UNIVERSITI PUTRA MALAYSIA

MOLECULAR MODELLING OF BERBERINE DERIVATIVES AS INHIBITORS OF ONCOGENIC SIGNALLING PATHWAYS IN BREAST CANCER CELL LINES

PARHAM JABBARZADEH KABOLI

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By

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

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DEDICATIONS

This thesis is dedicated to:

My late father, R.I.P., the best teacher I have ever had in my life who never stopped giving of himself in countless ways,

My dearest mother, the best lover in my life who gives me endless love, hopes and supports,

My sisters, Mahnoosh, Hanieh, and Sanaz, who have given me their genuine kindness,

My brothers-in-law, Mohammad and Ali Reza, who empower the family with their hope and energy,

My cute nephews, Aria and Pouya, smart and lovely, and the next generation of gentlemen,

My sweet niece, Parmis, a beautiful princess,

My late grandparents and all people gave me love and happiness,

and

You, as the valuable reader with the very best wishes in your academic journey.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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December 2017

Chair: Professor Patimah Binti Ismail, PhD
Faculty: Medicine and Health Sciences

Berberine (BBR) is an alkaloid that is widely distributed in different plant species. Several studies have been carried out on the anti-cancer effects of BBR but direct targets of BBR are unknown. In the development of approximately 20-25% of all cancers, altered hedgehog (Hh) signalling is involved where the smoothened (Smo) transmembrane receptor triggers Hh signalling pathway towards *Gli1* gene expression. Besides Smo receptor, BRAF mutations have been also detected in 7% of all cancers and 66% of melanomas; as such, the FDA has approved a few BRAF inhibitor drugs to date. However, BRAF can activate CRAF leading to resistance to BRAF inhibitors. The aim of this study is to model and compare the effects of BBR against key proteins involved in Hedgehog, MAP kinase, and PI3K pathways using *in silico* and *in vitro* approaches. BBR is found to interact with Lys395 of Smo receptor via hydrogen bonding and cation-π interactions [Score= -8.72 kcal/mol (Smo)]. In addition, π-π interactions between BBR aromatic rings and two aromatic residues in the Smo transmembrane domain, Tyr394 and Phe484, are noted. Target specific binding efficiency indices using an *in-silico* approach are calculated to plot the Smo-specific binding potency of each ligand. In addition, interactions of BBR derivatives (total number= 485 derivatives) against BRAF and CRAF kinases are modelled and predicted using an *in silico*-based approach. The Adenosine Triphosphate (ATP) is modelled in order to analyse and identify the residues important in BRAF docking. Results show Lys483 and Asp594 are the most important residues involved in both ATP and BBR binding [Scores= -11.50 kcal/mol (ATP); -8.50 kcal/mol (BBR)]. In addition to these polar residues, Trp530 and Phe583 are also applicable to the molecular docking of BRAF. The Asp593 is excluded from the enzyme cavity, while Phe594 is included inside the cavity, making the enzyme inactive. Finally, three alternatives

for BBR are identified with dual RAF inhibition effects. Direct effects of BBR derivatives against BRAF and CRAF kinases have not yet been reported previously, and thus, for the first time, three protoberberines are reported as lead compounds against RAF kinases [BBR-7 (Score= -9.75 kcal/mol), BBR-9 (Score= -9.76 kcal/mol), and BBR-10 (Score= -9.27 kcal/mol)]. In the current study, molecular docking and molecular dynamics simulations are also used for EGFR, p38 MAPK, ERK1/2 and AKT. The effects of BBR on MAP kinase and PI3K pathways are evaluated using Immuno-florescence assays and the amounts of phosphorylated kinases are compared with total kinases after they are treated with different concentrations of BBR [IC_{50}s= 7μM (EGFR), 45μM (ERK1/2), 60μM (AKT), 75μM (p38)]. BBR interacts accurately with EGFR, AKT, P38, and ERK1/2 active sites *in silico*, and decreases the level of active forms of corresponding enzymes in studied cell lines [Scores= -7.22 kcal/mol (EGFR), -7.51 kcal/mol (ERK), -7.78kcal/mol (AKT), -7.42 kcal/mol (p38)]. In addition, it is observed that BBR has toxicity effects which leads to cyto-necrotic effect at longer treatment time. This study predicts the role of BBR as an inhibitor of Smo receptor, suggesting its effectiveness in hedgehog signalling during cancer treatment. It concludes that BBR through its multi-kinase inhibitory effects may be a useful replacement for lapatinib, an EGFR inhibitor which may sometimes cause drug resistance in patients.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MODEL MOLEKULAR DERIVATIF BERBERIN SEBAGAI PENGHAMBAT LALUAN SIGNAL ONKOGENIK DALAM GARISAN SEL KANSER BUAH DADA

