UNIVERSITI PUTRA MALAYSIA

EFFECTS OF DISRUPTING INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR (IGF-1R) SIGNAL TRANSDUCTION PATHWAYS WITH INHIBITORS OF IGF-1R, EGFR, PI3K, MEK, AND MTOR IN BREAST CANCER CELL LINES

AYUNADIRAH BINTI AYUB

IB 2014 27

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By

AYUNADIRAH BINTI AYUB

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

June 2014

DEDICATION

Specially dedicated to,

My loving parents, family, and awesome friends,

For their invaluable love, understanding encouragement, and patience.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

EFFECTS OF DISRUPTING INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR (IGF-1R) SIGNAL TRANSDUCTION PATHWAYS WITH INHIBITORS OF IGF-1R, EGFR, PI3K, MEK, AND MTOR IN BREAST CANCER CELL LINES

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AYUNADIRAH BINTI AYUB

June 2014

Chairman : Professor Seow Heng Fong,PhD
Institute : Bioscience

Insulin-like growth factor receptor 1 (IGF-1R) has been shown to be overexpressed in patients with breast cancer. IGF-1R signaling pathway is known to activate numerous kinases, and proteins associated with cell growth and survival. Targeting these kinases and their pathways are promising therapeutic approaches compared to disruption of IGF-1R alone. The general objective of this study was to determine the appropriate combination of small molecules inhibitors which can synergize to inhibit the activation of IGF-1R and its downstream pathways in order to decrease the viability and growth of breast cancer cell lines. The specific objectives of this study were (i) to determine the effect of IGF-1R inhibitor (NVP-AEW541) and EGFR inhibitor (Gefitinib) on the viability of breast cancer cells, (ii) to determine the effects of KU0063794 (MTOR inhibitor), PD0325901 (MEK inhibitor), and NVP-BKM120 (PI3K inhibitor) which are small molecule inhibitors that target the downstream molecules of IGF-1R pathway on breast cancer cell viability, and (iii) to determine the efficacy of inhibitor combinations on cell viability, apoptosis, cell cycle, and the expression of phosphorylated biomolecules related to the targeted signaling pathways. Combinations of several inhibitors (NVP-AEW541, KU0063794, PD0325901, NVP-BKM120, and Gefitinib, simplified as AEW, KU, PD, BKM, and Gefitinib) that target different molecules in the IGF-1R pathway were compared with single (mono) inhibitor treatment. All combinations tested on MCF-7 and MDA-MB-231 cells can induced cell apoptosis and G1 and G2/M phase arrest. Furthermore, BKM+AEW, KU+BKM, and KU+AEW exhibited cytotoxic activity in MCF-7, while KU+AEW, PD+BKM, PD+KU, and PD+AEW led to complete cytostasis in MDA-MB-231 cells. The inhibitors also inhibited the activation of IGF-1R and its downstream pathways (PI3K/AKT, MTOR, MAPK/ERK), but there were exceptional new findings on the effects of BKM on MAPK/ERK pathway and PD on PI3K/AKT pathway in breast

cancer cell lines. The blockade of PI3K/AKT pathway by PI3K inhibitor (BKM) caused activation of MAPK/ERK pathway. However, the blockade of MAPK/ERK pathway by MEK inhibitor (PD) leads to activation of PI3K/AKT pathway through a negative feedback loop between PI3K/AKT and MAPK/ERK pathways. This study reveals that combination between KU and AEW exerted the most significant synergistic effect on growth inhibition of MCF-7 cells, whereas the most significant synergistic effect were found with PD and AEW in MDA-MB-231 cells. These two combinations yield the most significant synergistic effect on growth inhibition of the cells by blocking the IGF-1R downstream pathways which causes decreased in growth rate, induction of apoptosis, and cell cycle arrest. In addition, to the best of our knowledge, targeting MTOR and IGF-1R pathways by using KU and AEW is a potential new combination therapeutic strategy for breast cancer. The strategy of targeting IGF-1R pathway and its downstream molecules for breast cancer therapy is useful as they can exert synergism to reduce cell viability and to induce apoptosis and cell cycle arrest in breast cancer cells. The combination of inhibitors can also inhibit the activation of IGF-1R downstream pathways that may provide a therapeutic benefit in treating breast cancer through the pathways feedback as described in this study.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KESAN GANGGUAN LALUAN ISYARAT TRANSDUKSI RESEPTOR 1 FAKTOR PERTUMBUHAN MIRIP INSULIN (IGF-1R) DENGAN PERENCAT IGF-1R, EGFR, PI3K, MEK, DAN MTOR DALAM SEL KANSER PAYU DARA

