou and Talalay is widely used to determine the multiple drug effect (71). From the definition, CI value equal to 1 indicates an additive effect; less than 1 indicates synergy, and larger than 1 indicates antagonism.
Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells were exposed over a range of OSU-03012 and imatinib concentrations at a fixed ratio (1:1) for 48 hours. The cell viability obtained from MTS assays was used to determine fraction affected. As shown in Fig. 2.14, the CI values calculated in different fraction affected were significantly lower than 1 in two imatinib-resistant cell lines (p values are less than 0.05 from sign test). The CIs indicated synergistic interaction between OSU-03012 and imatinib in Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells.

2.6.4 Effects on Bcr-Abl and Akt

According to our hypothesis, PDK-1/Akt pathway plays a role in the synergistic effect with the combination of OSU-03012 and imatinib. Immunoblotting analysis showed that Bcr-Abl expression level was not changed after Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells were exposed to the drug combination (Fig. 2.15 A). However, while imatinib at less than 10 µM showed no sign to cause dephosphorylation of Akt (Fig. 2.15 B), the level of phosphor-Akt was further decreased with 5 µM OSU-03012 added with various concentrations of imatinib in both cell lines.
2.7 Discussion

Imatinib is a milestone in the drug development history. It represents the first successful example of molecularly targeted therapy for cancer treatments. When it is hoped that the development of imatinib can open the doors to more molecularly targeted drugs, the resistance to imatinib is the challenge need to be faced. Numbers of new therapeutic strategies to overcome imatinib resistance in CML have been reported. Identification of other novel Abl kinase inhibitors which are able to inhibit the mutant Abl kinase is one kind of the approaches, such as the discovery of PD180970, BMS-354825, and AP23464 (72-74). However, it is always potential that the alternative Abl kinase inhibitors might acquire resistance by other point mutations. Thus, the inhibitors which can lead to Bcr-Abl degradation might be the better approach. It has been shown to inhibit the growth of imatinib-resistant hematopoietic cells by this type of inhibitors, such as arsenic trioxide, or heat shock protein 90 (Hsp90) inhibitors, geldanamycin and 17-allyaminogelanamycin (17-AAG) (75, 76). The combinations of imatinib with other chemotherapeutic agents have also been reported to show synergistic effects in anti-proliferation in CML, such as arsenic trioxide, the hypomethylation agent, decitabine, the farnesyl transferase inhibitor, SCH66336, and the histone deacetylase inhibitors,
suberoylanilide hydroxamic acid (SAHA) and butyrate (69, 77-79). The combinations of different chemotherapeutic agents are another approach shown to be able to induce apoptosis in imatinib-resistant cells, such as the proteasome inhibitor, bortezomib, in combination with flavopiridol, or with SAHA (80, 81). However, many of these strategies remain fail to overcome the imatinib resistance caused by T315I mutation.

Due to the critical role of PI3K/Akt pathway, we hypothesize that inhibition of PDK-1/Akt signaling could reduce the apoptosis threshold, and restore the ability of imatinib to induce apoptosis in resistant cells. According to the experimental results, the susceptibility of Bcr-Abl positive cell lines to the PDK-1 inhibitor, OSU-03012, is similar, irrespective of the Bcr-Abl mutations. Moreover, co-treatment with OSU-03012 and imatinib is able to achieve synergistic effect of anti-proliferation by inducing apoptosis in resistant cell lines, especially the highly resistant Ba/F3p210^{T315I} cells.

Our data indicated that the mechanistic synergism between OSU-03012 and imatinib can be explained by the phospho-Akt level which regulates apoptosis. Although imatinib retains residual activity to inhibit Bcr-Abl kinase in the resistant cell lines, the inhibition at therapeutically achievable concentrations is not enough to cause Akt dephosphorylation which is important for triggering apoptotic signaling. However, decreasing phospho-Akt
by OSU-03012 causes the cells to lose protection effect from Akt and leads to the reduction of apoptosis threshold. Therefore, OSU-03012 sensitizes imatinib-resistant cells to the remaining anti-proliferation activity of imatinib.

Within therapeutically achievable concentrations, the combination of 5 µM imatinib and 5 µM OSU-03012 is able to decrease cell viability to less than 5% or cause 80% apoptotic cell death in both Ba/F3p210E255K and Ba/F3p210T315I cells. Thus, this combination represents a workable strategy in treating imatinib-resistant CML.
Fig. 2.1 The Ph chromosome.
Fig. 2.2 The signaling transduction by the Bcr-Abl protein (57).
Fig. 2.3 The inhibition mechanism of imatinib. (A) The structure of imatinib. (B) The competition between imatinib and ATP
**Fig. 2.4** Frequency of Bcr-Abl mutations detected in clinical specimens (n = 177) (60).
Fig. 2.5 Protein expression levels of Bcr-Abl and Akt in parental or Bcr-Abl positive cells.
Fig. 2.6 Differential susceptibility in parental and Bcr-Abl positive cells to imatinib. Dose-response curves were obtained by MTS assays after 48 hr treatment of imatinib. Each data point represents means ± S.D. (n = 6).
Fig. 2.7 Dose-dependent effect of imatinib on inducing apoptotic cell death, Akt dephosphorylation and cytochrome c release in Bcr-Abl positive cells. (A) Flow cytometric analysis of apoptotic death after treating with DMSO vehicle, or imatinib for 48 hours. (B) Dose-dependent effect of imatinib on phospho-Thr\textsuperscript{308}-Akt (C) Dose-dependent effect of imatinib on cytochrome c release after imatinib treatment for 36 hours. Results are representative of at least three independent experiments.
Fig. 2.8 Dose-dependent effect of imatinib on Akt dephosphorylation and cytochrome c release in Bcr-Abl positive cells. (A) Dose-dependent effect of imatinib on phospho-Thr$^{308}$-Akt after treating with imatinib for 36 hours. (B) Dose-dependent effect of imatinib on cytochrome c release after imatinib treatment for 36 hours. Results are representative of at least three independent experiments.
Fig. 2.9 Similar susceptibility in Bcr-Abl positive cells to OSU-03012. Dose-response curves were obtained by MTS assays after 48 hours treatment of imatinib. Each data point represents means ± S.D. (n = 6).
Fig. 2.10 Dose-dependent effect of OSU-03012 on inducing apoptotic cell death in Bcr-Abl positive cells. Flow cytometric analysis of apoptotic death after treating with DMSO vehicle, or OSU-03012 for 48 hours.
Fig. 2.11 Dose-dependent effect of OSU-03012 on Akt dephosphorylation, cytochrome c release and PARP cleavage in Bcr-Abl positive cells. (A) Dose-dependent effect of OSU-03012 on phospho-Thr\textsuperscript{308}-Akt after OSU-03012 treatment for 36 hours. (B) Effects of 5 µM OSU-03012 on cytochrome c release, and PARP cleavage after OSU-03012 treatment for 36 hours. Results are representative of at least three independent experiments.
Fig. 2.12 OSU-03012 sensitizes Bcr-Abl positive cells to imatinib-mediated anti-proliferation. Upper-left panel: Dose-response curves obtained by MTS assays after 48 hours exposure Ba/F3p210E255K and Ba/F3p210T315I to varying concentration of imatinib with (solid curves) or without (dotted curves) 5 µM OSU-03012. Upper-right panel: Dose-response curves obtained by MTS assays after 48 hours exposure Ba/F3p210E255K and Ba/F3p210T315I to varying concentration of OSU-03012 with (solid curves), or without (dotted curves) 5 µM imatinib. Lower-left: Dose-response curves obtained by MTS assays after 48 hours exposure 32D and Ba/F3 to varying concentration of imatinib with 5 µM OSU-03012. Each data point represents means ± S.D. (n = 6).
Fig. 2.13 OSU-03012 sensitizes Bcr-Abl positive cells to imatinib-induced apoptotic cell death. Flow cytometric analysis of apoptotic death in Ba/F3p210^{E255K} (upper panels) and Ba/F3p210^{T315I} (lower panels) cells treated with 1, 2.5, and 5 µM imatinib in combination with 5 µM OSU-03012 for 48 hours. Results are representative of at least three independent experiments.
Fig. 2.14 Combination index values from combination treatment. Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells were treated with varying concentrations of OSU-03012 and imatinib at a fixed ratio (1:1) for 48 hours. Fraction affected for cell viability was used for CI values determination. CI values less than 1 are considered as synergism. Mutually non-exclusive CI for combination at the IC\textsubscript{50} was 0.602 for Ba/F3p210\textsuperscript{E255K} and 0.649 for Ba/F3p210\textsuperscript{T315I}. The P values for sign test comparing CI equal to 1 versus CI less than 1 are 0.016 for Ba/F3p210\textsuperscript{E255K} and 0.008 for Ba/F3p210\textsuperscript{T315I}. 

