ORIGINAL ARTICLE

Drug Repurposing of Clinically Approved Drugs to Target Epithelial-mesenchymal Transition Using Molecular Docking Approach

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ABSTRACT

Introduction: Epithelial-mesenchymal transition (EMT) is a process of epithelial transformation into mesenchymal cells. It is also a process that contributes to the progression of fibrosis and cancer metastasis. Transforming growth factor-beta (TGF-β), as a potent inducer of EMT, has therefore became a potential therapeutic target. However, clinical developments of TGF-β inhibitors have been un-successful due to safety risks. Hence, drug repurposing of existing safe-to-use drugs could over-come this issue. **Methods:** In this study, the TGF-β receptor type 1 (ALK5) was selected as the target protein. Molecular docking was performed using known ALK5 inhibitors as positive controls. Clinical drugs with similar binding affinity and amino acid interaction were selected for in vitro experimental validation. **Results:** ALK5 inhibitor demonstrated binding affinities ranging from -11.2 to -9.5 kcal/mol. Analysis of amino acid interaction revealed that Val219, Ala230, Lys232, and Leu340 amino acid residues are crucial for binding. Subsequent screening of clinically approved drugs against ALK5 showed top five potential drugs (ergotamine, telmisartan, saquinavir, indinavir, and nelfinavir). The selected drugs were tested in TGF-β1-induced normal human bronchial epithelial cell line, BEAS-2B. Western blot analysis showed that 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). **Conclusion:** Based on these experimental outcome, it is postulated that the results from molecular docking were false positives. The tested drugs in this study could serve as negative controls in future screening against ALK5 protein.
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INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a process where epithelial cells acquire mesenchymal traits while losing their epithelial phenotype. This process contributes to pathological conditions like fibrosis and cancer (1). Epithelial cells transform into mesenchymal cell types like fibroblasts and myofibroblasts for the production and deposition of extracellular matrix components such as collagen. Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that is known as a potent inducer of the EMT process. TGF-βhas many important physiological roles like regulation of inflammation, proliferation, differentiation, and apoptosis. However, it can become problematic if the level of TGF-β goes unchecked. Individuals with chronic inflammation and elevated levels of TGF-β are subject to uncontrolled wound healing. As a result, the mesenchymal cells and extracellular matrix components will replace normal parenchymal tissues. While TGF-β can suppress the tumour growth in the early phase of neoplasia, TGF-β induction of EMT can promote cancer metastasis in the later phase of a malignant tumour (2). All these problems have highlighted the importance to develop a therapy that can inhibit the EMT process by targeting TGF-β activity.

Drug repurposing or repositioning is a process of identifying new therapeutic uses for existing clinically approved drugs. 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 lead to elucidation of the mechanism of action for old drugs and may also lead to discovery of novel therapeutic targets of 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 (3). Taking into consideration that the success rate from development to approval for novel drugs (11%) is lower than repurposed drugs (30%) globally (4), 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 a drug candidate for a new indication of interest. Recently, Ab Ghani and colleagues 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 sites from PDB repository with the query protein (5). 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 utilises 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 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. Enzyme-based assays can be carried out to assess the binding of the selected drugs to the protein of interest in vitro; while cell-based assay can demonstrate whether the selected drugs can affect the phenotype relevant to the disease model.

Activin receptor-like kinase 5 (ALK5) is the major type 1 receptor of TGF-β. Its inhibition can prevent activation of the TGF-β receptor and downstream signalling. ALK5 inhibitors are known to inhibit the EMT process via suppression of TGF-β signalling. Notable ALK5 inhibitors such as vactosertib and galunisertib display great potential in the inhibition of TGF-β-mediated EMT (6-7). Since EMT is known to contribute to cancer metastasis, vactosertib and galunisertib were eventually investigated in clinical trials that involve treatment of cancers (8-9). Despite the efforts, none of the ALK5 inhibitors reported have been approved for clinical use. In this study, we used a drug repurposing approach to identify potential ALK5 inhibitors by performing virtual screening of clinically approved drugs followed by experimental validation using cell-based assay.

MATERIALS AND METHODS

Protein Structure Retrieval

TGF-β receptor type 1 kinase domain (ALK5) was selected as the target protein of interest in this study. A ligand-bound protein structure is more suitable than ligand-free protein structure in molecular docking due to better defined geometries in binding pocket of ligand-bound structure (10). From the Protein Data Bank (PDB), the 3D structure with the PDBID of 3TZM was chosen. The structure has the presence of co-crystallized ligand SB431542 (Ligand ID: 085), a known inhibitor of ALK5. The structure has a resolution of 1.7 E, which is considered high resolution.

Ligand Structure Retrieval

PDBID 3TZM was entered as the query protein structure in the Drug ReposER web server. The structure was subjected to search against a database of known drug binding site for 3D amino acids patterns that are similar to the known drug binding sites in other proteins. Results of matched patterns of amino acid in query protein against known drug binding site was generated after the search is complete. The corresponding ligands in each of the drug binding site was retrieved from the search results. Then, the structures of ligands were downloaded from the drug bank.