Oleh

PARHAM JABBARZADEH KABOLI

Disember 2017

Pengerusi: Profesor Patimah Bt. Ismail, PhD
Fakulti: Perubatan dan Kesihatan

Berberin (BBR), merupakan alkaloid yang didapati dalam spesies tumbuhan yang berlainan. Banyak kajian telah dilakukan untuk mengetahui secara mendalam kesan anti-kanser BBR namun sasaran langsung BBR tidak berjaya diketahui. Dalam 20-25% kes kanser, signal Hh adalah terlibat di mana reseptor transmembrane Smo mengaktifkan laluan isyarat Hh ke arah ekspresi gen Gli1. Selain reseptor Smo, mutasi BRAF juga dikesan dalam 7% daripada semua kanser dan 66% kes melanoma. Oleh yang demikian, FDA telah meluluskan beberapa ubat perencat BRAF. Walau bagaimanapun, BRAF boleh mengaktifkan CRAF yang memberi rintangan terhadap perencat BRAF. Kajian ini menggunakan pendekatan *silico* dan *in vitro* untuk memodel dan membandingkan kesan BBR terhadap protein utama yang terlibat dalam Hh, MAP kinase, dan laluan PI3K BBR didapati berinteraksi dengan Lys395 dari reseptor Smo melalui ikatan hidrogen dan interaksi kation-π [Skor= -8.72 kcal/mol (Smo)]. Di samping itu, interaksi π-π antara BBR aromatic ring dan dua residu aromatik dalam domain transmembran Smo, Tyr394 dan Phe484, telah diperhatikan. Indeks yang berdasarkan pengikat sasaran tertentu efisen menggunakan pendekatan dalam silico untuk memplot potensi pengikat khusus Smo bagi setiap ligan. Di samping itu, interaksi derivatif BBR (jumlah = 485 derivatif) terhadap kinas BRAF dan CRAF dimodelkan dan diramalkan menggunakan pendekatan berasaskan siliko. Untuk menganalisis dan mengenal pasti residu penting dalam dok BRAF, ATP dimodelkan dan didapati bahawa Lys483 dan Asp594 adalah residu terpenting yang terlibat dalam ATP dan pengikat BBR [Skor= -11.50 kcal/mol (ATP); -8.50 kcal/mol (BBR)]. Sebagai tambahan kepada residu polar ini, Trp530 dan Phe583 juga boleh digunakan untuk mengikat molekul

BRAF. Asp593 dikecualikan daripada rongga enzim, manakala Phe594 digunakan di dalam rongga, dan kesannya adalah enzim ini menjadi tidak aktif. Tiga alternatif untuk BBR dikenal pasti yang mempuyai kesan penghambatan RAF [BBR-7 (Skor= -9.75 kcal/mol), BBR-9 (Skor= -9.76 kcal/mol), and BBR-10 (Skor= -9.27 kcal/mol)]. Kesan langsung derivatif BBR terhadap kinase BRAF dan CRAF belum dilaporkan sebelum ini, dan oleh itu, untuk pertama kalinya, tiga protoberberin dilaporkan sebagai sebatian utama terhadap kinas RAF. Dalam kajian ini, simulasi dok molekul dan molekul dinamik juga digunakan untuk EGFR, p38 MAPK, ERK1 / 2 dan AKT. Kesan BBR pada laluan kinas MAP dan PI3K dinilai menggunakan ujian Immuno-florescence dan jumlah fosforilasi kinas dibandingkan dengan jumlah kinas selepas rawatan menggunakan kepekatan BBR yang berlainan [IC_{50}= 7μM (EGFR), 45μM (ERK1/2), 60μM (AKT), 75μM (p38)]. Interaksi BBR memberi keputusan adalah tepat dalam eksperimen siliko di EGFR, AKT, P38, dan ERK1 / 2, dan berjaya mengurangkan keaktifan enzim yang di lihat dalam garisan sel yang yang dikaji [Skor= -7.22 kcal/mol (EGFR), -7.51 kcal/mol (ERK), -7.78kcal/mol (AKT), -7.42 kcal/mol (p38)]. Di samping itu, kita perhatikan bahawa BBR mempunyai kesan toksik yang menyebabkan kesan sito-nekrotik jika jangka masa rawatan adalah lebih lama. Kajian ini meramalkan peranan BBR sebagai perencat reseptor Smo yang menunjukkan keberkesanannya dalam isyarat Hh dalam rawatan kanser. Kita boleh menyimpulkan bahawa BBR melalui kesan perencatan pelbagai-kinas adalah pengganti yang efisen untuk lapatinib, perencat EGFR yang kadang-kadang boleh menyebabkan rintangan ubat kepada pesakit.