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Fig. 2.15 Combination effect of OSU-03012 and imatinib at different concentrations on Bcr-Abl expression (A) and Akt phosphorylation (B) in Ba/F3p210<sup>E255K</sup> and Ba/F3p210<sup>T315I</sup> cells. The immunoblots are representative of three independent experiments.
CHAPTER 3

OVERCOMING TRASTUZUMAB RESISTANCE IN HER2/NEU OVER-
EXPRESSING BREAST CANCER CELLS

3.1 Background

3.1.1 Breast Cancer

The breasts are made up of both ducts and lobes. Each breast consists of 15 to 20 lobes, with many smaller sections, called lobules. Lobes are the glands in which the milk is produced. The duct is the thin tube that connects to each lobe and opens separately on the nipple. A fatty tissue layer between the surface skin and the underlying muscles on the front of the chest where the breast develops is the other key component in the breast which is not involved in the milk production and contributes to the size of the breast.
Breast cancer is the cancer type that the malignant cells are found in the breast tissue. The most common type of breast cancer is the one found in the cells of the ducts, called ductal cancer. Cancer that begins in the lobes or lobules is called lobular carcinoma. Inflammatory breast cancer is an uncommon type of breast cancer that causes the swelling of breast. With the progression of breast cancer, the cancer cells can metastasize to the lymph system, lungs, liver, heart, bones or brain.

According to the report from the American Cancer Society (82), breast cancer in the United States is the most common form of cancer and the second leading cause of cancer death in women. Estimated 58,490 new cases of in situ (or non-invasive) breast cancer and 211,240 of new cases of infiltrating (or invasive) breast cancer which are spread out of the normal breast tissue barriers are expected among the women in the United States. At the same time, about 40,110 women and 470 men will die from breast cancer in the United States in 2005. However, the death rates from breast cancer have been shown to decline, due to increased awareness, the available early detection, and improved treatments.

The risk factors for breast cancer are including aging, gender, genetic mutation, and hormone replacement therapy (HRT). The risk to develop breast cancer increases when
people get older. Although men develop breast cancer, it is about 100 times more common among women than men. Furthermore, inherited genetic mutations in \textit{BRCA1}, \textit{BRCA2}, \textit{CHEK-2} or \textit{p53} tumor suppressor genes are linked to around 5\% of all breast cancer cases. HRT is a treatment with the drugs that contain estrogen alone or the combination of estrogen with progestin, and is used to relieve the symptoms of menopause in post-menopausal women. However, a major clinical trial suggests HRT is associated with increased risk of breast cancer, delay diagnosis, and more abnormal mammograms (83).

There are four basic stages of breast cancer which are defined by the size of tumor and the metastasis status. Stage I to III are the stages that tumor does not spread outside the breast and with the dimension less than 2 cm, between 2 to 5 cm and more than 5 cm, respectively. Stage IV indicates the tumor of any size with metastasis. A five-year survival rate decreases as the progression of breast cancer. The Stage I breast cancer survival rate is 92\%, and there is still 71\% survival rate in Stage II. However, Stage III survival rate is lower to 39\%, and the Stage IV survival rate is only 11\%. Therefore, it is very important for early detection to improve the chances that breast cancer can be treated successfully. So far, mammography, an X-ray of the breast, is a helpful early
detection tool which can detect cancer before physical symptoms occur. It is recommended to perform a mammogram screening once every year for the women over 40 years old.

The main choice for breast cancer treatments is the surgery which removes the tumor locally (lumpectomy) or large amounts of the breast tissue (mastectomy). Except surgery, it is possible that the treatments may involve radiotherapy, chemotherapy or hormone therapy. Because that breast cancer cells in early stage require estrogen to grow and reproduce, tamoxifen, a selective estrogen receptor antagonist, or inhibitors of aromatase which is a key enzyme in estrogen bio-synthesis are used for hormone therapy to block the supply of estrogen. Recently, immunotherapy with trastuzumab, a monoclonal antibody, is approved by FDA to be used in progressive breast cancer with HER2/neu expression.

