UNIVERSITI PUTRA MALAYSIA

DRUG REPURPOSING OF CLINICALLY-APPROVED DRUGS TO TARGET EPITHELIAL-MESENCHYMAL TRANSITION USING IN SILICO ANALYSIS

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By

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

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Chair : Professor Daud Ahmad Israf Ali, PhD
Faculty : Medicine and Health Science

Epithelial-mesenchymal transition (EMT) is a process where epithelial cells transform into mesenchymal cells type like fibroblasts and myofibroblasts. In the process, the epithelial cells lose their epithelial phenotype with reduced apical-basal polarity, while acquiring new mesenchymal traits with increased invasiveness. Accumulation of the mesenchymal cells also leads to the deposition of collagen in the extracellular matrix (ECM). It is well established that EMT contributes to the progression of fibrosis and cancer diseases. Therefore, a therapeutic method that inhibits the EMT process would be required. Transforming growth factor-beta (TGF-β) is a potent inducer of the EMT process. Previous studies had demonstrated that inhibition of the TGF-β receptor type 1 (also named ALK5) could inhibit EMT. However, current progress on the clinical development of novel ALK5 inhibitor has not been encouraging, often due to safety concerns of the novel drug leads. Since traditional de novo drug discovery comes with high risks, pharmaceutical companies have begun to use drug repurposing strategy for drug development. Drug repurposing or repositioning is a strategy of finding new therapeutic purposes for current existing drugs in the clinical market. Due to the fact that these drugs had been established to be safe for use, it would reduce the concerns of safety risks in human. In this study, drug repurposing approach was used to identify clinically approved drugs that can inhibit the EMT process via targeting TGF-β activity. Initial computational screening of clinically approved drugs via molecular docking had revealed several drugs with strong binding affinity (-10.8 to -9.6 kcal/mol) to ALK5 based on the reference range of known ALK5 inhibitors ranging from -11.2 to -9.5 kcal/mol. The shortlisted drug candidates include ergotamine, telmisartan, saquinavir, indinavir, nelfinavir and celecoxib. Subsequently, these drugs were tested experimentally in normal human bronchial epithelial cell line, BEAS-2B induced by TGF-β1. In the experiments, the

morphology changes from cobblestone shape of epithelial cells towards elongated shape of mesenchymal cells were not prevented by the drug treatments. In addition, the drugs did not exhibit inhibitory effects on the downregulation of epithelial proteins (E-cadherin) and upregulation of mesenchymal proteins (vimentin and α-smooth muscle actin). Based on these observations, it is postulated that the results from molecular docking were false positives. It is recommended that future studies involving molecular docking method would require better optimization and improvement by performing cross-docking validation prior to screening and including the negative controls during screening. The tested drugs in this study could serve as negative controls in future screening against ALK5 protein.

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REPOSISI DADAH KLINIKAL UNTUK MENSASARKAN PERALIHAN EPITELIUM KEPADA MESENKIMA DENGAN ANALISIS *IN SILIKO*

Oleh

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Pengerusi : Professor Daud Ahmad Israf Ali, PhD
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Peralihan epitelium-mesenkima (EMT) merupakan satu proses dimana sel-sel epitelium berubah menjadi sel-sel mesenkima seperti fibroblas dan myofibroblas. Di dalam proses tersebut, sel-sel epitelium kehilangan fenotip epitelium mereka disebabkan oleh pengurangan polariti apikal-basal, sementara memperolehi ciri mesenkima yang baru dengan peningkatan invasif. Pengumpulan sel-sel mesenkima turut membawa kepada pemendapan kolagen di dalam matriks ekstrasel (ECM). Seperti yang diketahui umum, EMT merupakan penyumbang kepada perkembangan penyakit fibrosis dan kanser. Oleh itu, satu kaedah terapeutik yang boleh merencat proses EMT amat diperlukan. Transformasi faktor pertumbuhan-beta (TGF-β) merupakan pendorong kuat dalam proses EMT. Kajian terdahulu telah menunjukkan bahawa perencatan reseptor TGF- β jenis 1 (juga dipanggil ALK5) boleh merencat proses EMT. Namun begitu, kemajuan semasa perkembangan klinikal perencat baharu ALK5 tidak memberangsangkan, selalunya berpunca daripada masalah keselamatan mengenai dadah yang baharu. Memandangkan penemuan ubat secara tradisional de novo mempunyai risiko tinggi, syarikat farmaseutikal telah mula menggunakan strategi penggunaan semula ubat bagi pembuatan ubat. Penggunaan semula atau reposisi ubat merupakan strategi untuk mencari penggunaan terapeutik baru bagi ubat yang sedia ada di pasaran klinikal. Oleh kerana ubat-ubatan tersebut telah terbukti selamat untuk digunakan, ini akan mengurangkan kebimbangan terhadap risiko keselamatan pada manusia. Dalam kajian ini, penggunaan semula ubat telah digunakan untuk mengenal pasti ubat-ubat yang telah diluluskan secara klinikal yang dapat merencatkan proses EMT dengan mensasarkan aktiviti TGF-β. Saringan awal komputasi ubat yang telah diluluskan secara klinikal melalui pendokkan molekular mendedahkan beberapa ubat-ubatan mempunyai ikatan afiniti yang kuat dengan ALK5, termasuk ergotamine, telmisartan, saquinavir, indinavir, nelfinavir dan celecoxib. Seterusnya, ubat-ubatan tersebut diuji secara eksperimen pada sel bronkus manusia normal, BEAS-2B yang dirangsang oleh TGF-β1. Di dalam eksperimen ini, rawatan ubat tidak dapat menghalang