Oleh

AYUNADIRAH BINTI AYUB

Jun 2014

Pengerusi : Profesor Seow Heng Fong, PhD
Institut : Biosains

Reseptor 1 pertumbuhan faktor mirip Insulin (IGF-1R) telah menunjukkan ekspresi berlebihan pada pesakit kanser payudara. Laluan isyarat IGF-1R diketahui mengaktifkan banyak kinase, dan protein yang berkaitan dengan pertumbuhan dan kemandirian sel. Mensasarkan kinase dan laluan mereka adalah satu pendekatan terapeutik yang menjanjikan berbanding dengan gangguan kepada IGF-1R sahaja. Objektif umum kajian ini adalah untuk menentukan kombinasi perencat molekul kecil yang boleh mensinergikan untuk merencat pengaktifan IGF-1R dan laluan hilirannya untuk mengurangkan kebolehhidupan dan pertumbuhan sel kanser payudara. Objektif khusus kajian ini adalah (i) untuk menentukan kesan perencat IGF-1R (NVP-AEW541) dan perencat EGFR (Gefitinib) terhadap kebolehhidupan sel-sel kanser payudara, (ii) untuk menentukan kesan-kesan KU0063794 (perencat MTOR), PD0325901 (perencat MEK), and NVP-BKM120 (perencat PI3K) iaitu perencat molekul kecil yang mensasarkan molekul laluan hiliran IGF-1R pada kebolehhidupan sel kanser payudara, dan (iii) untuk menentukan keberkesanan rawatan gabungan pada kebolehhidupan sel, apoptosis, kitaran sel, dan ekspresi biomolekul terfosforil yang berkaitan dengan signal aliran sasaran. Kombinasi beberapa inhibitor (NVP-AEW541, KU0063794, PD0325901, NVP-BKM120, dan Gefitinib diringkaskan seperti AEW, KU, PD, BKM, dan Gefitinib) yang mensasarkan molekul yang berbeza dalam laluan IGF-1R telah dibandingkan dengan tunggal (mono) rawatan perencat. Semua kombinasi diuji kepada MCF-7 dan MDA-MB-231 sel mendorong apoptosis sel dan penahanan fasa G1 dan G2/M. Tambahan pula, BKM+AEW, KU+BKM, dan KU+AEW menunjukkan aktiviti sitotoksik dalam MCF-7, manakala KU+AEW, PD+BKM, PD+KU, dan PD+AEW membawa kepada sitostatik lengkap di MDA-MB-231 sel. Semua inhibitor juga menghalang pengaktifan IGF-1R dan laluan hiliran (PI3K/AKT, MTOR, MAPK/ERK), tetapi terdapat suatu penemuan baharu yang luar