3.1.2 HER2/neu in Breast Cancer

HER2/neu (or ErbB-2) belongs to the EGFR family. There are four structurally related EGFR receptors: EGFR (HER1, or ErbB-1), HER2/neu, ErbB-3 and ErbB-4, which can bind with over 30 different ligands to affect over 50 downstream effectors.
The receptors and ligands in the EGFR family form a complex system to regulate numerous cellular functions, such as differentiation, growth, adhesion and migration (Fig. 3.1) (84).

Following ligand binding, receptors in EGFR family usually form homodimers or heterodimers, and activate downstream signaling network. Unlike other members in EGFR family, no ligand for HER2/neu has been found, and there is also no HER2/neu homodimers. Therefore, it is suggested that HER2/neu acts as a co-receptor. By forming heterodimers with receptors in EGFR family, HER2/neu is able to involve in signaling transduction without a specific ligand. Moreover, it is shown that the ligand dissociation rate is slower in HER2/neu heterodimers; thereby stronger signaling is generated.

Usually, there are two copies of HER2/neu gene in the normal epithelial cells, and the expression levels of HER2/neu on the cell surface are low. However, the number of gene copies per cell is increased during tumorigenesis, and causes increased transcription in mRNA, which leads to a 10 to 100 fold increase in the number of HER2/neu expressing on the cell surface.

Gene amplification or HER2/neu over-expression has been demonstrated in breast, ovarian, bladder, gastric, and pancreatic cancers. Approximately 25 to 30% of breast
cancers over-express HER2/neu. Over-expression of HER2/neu leads to unregulation of downstream signaling and is associated with more aggressive breast cancer. It has also been suggested that HER2/neu over-expression may be linked to tamoxifen resistance (85). Thus, breast cancers with over-expressing HER2/neu are usually poor prognosis and do not respond well to chemotherapy.

Since HER2/neu is an important factor for controlling growth or survival in the breast cancer, the drugs targeting to HER2/neu may provide a successful way to treat breast cancer.

3.1.3 Trastuzumab

Trastuzumab, or so called Herceptin, is a recombinant monoclonal antibody developed by Genetech Inc. to target HER2/neu proteins expressing on the cell surface. The crystal structure shows that trastuzumab binds with HER2/neu in extracellular domain IV, and blocks HER2/neu to from heterodimers with other receptors in EGFR family (Fig. 3.2) (86). In 1998, FDA approved the use of trastuzumab in treating metastatic breast cancer. In approved clinical applications, trastuzumab is used as a single agent or in combination with paclitaxel.
After trastuzumab specifically binds to HER2/neu, the cells undergo G1-phase cell cycle arrest and apoptosis. Trastuzumab not only blocks the binding of growth factors to receptors, but triggers the endocytosis of HER2/neu on the cell surface (87). The inhibition and reduction of HER/neu sequentially causes down-regulation of PI3K/Akt signaling, and increases in the CDK inhibitor, p27, expression (88, 89). Besides, a recently published report claims that HER2/neu signaling down-regulation by trastuzumab is able to increase response to TRAIL which is relative to death receptor mediated apoptosis in SKBR3 cells, but decrease response in BT474 cells through PI3K/Akt pathway (90). Overall speaking, trastuzumab mediates the effects to down-regulate cell growth, or survival (91). In addition to anti-proliferation effect, trastuzumab also facilitates immune response through antibody-dependent cell-mediated cytotoxicity (92). The natural killer (NK) cells from immune system are attracted to the HER2/neu-antibody complex on the cell surface. The NK cells attach to trastuzumab, and destroy cancer cells. Moreover, recent clinical trail studies demonstrate that trastuzumab in combination with chemotherapeutic agents, such as adriamycin, or taxol, significantly increases disease-free survival for women with early-stage HER2/neu positive breast cancer.
Because the specific targeting of trastuzumab to HER2/neu, the expression levels of HER2/neu in the patients determinate the outcome of the treatment. So far, two main methods are used for testing HER2/neu positive breast cancer to select patients for trastuzumab treatment: immunohistochemistry (ICH) and fluorescence in-situ hybridization (FISH) assay. ICH detects the HER2/neu protein levels in the tumor sample the level is graded from 0 to 3+, and 3+ means HER2/neu positive. At the same time, FISH assay measures the amount of HER2/neu gene in each cell. Some studies have shown that HER2/neu gene amplification, measured by FISH assay, is the best predictive marker of response to trastuzumab therapy (93). Basically, increased response rate can be accomplished by more knowledge to determine with what HER2/neu expression levels might have better response to trastuzumab, and by the development of more accurate and convenient HER2/neu test. Recently, a research article demonstrating a valuable method to monitor serum HER2/neu

In monotherapy, trastuzumab is given through an intravenous infusion with 4 mg/kg loading dose, and weekly 2 mg/kg maintenance dose. The peak concentration is 110 mg/L and steady-state concentrations are 66 mg/L which will be reached by 20 weeks. However, the downside of trastuzumab therapy is cardiotoxicity, which occurs in 4% of
the patients receiving trastuzumab as single agent treatment. Although trastuzumab plus
adjuvant chemotherapy shows higher percentage of disease free patients, the incidence of
heart failure also increases (94).

Besides breast cancer, trastuzumab is also being studied in clinical trials for other
types of cancer, including osteosarcoma (a type of bone cancer), lung, pancreas, colon,
prostate, and bladder which most of them are epithelial cell original. It is believed that
these types of cancer with over-expression of HER2/neu protein can benefit from
trastuzumab treatment.

3.1.4 Mechanisms of Trastuzumab Resistance

The objective response rate to trastuzumab monotherapy is 12 to 34% for a median
duration of 9 months, by which point most patients become resistant to the treatment.
Thus, this indicates that patients either do not response or relapse shortly after an initial
response.

Therefore, even with HER2/neu over-expression, cancer cells still can escape from
the anti-proliferation effects of trastuzumab therapy by different resistant mechanisms.
Mechanisms of intrinsic or acquired trastuzumab resistance are not well understood.
Evidence from *in vitro* or *in vivo* indicates that the resistance is developed in the way to overcome trastuzumab-mediated anti-proliferation signaling, including up-regulation of insulin-like growth factor-I receptor (IGF-IR) expression (95), loss of PTEN function (96), down-regulation of p27 expression (97), and constituted Akt activation (98).