perubahan morfologi daripada epitilum sel berbentuk batu buntar ke bentuk sel mesenkima yang memanjang. Tambahan lagi, ubat-ubatan tersebut tidak menunjukkan kesan perencatan terhadap penurunan regulasi protein epitelium (E-cadherin) dan peningkatan regulasi protein mesenkima (vimentin dan otot lembut α-aktin). Berdasarkan pemerhatian ini, disimpulkan bahawa keputusan daripada pendokkan molekular merupakan positif palsu. Adalah disarankan agar kajian akan datang melibatkan pendokkan molekular memerlukan pengoptimuman dan peningkatan yang lebih baik. Ubat-ubatan yang diuji di dalam kajian ini boleh berfungsi sebagai kontrol negatif di dalam saringan terhadap protein ALK5 pada masa akan datang.

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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 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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LIST OF ABBREVIATIONS

ADT	Autodock Tools
AIDS	Acquired immunodeficiency syndrome
ALK	Activin receptor-like kinase
ASO	Antisense oligonucleotide
BCA	Bicinchoninic acid assay
BDL	Bile duct ligation
BEGM	Bronchial Epithelial Growth Medium
CCL4	Carbon tetrachloride
Co-Smad	Common partner Smad
COX-2	Cyclooxygenase 2
CTBP	C-terminal binding protein
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
FHD	First-in human dose
GSI	Glomerular Sclerosis Index
IL	Interleukin
I-Smad	Inhibitory Smad
LAP	Latency-associated peptide
LTBP	Latent TGF-β binding protein

MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PBS	Phosphate buffer saline
PDB	Protein Data Bank
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RMSD	Root mean square division
R-Smad	Receptor-regulated Smad
SDS-PAGE	Sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
shRNA	Short hairpin ribonucleic acid
TF	Transcription factor
TGF-β	Transforming growth factor-beta
T_{reg}	Regulatory T cell
TSP	Thrombospondin
TβR	TGF-β receptor
uPA	Urokinase-type plasminogen activator
UUO	Unilateral urethral obstruction
ZO	Zona occludens
α-sma	Alpha-smooth muscle actin

CHAPTER 1

INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a process where epithelial cells acquire mesenchymal traits while losing their epithelial phenotype. This process was first recognised in embryonic development, where the pluripotent epiblast forms the mesoderm and endoderm via EMT (Hay, 1995; Kim et al., 2018). The EMT process also plays a major role in wound healing. During the re-epithelialization phase of wound healing, the EMT process allows the epithelial cells to acquire the invasive phenotype of mesenchymal cells, so that the cells can migrate and close the wounds (Stone et al., 2016). The EMT process can be involved in pathological conditions such as fibrosis and cancer metastasis when it is not adequately regulated (Barriere et al., 2015). When EMT is adequately regulated, the epithelial cells transform into mesenchymal cell-types such as fibroblasts and myofibroblasts for the production and deposition of extracellular matrix (ECM) components that would help in the wound healing process. However, pathologically prolonged induction of EMT would result in persistent myofibroblast activation and excessive deposition of ECM components, which eventually lead to fibrosis (Stone et al., 2016). In cancer, the epithelial cells transform to a mesenchymal cell type with the ability of invasion and migration through ECM, which allows them to metastasise via the blood and lymphatics (Ramos et al., 2017).