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I certify that a Thesis Examination Committee has met on 19 December 2017 to conduct the final examination of Parham Jabbarzadeh Kaboli on his thesis entitled "Molecular Modelling of Berberine Derivatives as Inhibitors of Oncogenic Signalling Pathways 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 Doctor of Philosophy.

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

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- the research conducted and the writing of this thesis was under our supervision;
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Name of Member of Supervisory Committee:
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Signature: _____

Name of Member of Supervisory Committee:
Abdah Md Akim

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Å	Angstrom, 10^{-10} meter
ABC	ATP-binding cassette
ADME	Absorption Distribution Metabolism Excretion
AP	Alkaline phosphatase
ATB	Automated topology builder
BBB	Blood Brain Barrier
BBR	Berberine
BIRC	Baculoviral IAP repeat-containing *protein*
BRCA	Breast cancer protein
CCND1	Cyclin D1
CCND3	Cyclin D3
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cIAP	Cellular inhibitor of apoptosis
DMSO	Dimethyl sulfoxide
DS	Discovery studio
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EM	Energy minimization
ERBB	Human epidermal growth factor receptor
ERK	Extracellular signal–regulated kinase
FACS	Fluorescent associated cell sorting
FBS	Fetal bovine serum
FEN1	Flap structure-specific endonuclease 1
FF	Force field
GA	Genetic algorithm
Genrestr	Generate position restraints
Gromacs	GROningen MAchine for Chemical Simulations
GROMOS	GROningen MOlecular Simulation computer program package

hAGP	human alpha1-acid glycoprotein
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
HER2	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
HAS	Human serum albumin
IC50	Half inhibitory concentration
Ki	Inhibition constant
LEH	Ligand efficiency index
LEI	Ligand efficiency index
LGA	Lamarckian genetic algorithm
LJ	Lennard-Jones potential
LLE	Ligand lipophilicity efficiency index
M	Molar
MAPK	Mitogen activated protein kinase
MAPK1	Mitogen-activated protein kinase 1 (ERK2)
MAPK3	Mitogen-activated protein kinase 1 (ERK1)
MAPK14	Mitogen-activated protein kinase 14 (p38 MAPK)
MD	Molecular dynamics
µM	Micromolar, 10^{-6} molar
µm	Micrometer, 10^{-6} meter
MTOR	Mechanistic target of rapamycin (serine/threonine kinase)
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MW	Molecular weight
nBEI	Binding efficiency index based on non-hydrogen atoms
NHA	Number of non-hydrogen atoms
NIH	National institute of health
nM	Nanomolar, 10^{-9} molar
nm	Nanometer, 10^{-9} meter
ns	Nanosecond, 10^{-9} second
ps	Picosecond, 10^{-12} second
NPOL	Number of polar atoms

NPT	Constant number of atom, pressure, temperature
NSEI	Binding efficiency index based on polar surface area
nTDOF	Number of torsional degree of freedom
NVT	Constant number of atom, volume, temperature
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PDB	Protein data bank
PI	Propidium iodide
PI3K	Phosphatidylinositide 3-kinase
PK	Pharmacokinetics
pKi	-LogKi
Posres	Position restraints
RAF	Rapidly Accelerated Fibrosarcoma
RDF	Radial distribution function
Rg	Radius of gyration
RFC1	Replication factor C (activator 1) 1
RFC3	Replication factor C (activator 1) 3
RFC4	replication factor C (activator 1) 4
RFC5	Replication factor C (activator 1) 5
RMSD	Root-mean-square deviation
RMSF	Root-mean-square fluctuation
ROS	reactive oxygen species
SHC1	SHC (Src homology 2 domain containing) transforming protein 1
Smo	Smoothened receptor
SPC	Simple Point Charge water model
SPDBV	Swiss protein data bank visualizer
TM	Transmembrane
TORDOF	Torsional degree of freedom
TPSA	Topological polar surface area
vdW	van der Waals
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Brief background