Lu et al. found that human breast cancer cells over-expressing HER2/neu with substantial activated IGF-IR, MCF-7/HER2-18, are not inhibited by trastuzumab in the presence of serum or IGF-I (95). In contrast, SKBR3, a cell line over-expressing HER2/neu alone, is very sensitive to trastuzumab mediated anti-proliferation effects. Therefore, the study indicates that in breast cancer cells over-expressing HER2/neu, increased IGF-IR signalling reduces trastuzumab response, and anti-IGF-IR strategies may be used in overcoming trastuzumab resistance.

Nagata el al. from The University of Texas demonstrated that PTEN deficiency is very powerful to predict the response of trastuzumab treatment (96). The cells with reduced PTEN show less growth inhibition by trastuzumab. *In vivo* breast cancer xenografts in nude mice model and patient samples confirm the relation of PTEN reduction with trastuzumab. The authors suggest that the use of inhibitors of PI3K family protein might be helpful in PTEN-mediated trastuzumab resistance.
Also, Nahta et al. from M.D. Anderson Cancer Center created two trastuzumab-resistant pools from SKBR3, a cell line over-expressing HER2/neu. They found that in resistant cells, the CDK inhibitor, p27, is decreased, and the S-phase fraction in cell cycle is increased (97). Thus, it is propose that trastuzumab resistance may be associated with decreased p27 levels.

Moreover, Clark et al. from National Cancer Institute demonstrated that induction of Akt by trastuzumab treatment precedes apoptosis, and might be involved in resistance (98). Not only inducted Akt, by using a specific PI3K inhibitor, LY294002, the endogenous Akt activity is also proved to be able to promote breast cancer cell survival and resistance.

From the proposed mechanisms that lead to trastuzumab resistance, it can be easily found out that the trastuzumab-resistant mechanisms are highly associated with PI3K/Akt pathway.

3.2 Experimental Hypothesis

Numbers of published reports have demonstrated that PI3K/Akt pathway is important for the anti-cancer action mediated by trastuzumab (88, 89). Moreover,
dysfunctional PI3K/Akt pathway is the main mechanism associated with trastuzumab resistance (95-98). Therefore, it is proposed that the inhibitors targeting to PI3K/Akt pathway could enhance the sensitivity to trastuzumab and overcome trastuzumab resistance (96, 99).

Here, we would like to demonstrate that OSU-03012, a novel PDK-1 inhibitor, can block PDK-1/Akt signaling and thereby in combination with trastuzumab can achieve more anti-cancer effects in HER2/neu positive breast cancer. In chapter 2, we have shown that the combination of OSU-03012 with imatinib, a Bcr-Abl kinase inhibitor, was able to overcome imatinib resistance and to work synergistically through the mechanism of Akt inactivation that results to lower apoptosis threshold (100). Thus, it is logical for us to hypothesize that the combination treatment with OSU-03012 and trastuzumab could have similar synergistic interaction and is able to overcome trastuzumab resistance in SKBR3/IGF-IR cells.

3.3 In Vitro Cell Models

Four different breast cancer cell lines with different HER2/neu expression status were used. Among them, MDA-MB-231 cells represent the cells with low HER2/neu
expression. Meanwhile, BT-474, SKBR3, and SKBR3/IGF-IR cells are with high HER2/neu expression. However, SKBR3/IGF-IR cells which are SKBR3 cells transfected with IGF-IR have been shown to be resistant to trastuzumab treatment to act as a resistant phenotype. According to the published data, all of MDA-MB-231, BT474, and SKBR3 cells have functional PTEN expression. However, PTEN level is very low in MDA-MB-231 comparing with the other two cell lines, BT474 and SKBR3.

The basal level of HER2/neu and IGF-IR in four cell lines was confirmed by western blotting (Fig. 3.3). In line with literature reports, BT474, SKBR3 and SKBR3/IGFR-IR cells expressed high level of HER2/neu. The HER2/neu expression level is over 10-fold higher than in MDA-MB-231 cells. Moreover, in SKBR3/IGF-IR, the expression level of IGF-IR is 10 fold higher than the level in MDA-MB-231, BT474 or SKBR3. Furthermore, the level of activated Akt was shown to be correlated with the amount of HER2/neu and IGF-IR expression, while there was no much difference in total Akt level between four cell lines. Although MDA-MB-231 cells with low PTEN expression should have high activated Akt, the amount of phosphor-Akt was still about 10 fold less comparing with BT474, SKBR3, and SKBR3/IGF-IR.
3.4 Sensitivity of Different HER2/neu Expression Breast Cancer Cell Lines to Trastuzumab

3.4.1 Anti-proliferation Effects

After treating cells with trastuzumab in culture medium with 10% FBS for 72 hours, the cell viability was measured by MTT assays (Fig. 3.4). Because lack of HER2/neu expression, the dose-response curve showed that MDA-MB-231 cells were insensitive to trastuzumab mediated anti-proliferation effects, even the concentration as high as 50 µg/ml. However, in BT-474 and SKBR3 cells with HER2/neu over-expression, treating with 20 µg/ml trastuzumab induced 30 to 50% reduction in cell proliferation. Meanwhile, SKBR3/IGF-IR cells were shown to be a resistant phenotype that is insensitive to trastuzumab treatment at concentration higher than 50 µg/ml as a result of IGF-IR over-expression (95).

3.4.2 Immunobloting

Since previous reports demonstrated that the anti-proliferation effects of trastuzumab were through the decrease of HER2/neu expression, down-regualted Akt signaling, and increase of the CDK inhibitor, p27 (87-89), we examined these key
molecules by western blotting to evaluate the mechanisms of drug effects. Additionally, the proteins which are associated with PI3K/PDK-1/Akt pathway were also checked, such as p70\textsuperscript{S6K} which is the downstream molecule of PDK-1, and phosphor-p27. As shown in Fig. 3.3, in BT474 and SKBR3 cells, trastuzumab within 20 µg/ml caused dose-dependent decrease in HER2/neu expression, increase in p27 expression and decrease of Akt phosphorylation without changing total Akt level, which were in line with the published data. Moreover, phospho-p27 which is selective phosphorylated by Akt, was down-regulated alone with a lesser extent decrease of phospho-p70\textsuperscript{S6K} (Fig. 3.5). However, none of these effects was found in MDA-MB-231 or trastuzumab-resistant SKBR3/IGF-IR cells within the dose range examined. The western blotting data here were correlated with the MTT assays that the anti-proliferation effects of trastuzumab only occurred in BT474 and SKBR3 cells.