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is known to be a potent inducer of the EMT process. TGF-β has many important physiological roles including regulation of inflammation, proliferation, differentiation, and apoptosis (Prud'homme, 2007). However, it can become problematic if the level of TGF-β goes unchecked. Chronic inflammation and elevated levels of TGF-β can lead to excessive induction of the EMT process. As a result, the mesenchymal cells and ECM components will replace the normal parenchymal tissue. While TGF-β can suppress the tumour growth in the early phase of neoplasia, excessive TGF-β induction of EMT can promote cancer metastasis in the later phase of malignant tumour development (Prud'homme, 2007). All these problems have highlighted the importance to develop a new therapy that can inhibit the EMT process by targeting TGF-β activity.

To date, various research projects have tried to develop novel inhibitors of TGF-β for the treatment of fibrosis and cancer, but none so far have reached the market. This highlights the difficulty of developing a new drug, which is costly, time-consuming and has a low success rate (Xu et al., 2015). In order to overcome this issue, the drug discovery fraternity have turned to another strategy, known as drug repurposing. Drug repurposing or repositioning is a process of identifying new therapeutic uses for existing clinically-approved drugs (GNS et al., 2019). Since an existing drug has already been tested in humans, it is less likely to fail in clinical trials due to toxicity issues. Moreover, numerous preclinical and clinical data would be available for an existing drug. As a result,

the repurposing process will be relatively less time-consuming and also less costly as compared to the traditional *de novo* drug discovery process. Aside from the benefits of saving valuable resources in drug development, drug repurposing can help to deepen the understanding of the mechanism of action for old drugs and sometimes also lead to discovery of novel therapeutic targets of the diseases. One notable example of drug repurposing is sildenafil, a phosphodiesterase type 5A inhibitor that was originally developed for angina pectoris. Its initial clinical trial revealed little efficacy on the cardiovascular system, and a side effect of penile erection was reported at high doses. Eventually, the investigators repurposed sildenafil for the treatment of erectile dysfunction, and later successfully marketed it as Viagra (Kass et al., 2007).

Taking into consideration that the success rate from development to approval for novel drugs (11%) is lower than repurposed drugs (30%) globally (Fetro and Scherman, 2020), increased efforts have been focused on the repurposing of clinically approved drugs in the recent years. There are computational and experimental approaches that could be used in order to identify the drug candidate for a new indication of interest. Recently, Ab Ghani et al. (2019) have designed a web server known as Drug Repositioning Exploration Resource (Drug ReposER) that can identify potential alternative targets of known drugs by comparison of the three dimensional amino acid arrangement of known drug binding site from the Protein Data Bank (PDB) repository with the query protein. The concept is that when the binding site of two different proteins share similarity in amino acid arrangement, a drug that is known to bind to the first protein would likely bind to the other protein as well. Molecular docking is another computational tool that can predict how two molecules (for example a ligand and a receptor) can form stable binding. This method utilise docking algorithms to predict the binding affinity of a ligand to the binding site of the protein target. Therefore, a large number of drugs can be screened against a protein of interest that is involved in a disease by using the molecular docking tool. This would allow the identification of drugs with the best affinity (in comparison to positive controls) towards the protein of interest based on the result of molecular docking prediction. Subsequently, experiments can be performed to verify the results of molecular docking. This can be carried out with cell-based assays to demonstrate whether the selected drugs can affect the cell phenotype relevant to the disease model. In this study, computational methods (in silico) were used to screen clinically approved drugs for potential inhibition of the TGF-β type I receptor. The identified drugs were further examined in subsequent cell-based assays that employed a TGF-β1-induced model of the bronchial epithelial cell line, BEAS-2B. In past studies, molecular docking and *in vitro* screening of new compounds were used as the first steps in the development of TGF-β inhibitors but many hit compounds eventually failed at the clinical stage. In current study, a new drug repurposing approach by combining the bioinformatics-based method (Drug ReposER) and molecular docking was used to screen for potential TGF-β inhibitors among the clinically approved drugs. This approach would expedite the development of new TGF-β inhibitors after successful identification of potential drug candidate.

1.1 General objective

To identify existing drugs that inhibit TGF-β activity upon the bronchial epithelial cell line, BEAS-2B

1.2 Specific objectives

i. To identify potential drug candidates with high binding affinity to TGF-β type 1 receptor by using molecular docking

ii. To determine the effects of selected drug candidates on the cell morphology of TGF-β1-induced BEAS-2B cells

iii. To determine the effect of selected drug candidates on the EMT markers of TGF-β1-induced BEAS-2B cells

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