Breast cancer is the most frequently diagnosed cancer in women, and is the second most common cancer worldwide. In 2012, 25% (1.67 million) of all new cancer cases and 15% (522,000) of all cancer deaths in women were due to breast cancer (Tao et al., 2015). Based on the data provided between 2000 and 2014, an estimated 252,710 new cases of invasive breast cancer will be diagnosed among women and 2,470 cases will be diagnosed in men in 2017. In addition, 63,410 cases of in situ breast carcinoma will be diagnosed among women. Approximately 40,610 women and 460 men are expected to die from breast cancer in 2017 (American Cancer Society, 2017) (Figure 1.1). Breast cancer is also the most common cancer in South-East Asian females. A woman in Malaysia had a 1 in 20 chance of developing breast cancer in her lifetime. In Malaysia, 37 individuals per 100000 of population for breast cancer cases, and 14.7 individuals per 100000 of population for death related to breast cancer have been reported (Miao et al., 2014).

Berberine can be isolated from the stems and roots of several plants, such as *Berberis vulgaris* and *Coptis chinensis* (Tomosaka et al., 2008; Zovko Končić et al., 2010; Abd El-Wahab et al., 2013). Berberine is a nitrogenous cyclic compound (Figure 1.2) with a structure that is highly similar to that of intercalating agents (e.g., ethidium). Intercalating agents are often used as nucleic acid dyes to study cell functions, and berberine is a well-known alkaloid drug that is commonly used as a fluorescent dye (Wang et al., 2012).

Berberine (Figure 1.2) induces apoptosis and inhibits cell proliferation in various cell lines derived from breast, lung, colon, and liver cancer. However, berberine has been shown to have synergistic effects on cells treated in combination with more toxic drugs, including vincristine and irinotecan (Kars et al., 2008). Previous studies showed that drug resistance was decreased by combination treatments with berberine (Sun et al., 2009; Zhou et al., 2012; Kukula-Koch et al., 2013).

Despite these findings, berberine efficacy and the molecular regulators that are targeted by berberine remain unclear. This thesis aims to provide an extensive analysis of berberine effects on various molecular mechanisms. In addition, this thesis also covers the metabolism, toxicity and adverse effects of berberine by using of molecular simulation of pharmacodynamics and pharmacokinetics for different berberine derivatives to provide more effective drug candidates than berberine.