For apoptosis induced by trastuzumab, PARP cleavage was used as the evidence (Fig. 3.5). Similar to MTT assays, trastuzumab only induced cell apoptosis in HER2/neu over-expressing BT474 and SKBR3 cells, but not in MDA-MB-231 cells or resistant SKBR3/IGF-IR cells.
3.5 Sensitivity of HER2/neu Expression Breast Cancer Cell Lines to OSU-03012

3.5.1 Anti-proliferation Effect

Despite the sensitivity to trastuzumab was differed, these four cell lines showed similar degree of the anti-proliferation effects mediated by OSU-03012 based on MTT assays (Fig. 3.6). The IC\textsubscript{50} values from the median dose-response curves were: MDA-MB-231, 3.8 ± 0.4 µM; BT-474, 3.9 ± 0.4 µM; SKBR3, 3.0 ± 0.2 µM; SKBR3/IGF-IR, 3.2 ± 0.1 µM. The results indicated the effects of OSU-030212 were not associated with the expression level of HER2/neu, and lack of cross-resistance to OSU-03012 in trastuzumab-resistant SKBR3/IGF-IR cells.

3.5.2 Immunobloting

All of the proteins examined in the trastuzumab treatment were checked in the OSU-03012 treatment with the same condition (Fig 3.7). Since OSU-03012 inhibited the PDK-1/Akt signaling pathway, the proteins which are downstream of PDK-1 have been shown to be dephosphorylated without changing the expression level, such as the PDK-1 substrates, Akt and p70\textsuperscript{S6K}, and the Akt substrate, p27. Unlike only BT474 and SKBR3 cells responded to trastuzumab, all four cell lines treated with OSU-03012 showed
comparable response. PARP cleavage as the evidence of apoptosis suggested that the cell
death induced by OSU-03012 was irrelevant to the HER2/neu expression status or
resistant phenotype. Moreover, apoptosis might be attributed to PDK-1/Akt inhibition.
However, in contrast to trastuzumab, OSU-03012 had no effect on up-regulation of p27
expression.

Interestingly, from western blot analysis, we noticed that OSU-03012 treatment was
able to decrease HER2/neu expression level through the unknown mechanism.

3.5.3 Immunohistochemical Staining

In order to further investigate the down-regulation of HER2/neu mediated by OSU-
03012, immunocytochemical staining was performed to analyze the cellular localization
of HER2/neu. Fig 3.8 demonstrated the cytoplasmic translocation of HER2/neu after 5
µM OSU-03012 treatment in both SKBR3 and SKBR/IGF-IR cells within 24 hours. This
phenomenon provided a link between the receptor internalization and HER2/neu down-
regulation. Moreover, in the follow up study, we found the similar cytoplasmic
translocation of IGF-IR and EGFR after OSU-03012 treatment. Although the mechanism
is not yet clear, it is possible that casitas b-lineage lymphoma (c-Cbl), an E3 ubiquitin
ligase regulating the endocytosis and degradation of tyrosine kinase, might play a role.

Recently, a published report claimed the exist of the crosstalk between Akt and APS, an adaptor protein essential for c-Cbl tyrosine phosphorylation (101).

3.6 OSU-03012 Sensitizes HER2/neu Expressing Cells to Trastuzumab

3.6.1 Anti-proliferation Effects

To test our hypothesis that PDK-1 inhibitor, OSU-03012, can sensitize HER2/neu positive breast cancer cells to trastuzumab treatment, MDA-MB-231, BT474, SKBR3, and SKBR3/IGF-IR cells were co-treated with varying concentrations of trastuzumab in the presence of 2.5 µM or 5 µM OSU-03012 for 72 hours. The dose-response curves of MTT assays indicated that in the presence of 2.5 µM or 5 µM OSU-03012 was able to enhance anti-proliferation effects mediated by trastuzumab in these four cell lines (Fig. 3.9).

3.6.2 Combination Index

The combination effect between OSU-03012 and trastuzumab was evaluated with CI values. The fraction affected of cell viability was carried out by exposing cells to a range
of OSU-03012 and trastuzumab concentrations at a fixed ratio (1:1; μM:μg/ml). As shown in Fig. 3.10, the CI values in different fraction affected were all lower than 1 in BT474, SKBR3, and SKBR3/IGF-IR cells. The CIs calculated from the combination treatment at IC$_{50}$, which means 50% inhibition of cell viability, were 0.96, 0.50, 0.71, and 0.68 for MDA-MB-231, BT474, SKBR3, and SKBR3/IGF-IR cells, respectively. These findings indicated that co-treatment with OSU-03012 and trastuzumab lead to synergistic effect in HER2/neu over-expressing cells, even in trastuzumab-resistant SKBR3/IGF-IR cells. However, this combination treatment yielded only an additive or less than additive effect in MDA-MB-231 cells.

3.6.3 Immunobloting

In order to mechanistically explain the combination effects, various proteins in HER2/neu and PI3K/Akt pathways were examined, including HER2/neu, Akt, phospho-Akt, p27, and phospho-p27 (Fig. 3.11).

Except MDA-MB-231, the other three cell lines with HER2/neu over-expression showed more than additive effect with the combination treatment in HER/neu degradation, Akt and p27 dephosphorylation and p27 up-regulation. Especially in
SKBR3/IGF-IR cells, a trastuzumab resistant phenotype, trastuzumab at 20 µg/ml alone had little effects on the proteins we tested. However, in combine with 2.5 µM or 5 µM OSU-03012, less than 10 µg/ml trastuzumab not only enhanced the effects of OSU-03012, but restored the ability to up-regulate p27 expression.

PARP cleavage provided the evidence for apoptosis induced by combination treatment.

3.7 Discussion

Trastuzumab has been shown to be effective in treating HER2/neu over-expressing metastatic breast cancer, but resistance limits trastuzumab to benefit more patients with HER2/neu over-expression. Although how patients acquire trastuzumab resistance is still not clear, all of the evidences published indicate a significant role of PI3K/Akt signaling. Based on our previous study that OSU-03012, a novel PDK-1 inhibitor, is able to work synergistically with imatinib to treat imatinib-resistant cells, we hypothesize that the combination of trastuzumab with OSU-03012 represents a new strategy to enhance the anti-proliferation effect and overcome resistance.
Our experimental results showed that the four different breast cancer cell lines have similar susceptibility to OSU-03012 irrespective of the HER2/neu expression status. The $IC_{50}$ values are around 3 to 4 $\mu$M. However, only HER2/neu positive cells, but not resistant phenotype, BT474 and SKBR3, are sensitive to the trastuzumab treatment. The maximum cell viability reduction when treating with higher than 20 $\mu$g/ml trastuzumab for 72 hours is approximately 50%.