biasa pada kesan BKM pada laluan MAPK/ERK dan PD pada laluan PI3K/AKT di sel kanser payudara. Sekatan laluan PI3K/AKT oleh perencat PI3K (BKM) menyebabkan pengaktifan laluan MAPK/ERK. Sebaliknya, sekatan laluan MAPK/ERK oleh perencat MEK (PD) mengarah kepada pengaktifan laluan PI3K/AKT melalui gelung maklum balas negatif antara laluan PI3K/AKT dan MAPK/ERK. Kajian ini menperlihatkan bahawa kombinasi diantara KU dan AEW menunjukkan kesan sinergi yang ketara dalam perencatan pertumbuhan sel MCF-7, manakala kesan sinergi yang paling ketara ditemui diantara PD dan AEW di sel MDA-MB-231. Kedua-dua kombinasi menghasilkan kesan sinergi yang paling ketara pada perencatan pertumbuhan sel dengan menyekat laluan hiliran IGF-1R yang menyebabkan penurunan pada kadar pertumbuhan sel, apoptosis induksi, dan penahanan kitaran sel. Sebagai tambahan, sepanjang pengetahuan kami, mensasarkan laluan MTOR dan IGF-1R dengan menggunakan KU dan AEW adalah strategi kombinasi teraputik baharu yang berpotensi untuk kanser payudara. Strategi mensasarkan laluan IGF-1R dan molekul hiliran untuk terapi kanser payudara adalah berguna kerana ia boleh mendorong sinergi untuk mengurangkan kebolehhidupan sel dan untuk mendorong apoptosis dan penahanan kitaran sel dalam sel-sel kanser payudara. Gabungan inhibitor juga boleh menghalang pengaktifan IGF-1R laluan hiliran yang boleh menyediakan satu kebaikan terapeutik dalam merawat kanser payudara melalui maklum balas laluan seperti yang telah diterangkan dalam kajian ini.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deep and sincere gratitude to my supervisor, Prof. Dr. Seow Heng Fong for trusting and allowing me to start this project. Her generous guidance, advice, endless support and encouragement throughout the period of this project have been the foundation and driving force for this thesis. Her careful review and constructive criticism have been crucially important for this thesis.

My sincere gratitude is also accorded to my co-supervisor, Dr. Yip Wai Kien for his full support, constructive advice, priceless comments, and sharing his expertise in this research throughout the entire course of this research.

I would like to acknowledge and thank all the staffs in the Immunology Laboratory, especially Ms. Siti Aishah, Mrs. Marsitah, Mrs. Siti Ezura, and Mr. Anthonysamy for their assistance in laboratory management.

I am indebted to my lab mate, especially my work partner, Mr. Andrew Goh Tse Hau for his collaboration and endless support in every experiments conducted in this study. Not forgetting the other lab mates, Mr. Ng Chin Tat, Mr. Samuel Khoo Leon Juan, and Ms. Iris Goh Wen Li for sharing everyday joys and miseries in the making of science.

Finally, yet importantly, no word to say how grateful I am to have parents and family members who always support and encourage me to do my very best in all aspects of life and not to forget, supported me financially to complete this thesis. I also would like to thank Allah s.w.t. for everything. His blessings are countless that I cannot even write them down.

I certify that a Thesis Examination Committee has met on 27 June 2014 to conduct the final examination of Ayunadirah binti Ayub on her thesis entitled "Effects of Disrupting Insulin-Like Growth Factor- 1 Receptor (IGF-1R) Signal Transduction Pathways with Inhibitors of IGF-1R, EGFR, PI3K, MEK, and MTOR in Breast Cancer Cell Lines" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Name of Chairman of Supervisory Committee: Professor Dr. Seow Heng Fong

Signature: _____
Name of Member of Supervisory Committee: Dr. Yip Wai Kien

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LIST OF ABBREVIATIONS

5-FU	Fluorouracil
AKT	v-akt murine thymoma viral oncogene homolog
ATP	Adenosine triphosphate
BAD	BCL-2-associated death domain
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
GSK3	Glycogen synthase kinase 3
HER2	Human epidermal growth factor receptor 2
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2R	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding protein
IRS	Insulin receptor
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase

MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEK	MAPK/ERK
MTOR	Mammalian target of rapamycin
MTORC1	Mammalian target of rapamycin complex 1
MTORC2	Mammalian target of rapamycin complex 2
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NF-kB	Nuclear factor-kB
PBS	Phosphate buffered saline
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate
PKB	Protein kinase B
RAF	v-raf-1 murine leukemia viral oncogene homolog
RTK	Receptor tyrosine kinase
S473	Serine 473
SDS	Sodium dodecyl sulfate
Thr308	Threonine 308

CHAPTER 1

INTRODUCTION

Cancer is a diverse disease with many molecular drivers that control growth, survival as well as response to treatment. Breast cancer is the second most common cancer worldwide and is the leading cause of cancer-related deaths among women (Ferlay *et al*., 2010). Surgery, radiotherapy and chemotherapy are commonly used in breast cancer treatment. While high survival rates are reported for early stage of this disease, the prognosis for patients with metastatic disease remains poor. Clinical trials with new targeted drugs are disappointing in treating metastatic or recurrent breast cancers. Consequently, further understanding of the molecular and cellular changes of metastatic breast cancer is important in the development of new and promising therapies.