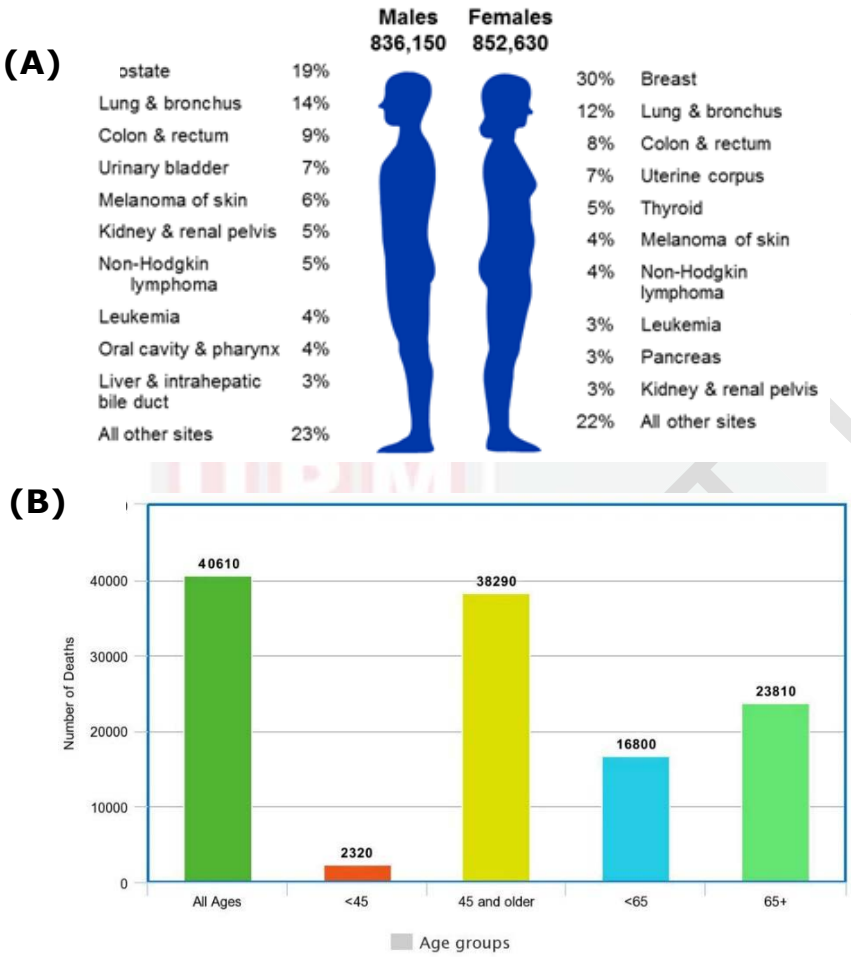

Figure 1.1: Prevalence of breast cancer and other kinds of cancer in the United States
(A) Estimated new cancer cases in US in 2017; **(B)** Estimated number of deaths by age for female breast cancer.
Source: (American Cancer Society, 2017)

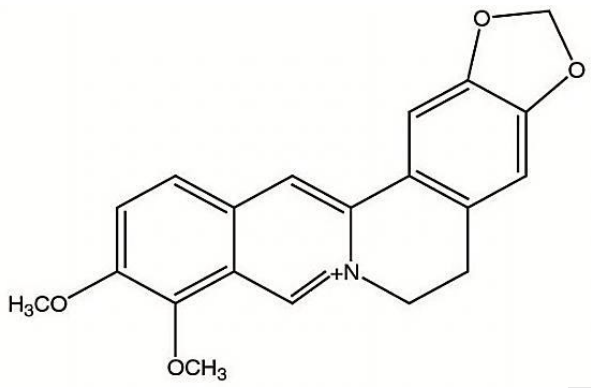

Figure 1.2: Berberine structure
Berberine is an alkaloid containing three aromatic rings and five polar atoms. Topological Polar Surface Area (TPSA) is 40.8 $Å^2$.

1.2 Problem statement

There are many research gaps associated with anticancer effects of berberine. By damaging and mutating the DNA, oxidative stress, radiation, and mutagens turn normal cells to cancerous forms. As a result, while these mutations change certain genes that control critical biological processes, one or several kinds of cancer occur. The use of anti-oxidant compounds, which is broadly paid attention as an anti-cancer treatment, is a long-term treatment method. Whilst a cancer case observed, however, a significant and short-term treatment way must be found. Drugs which control genes and proteins that are not working properly must be discovered. Consequently, effects of drugs on genes that can cause cancer must be studied.

While drug candidates are studied by different experimental methods to evaluate its specific target, it is generally hard to spend millions of dollars to pass different levels of approval steps: preclinical, and phase 1-3 clinical phases. The challenge of *in silico* studies is the time, often several months, required for molecular modelling and molecular dynamics simulations. Accordingly, the mentioned mixed strategy of *in silico* and *in vitro* investigations not only save the budget and increase the number of drug candidates before starting the laboratorial works, but it can also lead to the more effective drug candidates; meanwhile, the toxicity and distributions of candidates can be also modeled.

There is a lack of such software, at least as a well-known and universal tool, to compute the ligand binding efficiency index and several other parameters from the thousands of input compounds. At the moment, the current tools of drug discovery analyze separate steps of drug discovery and many parameters should be manually calculated. This makes them

difficult to use for thousands of molecules, and the researchers must be involved during throughout of the procedures.

1.3 Objectives

1.3.1 General objective

To determine the effects of berberine and its derivatives on different signalling pathways inovolved in breast cancer.

1.3.2 Specific objectives

a. To identify more effective berberine derivatives against smoothened receptor.

b. To identify more effective berberine derivatives as multi-kinase inhibitors for cancer treatment.

c. To determine anti-tumour activity of berberine in breast cancer cell lines.

1.4 Hypothesis

a. Berberine inhibits smoothened receptor.

b. Berberine derivatives are multi-kinase inhibitors in cancer treatment.

c. Berberine is anti-tumour in breast cancer cell lines.

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