The combination treatment with OSU-03012 and trastuzumab is able to achieve synergistic effect in anti-proliferation within HER2/neu positive breast cancer cells, but not in HER2/neu negative MDA-MB-231 cells. This synergistic effect is especially noteworthy in SKBR3/IGF-IR cells, which represent as a trastuzumab-resistant phenotype. In SKBR3/IGF-IR cells, trastuzumab retains its ability to bind with HER2/neu and to activate downstream apoptotic signaling, but the survival signaling through alternative tyrosine kinase pathway, IGF-IR, neutralizes the effect from trastuzumab. Since OSU-03012 blocked the PDK-1/Akt pathway which is important in both HER2/neu and IGF-IR signaling and controls cell survival, the resistant cells lost the anti-apoptotic protection provided by IGF-IR signaling and regained the sensitivity to trastuzumab.
Comparing to the effect of the treatment with trastuzumab or OSU-03012 alone, co-treatment strongly enhances the anti-proliferation effects, except in MDA-MB-231 cells. With 20 µg/ml trastuzumab, the cell viability reduction is 30 to 50% in BT474 and SKBR3 cells, and no effect is found in SKBR3/IGF-IR cells. Meanwhile, 2.5 µM or 5 µM OSU-03012 causes approximately 30% and 60% cell viability reduction, respectively, in HER2/neu positive cells. However, the combination of 5 mM OSU-03012 with 10 mg/ml trastuzumab causes greater than 90% anti-proliferation effect in BT474 and SKBR3 cells and, more importantly, 80% in SKBR3/IGF-IR cells.

The mechanistic synergism between OSU-03012 and imatinib can be illustrated with the dephosphorylation of Akt, p27 expression and HER2/neu degradation. OSU-03012 is able to cause Akt dephosphorylation by inhibiting PDK-1/Akt pathway. The inactivation of Akt leads to lower apoptotic threshold, and sensitizes the cell to trastuzumab-induced anti-proliferation effects. Consequently, inactivated Akt results to the dephosphorylation of p27 which slows the degradation and increases nucleus p27. Thus, OSU-03012 may strength the ability of trastuzumab to induce p27 expression in BT474 and SKBR3 cells or restore it in SKBR3/IGF-IR cells. Moreover, the finding of OSU-03012 to mediate HER2/neu internalization facilitates the degradation of HER2/neu with trastuzumab.
The pharmacokinetic study indicated that a steady-state serum concentration of 60 to 80 µg/ml trastuzumab can be reached, when dosing with 2 mg/kg weekly or 6 mg/kg every three week (102). Within these concentrations, in the presence of 5 µM OSU-03012 can increase the anti-proliferation effects from 50% to over 90% in BT474 and SKBR3 cells, or restore the anti-proliferation sensitivity to achieve 80% cell viability decrease in trastuzumab-resistant cells, SKBR3/IGF-IR. Thus, this combination represents a novel strategy not only to increase the efficacy of trastuzumab in HER2/neu positive breast cancer treatment, but also to overcome trastuzumab resistance.
Fig. 3.1 Schematic of the complex EGFR family of receptors and ligands in regulating cellular functions (84).
Fig. 3.2 The structure of rat and human sHER2/neu. (A) Ribbon diagram of rat sHER2/neu. (B) Ribbon diagram of the human sHER2/neu and trastuzumab complex (86).
**Fig. 3.3** Protein expression levels of HER2/neu, IGF-IR and Akt in four different breast cancer cell lines.
Fig. 3.4 Differential susceptibility in four different breast cancer cells to trastuzumab. Dose-response curves were obtained by MTT assays after 72 hours treatment of trastuzumab.
**Fig. 3.5** Dose-dependent effects on the expression and/or phosphorylation levels of HER2/neu, Akt, p27, and p70{superscript}S6K, and PARP cleavage in MDA-MB-231, BT474, SKBR3, and SKBR3/IGF-IR cells after treating trastuzumab for 72 hours. These immunoblots are representatives of three independent experiments.
Fig. 3.6 Similar susceptibility in four different breast cancer cells to trastuzumab. Dose-response curves were obtained by MTT assays after 72 hours treatment of OSU-03012. Each data point represents means ± S.D. (n = 6).
**Fig. 3.7** Dose-dependent effect on the expression and/or phosphorylation levels of HER2/neu, Akt, p27, and p70\(^{S6K}\), and PARP cleavage in MDA-MB-231, BT474, SKBR3, and SKBR3/IGF-IR cells after treating OSU-03012 for 72 hours. These immunoblots are representative of three independent experiments.
**Fig. 3.8** Immunocytochemical blots of HER2/neu localization in SKBR3 and SKBR3/IGF-IR cells. The cells were treated with DMSO vehicle or 5 µM OSU-03012 for 24 hours. (provided by Shu-Chuan Weng)
Fig. 3.9 OSU-03012 sensitizes BT474, SKBR3, and SKBR3/IGF-IR cells, but not MDA-MB-231 cells, to trastuzumab mediated anti-proliferation effects. Dose-response curves obtained by MTT assays after 72 hours treatment of varying concentrations of trastuzumab in combination with 2.5 or 5 µM OSU-03012. Each data point represents means ± SD (n = 6).
Fig. 3.10 Combination index values from combination treatment. The breast cancer cells were treated with varying concentrations of OSU-03012 and trastuzumab at a fixed ratio (1:1; μM:μg/ml) for 72 hours. Fraction affected for cell viability was used for CI values determination. CI values less than 1 are considered as synergism. Mutually non-exclusive CI for combination at the IC₅₀ was 0.962 for MDA-MB-231 (not shown), 0.501 for BT474, 0.712 for SKBR3, and 0.677 for SKBR3/IGF-IR.
**Fig. 3.11** Combination effect of OSU-03012 and trastuzumab at different concentrations on the expression and/or phosphorylation level of HER2/neu, Akt, and p27, and PARP cleavage in MDA-MB-231, BT474, SKBR3, and SKBR3/IGF-IR cells after 72 hours treatment. These immunoblots are representatives of three independent experiments.
CHAPTER 4

CONCLUSION AND FUTURE WORK

4.1 Conclusion

Due to the recent understanding in cancer biology, the development of selective molecular targeting agents obtains some promising progression (1-3). However, the limitations for the molecular targeting therapy are toxicity and resistance. Although the targeting therapy is less toxic than traditional chemotherapeutic agents, in certain normal tissue, the targeted enzymes might also important for certain normal biological functions. It has been reported that imatinib casues defection in cell-mediated immunity by blocking c-Abl signals (103). Cardiotoxicity caused by trastuzumab is also relative the functions of HER2/neu in normal tissue (94).
Together with toxicity and resistance we have mentioned previously, the new strategies for clinical use of molecular targeting agents need to be developed. Combination therapy might provide the answer for solving the problems of toxicity and resistance (104, 105). With combination therapy, the lower dosage for individual drugs is used to achieve the same therapeutic effects with single agent treatment, and leads to less side effects caused by toxicity. Furthermore, by using two or more agents with different mechanisms of action, it reduces the chance that cancer cells escape from the killing effect of therapeutic agents and develop drug resistance.