Insulin-like growth factor (IGF) plays a key role in signal transduction pathways that control growth of breast cancer cells (Sachdev and Yee, 2001). Deregulation of multiple molecules of IGF signaling pathway is one of the important molecular abnormalities in breast cancer (Belfiore and Frasca, 2008). High circulating levels of insulin-like growth factor 1 (IGF-1) concentration has been reported to increase the risk of breast cancer in women (Sachdev and Yee, 2007) predominantly at pre-menopausal ages (Hankinson *et al*., 1998). In addition, epidermal growth factor (EGF) is also one of the growth factors, other than IGF, that modulate the growth of the cells. Breast cancer is one of the diseases that has been reported to have mutations in EGFR (Bogdan and Klambt, 2001).

The activation of insulin-like growth factor 1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR) lead to activation of downstream signaling pathways such as phosphatidylinositol 3-kinase (PI3K), AKT, mammalian target of rapamycin (mTOR), and mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) which have been found in a variety of human cancers, including breast cancer. These activated signaling pathways lead to enhanced proliferation, survival and metastasis in breast cancer cells as well as inhibit apoptosis (Sachdev and Yee, 2007). IGF is one of many signaling pathways in cancer cells. Thus, it is more likely that effective IGF-1R targeting in breast cancer must occur in parallel with the blockade of other activated signaling pathways. One of the earlier studies has explored the effects of combination between DALO (humanized monoclonal antibody of IGF-1R) and RIDA (rapamycin analog MTOR inhibitor) (Di Cosimo *et al*., 2010) in preclinical models and patients of breast cancer. The dual IGF-1R/MTOR inhibition resulted in synergistic antitumor activity and is more effective in treating the patients as compared to single treatment. Therefore, interrupting these signaling pathways appears to be a rational approach for new cancer treatment regimens. Targeting both IGF-1R and its downstream pathways may be a strategy to maximally inhibit the growth in breast

cancer cells. Small molecule inhibitors targeting the significant molecules of these pathways can be applied to inhibit proliferation and survival of breast cancer cells.

Earlier studies have shown inhibition of IGF-1R, EGFR, PI3K/AKT, MTOR, and MAPK/ERK pathways by using monoclonal antibodies or specific drugs (Scotlandi *et al*., 2005), but the use of small molecule inhibitors is still lacking. Existing literature on the effects of small molecule inhibitors, especially in combination treatment, is still inadequate. Hence, five small molecule inhibitors have been selected and used in this study to investigate their effects on two breast cancer cell lines, MCF-7 and MDA-MB-231 which are different in the expression of estrogen receptor (ER), progesterone receptor (PR), and ERBB2 (or known as human epidermal growth factor receptor 2 [HER-2]). MCF-7 is a model for ER-positive and PR-positive tumors, while MDA-MB-231 is a triple-negative breast cancer (lack of ER, PR, and ERBB2) model. The differences in the expression of these receptors provide evidence on the heterogeneity of breast cancer. This disease can be classified into different subtypes such as luminal (MCF-7) and basal (MDA-MB-231). It is important to study cells with different subtypes because each subtype has different response to treatment (Sørlie *et al*., 2001).

The general objective of this study was to determine the appropriate combination of small molecules inhibitors which can synergize to inhibit the activation of IGF-1R and its downstream pathways in order to decrease the viability of breast cancer cell lines.

The specific objectives of this study were:

1. To determine the effect of IGF-1R inhibitor (NVP-AEW541) and EGFR inhibitor (Gefitinib) on the viability of breast cancer cells.
2. To determine the effects of KU0063794 (MTOR inhibitor), PD0325901 (MEK inhibitor), and NVP-BKM120 (PI3K inhibitor) which are small molecule inhibitors that target the downstream molecules of IGF-1R pathway on breast cancer cell viability.
3. To determine the efficacy of inhibitor combinations on cell viability, apoptosis, cell cycle, and the expression of phosphorylated biomolecules related to the targeted signaling pathways.

The hypothesis of this study is that, combination of inhibitors of IGF-1R and other downstream pathways can synergize to reduce viability of breast cancer cells.

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