The combination of the PDK-1 inhibitor, OSU-03012, with imatinab or trastuzumab has been proved as the potential therapeutic applications in CML or breast cancer. The combination therapy is not only able to enhance the drug effects, but also overcome the drug resistant phenotypes. Our discovery agrees with other reports that PI3K/Akt pathway is critical for cell survival and proliferation (13, 106, 107). By blocking PI3K/Akt signals, we demonstrate the synergistic effects of combination therapy can be achieved through lowering apoptosis threshold by deactivating Akt, inducing receptor degradation, or interfering cellular localization of the CDK inhibitor, p27.
4.2 Future Work

The study of the PDK-1 inhibitor, OSU-03012, combined with other chemotherapeutic agents are currently undergoing in our laboratory, such as combining with gefitinib in non-small lung cancer, tomaxifan in estrogen receptor positive or negative breast cancer, or cisplatin in ovarian cancer. The preliminary data of these combination treatments show promising synergistic or additive effects in inhibiting cancer growth. Except to combine with chemotherapeutic agents, it may be benefit to more cancer patients by studying the combination between PDK-1 inhibitor and radiotherapy.

Moreover, if suitable models were available, it would be worthwhile to show whether or not that the combination of OSU-03012 with imatinab or trastuzumab could prevent cancer from relapse or delay the occurrence of drug resistance.
CHAPTER 5

MATERIALS AND METHODS

5.1 Materials and Reagents

Imatinib mesylate, also known as STI-571, was obtained from commercial Gleevec® capsules (Novartis Pharmaceuticals, East Hanover, NJ) by solvent extraction followed by recrystallization. Trastuzumab, also known as Herceptin, was purchased from Genentech Inc. (San Francisco, CA). The PDK-1 inhibitor OSU-03012 was synthesized as described (34). Rabbit monoclonal anti-PARP was purchased from Cell Signaling Technology Inc. (Beverly, MA). Rabbit antibodies against HER2/neu, phospho-Thr\(^{308}\)-Akt, Akt and p27, and mouse antibody against p70\(^{S6K}\) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-phospho-Thr\(^{229}\)-p70\(^{S6K}\) and anti-phospho-Thr\(^{157}\)-p27 were from R&D Systems Inc. (Minneapolis, MN).
Mouse monoclonal anti-cytochrome c, anti-Bcr, and anti-actin were from BD Pharmingen (San Diego, CA), Oncogene (Boston, MA), and ICN Biomedicals Inc. (Costa Mesa, CA), respectively. Goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 647 goat anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA). MDA-MB-231, BT474, and SKBR3 cell lines were from American Type Culture Collection (Manassa, VA). SKBR3/IGF-IR cell was kindly gift from Dr. Michael Pollak (McGill University, Montreal, Quebec, Canada). 32D, Ba/F3, Ba/F3p210Bcr-Abl and imatinib-resistant Ba/F3p210 mutant cell lines including Ba/F3p210E255K, and Ba/F3p210T315I were generated as previously reported and as gift from Dr. Brian Druker (Oregon Health and Science University, Portland, OR) (70).

5.2 Cell Culture

Cells were grown in 75-cm² plastic tissue culture flasks at 37°C in a humidified incubator (5% CO₂). 32D and Ba/F3 cells were grown in RPMI 1640 medium supplemented with 10% FBS (Gibco, Carlsbad, CA); 15% Walter and Eliza Hall Institute (WEHI)-conditional media as an interleukin 3 (IL-3) source; and 50 units/ml penicillin G,
50 μg/ml streptomycin, and 10 μg/ml gentamycin (Sigma, St Louis, MO). Ba/F3p210^{Bcr-Abl} and imatinib-resistant Ba/F3p210 mutant cell lines, Ba/F3p210^{E255K}, and Ba/F3p210^{T315I}, were cultured in RPMI 1640 medium containing 10% FBS, 50 units/ml penicillin G, 50 μg/ml streptomycin, and 10 μg/ml gentamycin. Breast cancer cell lines, MDA-MB-231, BT474, SKRB3, and SKBR3/IGF-IR, were grown in Dulbecco's minimal essential medium/Ham's F12 (DMEM/F12; 1:1) medium containing 10% FBS and 10 μg/ml gentamycin. All of the Cells were passaged at 1:4 dilution with fresh medium once every 4 days.

5.3 Cell Proliferation Assay

5.3.1 MTT Assay

Cells (3,000) were grown in 10% FBS-supplemented DMEM/F12 medium in 96-well, flat-bottomed plates, and exposed to various concentrations of individual agents or combination of drugs dissolved in DMSO (final concentration ≤ 0.1%) in the same medium. Control groups received DMSO vehicle at a concentration equal to that in drug-treated cells. After 72 hours treatment, medium was removed and replaced by 200 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (TCI America;
Portland, OR) (0.5 mg/ml) in DMEM/F12 medium, and cells were incubated in the CO₂ incubator at 37 °C for 3 hours. After discarding the MTT solution, the reduced MTT dye was solubilized with 200 µl/well DMSO. The converted dye was analyzed by measuring the absorbance at 570 nm in a plate reader. The Dm (or IC₅₀) value which represents the drug concentration required for 50% growth inhibition for single drug was calculated by the CalcuSyn software (Biosoft, Cambridge, UK) with the median-effect method.

5.3.2 MTS Assay

Cells (5,000) were grown in 10% FBS-supplemented RPMI 1640 medium in 96-well, flat-bottomed plates, and exposed to various concentrations of individual agents or combination of drugs dissolved in DMSO (final concentration ≤ 0.1%) in the same medium. Control groups received DMSO vehicle at a concentration equal to that in drug-treated cells. After 48 hours treatment, MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, WI) and the phenazine methosulfate (PMS) detection reagent were mixed at a ratio of 20:1 (MTS:PMS), and immediately added to the culture medium at a ratio of 1:5. Cells were further incubated in the CO₂ incubator at 37 °C for 3 hours, and the production of
formazan was analyzed by measuring the absorbance at 492 nm in a plate reader. The Dm (or IC<sub>50</sub>) value was calculated by the Calcusyn software (Biosoft, Cambridge, UK) with the median-effect method.

5.4 Immunoblotting

The general procedure for the Western blot analysis was performed as follows. Cells were scratched from culture flasks and collected by centrifugation at 2000 x g, and resuspended in RIPA lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a mixture of protease inhibitor cocktail [100 µM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 nM aprotinin, 5 µM bestatin, 1.5 µM E-64 protease inhibitor, 2 µM leupeptin, 1 µM pepstatin A (Calbiochem, La Jolla, CA)] and phosphatase inhibitors (10 µM sodium fluoride, 5 µM sodium vanadate, and 10 µM β-glycerol phosphate). The mixture was sonicated for 5 sec, and protein contents were analyzed by using the Bradford assay kit (Bio-Rad). 50 µg total proteins were resolved in SDS-polyacrylamide gels on a Minigel apparatus, and transferred to a nitrocellulose membrane using a semi-dry transfer cell. After blocking with TBS containing 0.05% Tween 20 (TBST) containing 5% nonfat milk for 60 min, the membrane was incubated with the appropriate primary antibody at 1:1000 dilution in
TBST-1% nonfat milk at 4 °C overnight, and then washed 15 min three times with TBST. The membrane was probed with horseradish peroxidase-conjugated secondary antibody at (1:3000) for 1 hour at room temperature, and was then washed with TBST three times. The immunoblots were visualized by enhanced chemiluminescence.

5.5 Immunohistochemic Staining

SKBR3 and SKBR3/IGF-IR cells were treated with 5 µM OSU-03012 in 10% FBS medium. After 24 hours, washed with Dulbecco's PBS (0.9 mM CaCl2, 2.7 mM KCl, 1.5 mM KH2PO4, 0.5 mM MgCl2, 137 mM NaCl, and 8.1 mM Na2HPO4), fixed with 4% paraformaldehyde for 30 min at room temperature, and then washed with PBS. For HER2/neu staining, the cells were permeabilized with 0.1% Triton X-100 in PBS containing 1% FBS and then stained with a 1:100 dilution of rabbit antibodies against HER2/neu for 24 h at 4°C. For fluorescent microscopy, Alexa Fluor 647 goat anti-rabbit IgG was used for conjugating with anti-HER2/neu. All the antibodies were diluted in dilution solution (0.1% Triton X-100, 0.2% bovine serum albumin in PBS). The nuclear counter staining was performed using a DAPI-containing mounting medium (Vector Laboratories, Burlingame, CA) prior to examination. Images of immunocytochemically
labeled samples were observed using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Jena, Germany) with an Argon laser and a He-Ne laser, and appropriate filters (excitation wavelength: 633 nm for HER2/neu, and 543 nm for DAPI).

5.6 Apoptosis Analysis

Western blot analysis of PARP cleavage and the detection of cytochrome c release were used to exam drug-induced apoptotic cell death.

5.6.1 PARP Cleavage Assay

Drug-treated cells for 72 hours were collected, washed with ice-cold PBS, and resuspended in RIPA lysis buffer. Soluble cell lysates were collected after centrifugation at 10,000 x g for 5 min. Equivalent amounts of proteins (50 µg) from each lysate were resolved in 10% SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes, and analyzed by immunoblotting.

5.6.2 Cytochrome c Release Analysis

Cytosol-specific mitochondria-free lysates were prepared as previously described (108). Drug-treated cells were collected by centrifugation at 1000 x g for 5 min. The pellet fraction was recovered, placed on ice, and resuspended in 100 µl of a chilled...
hypotonic lysis solution (220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, and the aforementioned protease inhibitors cocktails). After 20-min incubation on ice, the mixture was centrifuged at 600 x g for 10 min. The supernatant was collected in a microcentrifuge tube and centrifuged at 14,000 x g for 30 min. An equivalent amount of protein (25 µg) from each supernatant was resolved by 15% SDS-polyacrylamide gel electrophoresis and blotted with anti-cytochrome c antibody by following the procedure described above.

5.7 Flow Cytometric Analysis

Fluorescein-conjugated Annexin V (Annexin V-FITC) and PI (BD Pharmingen) were used to quantify the percentage of cells undergoing apoptosis by following the protocol provided by the vendor. In short, after drug treatment, the cells were collected and resuspended in 1 ml binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 5 x 10⁶ cells/ml. 200 µl solution (1 x 10⁶) was transferred to a culture tube, to which were added annexin V-FITC and PI. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. Additional 800 µl of binding buffer was added to each tube and the samples were analyzed by flow cytometry.
5.8 Combination Index Analysis

The synergistic effect of multiple drugs was determined by the definition of Chou and Talalay (71). The software package CalcuSyn was used to calculate median effect and CIs. Cell lines were treated with combined drugs with a fixed molar ratio to adapt the software requirements and the drugs were assumed as totally independent modes of action and are therefore mutually non-exclusive. With a combination index (CI) value significantly less than 1 was defined as synergism.

The median effect equation is developed from Michael-Menten type and Hill type equations. The mathematic form of median effect equation is \( \frac{f_a}{f_u} = \left( \frac{D}{D_m} \right)^m \), where \( f_a \) means fraction affected by the dose, \( f_u \) is fraction unaffected, \( D \) is the dose of the drug, \( D_m \) means median effect which is similar to \( IC_{50}, ED_{50} \) or \( LD_{50} \), and \( m \) is the Hill-type coefficient for the sigmoidicity of the dose response curve.

The mutually non-exclusive combination index (CI) can be obtained by the equation:

\[
CI = \frac{D_1}{D_{x1}} + \frac{D_2}{D_{x2}} + \frac{D_1D_2}{D_{x1}D_{x2}}
\]

where \( D_1 \) or \( D_2 \) are the doses of Drug 1 and Drug 2 used in combination, \( D_{x1} \) or \( D_{x2} \) are the doses of Drug 1 and Drug 2 which are required to achieve \( x\% \) inhibition.
Practically, from dose response curve of the single drug, we can get the information of Dm and m values. After combination of two drugs, the fa and fu can be got (fa + fu = 1), and we can calculate from the median effect equation to get $D_{x1}$ or $D_{x2}$ which are essential for CI calculation.