Preparation of Proteins and Ligands

The protein 3TZM was loaded as the macromolecule structure in AutoDockTools 1.5.6 (ADT) (11). The co-crystallized ligand was removed from the structure. Then, water molecules were deleted from the macromolecule structure. Hydrogens were added to all residues followed by the addition of Kollman charges. Lastly, the non-polar hydrogens were merged. Each of the ligand file was also loaded in ADT and was prepared by the addition of Gasteiger charges. The prepared macromolecule and ligand structures were saved as PDBQT format, which is the required file format for Autodock Vina.

Molecular docking

Docking simulation was performed using Autodock Vina, which is a widely used molecular docking and virtual screening tool developed as the successor of Autodock (12). The search space was specified by using the grid box function in ADT. The entire binding site where the co-crystalized SB431542 bound to was covered in the grid box. The number of points in x, y, z-dimensions were 30*30*30. The centre coordinates for x, y and z were set to 3.355, 8.217, and 5.907 respectively. The default setting for the grid space was 1 E. The exhaustiveness value remained at the default of 8. After

each docking run, the binding pose with the best binding affinity (lowest binding energy) was considered the best. The best binding energy between the macromolecule and the ligand was recorded for each of the ligands. The top five ligands with the highest binding affinity were further analysed for their interaction with the amino acid residues at the binding site using Poseview (13). Self-docking was also performed for the validation of the docking process. The co-crystalized ligand was removed from the protein and redocked to the binding site by using the above mentioned parameters. Then, the root-mean-square deviation (RMSD) between the best docking pose and the original co-crystalized pose was calculated in PyMOL.

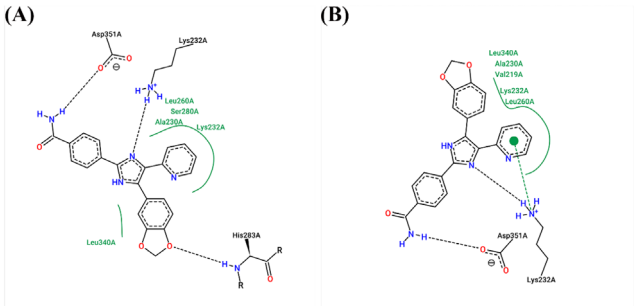

Figure 1: Poseview 2D interaction diagram of SB431542. Black dashed line: hydrogen bond; Green dashed line: ϖ interaction; Green line: hydrophobic contact. (A) Co-crystalized SB431542. (B) Redocked SB431542

Experimental validation

Normal human bronchial epithelial cell line, BEAS-2B was grown in Bronchial Epithelial Growth Media (BEGM) (Lonza, Switzerland). Seven cell groups were included for each set of experiment, which include normal untreated group, TGF-β1-induced group, positive control group treated with known inhibitor (SB431542), three pre-treatment drug groups and one co-treatment drug group. Pre-treatment of drugs were given at 1, 3, and 24 hours prior to TGF-β1 induction while the co-treatment group was treated at the same time as TGF-β1 induction. All the groups except for normal group were induced by TGF-β1 for four days. The cells were lysed in RIPA lysis buffer containing protease inhibitor cocktail (Nacalai Tesque, Japan) in 1:100 dilutions. Equal amounts of the extracted protein samples were loaded for sodium dodecycl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After protein separation by SDS-PAGE, the proteins were transferred to polyvinylidene fluoride (PVDF) membrane in a wet transfer system at 0.35 mA for 90 minutes. The blots were blocked for 1 hour with 5% bovine serum albumin (BSA) before incubation in primary antibody overnight at 4 °C. Secondary antibody incubation was carried out for 1 hour at room temperature. Chemiluminescence signal was then detected using WesternBright ECL kit (Advansta, USA) in gel documentation system (Vilber Lourmat, Germany). The antibodies used in this study include anti-rabbit HRP-linked IgG antibody (CST, USA), E-cadherin rabbit monoclonal antibody (CST, USA), alpha-smooth muscle actin (α-sma) rabbit monoclonal antibody (CST, USA), α-tubulin rabbit monoclonal antibody (CST, USA), and vimentin rabbit monoclonal antibody (Santa Cruz, USA).

RESULTS

The protein structure with PDBID 3TZM was selected in this study. Co-crystalized ligand SB431542 resides in the active site of the kinase domain. SB431542 was first discovered as a small molecule ALK5 inhibitor that can block the TGF-β response (14). Fig. 1A demonstrates the interaction between the inhibitor and the amino acid residues of the active site. The inhibitor forms hydrogen bonding with several amino acids including Lys232, His283 and Asp351. There are also hydrophobic interactions between the inhibitor and Ala230, Lys232, Leu260, Ser280, and Leu340. Most of the amino acid interactions demonstrated by the crystal structure pose were reproduced by the best docking pose (Fig. 1B), except for the hydrogen bonding with His283 and hydrophobic interaction with Ser280. There are also a few additional interactions that were not originally observed in the co-crystal structure, namely a hydrophobic interaction with Val219 and also ϖ interaction with Lys232 in addition to the hydrogen bonding. Superimposition of co-crystalized ligand and redocked ligand showed similar binding pose upon visual inspection (Fig. 2). Comparison of the best docking pose with the crystal structure pose showed RMSD of 1.065. Since the RMSD is less than 2.0 E, the docking simulation is considered a success (15).

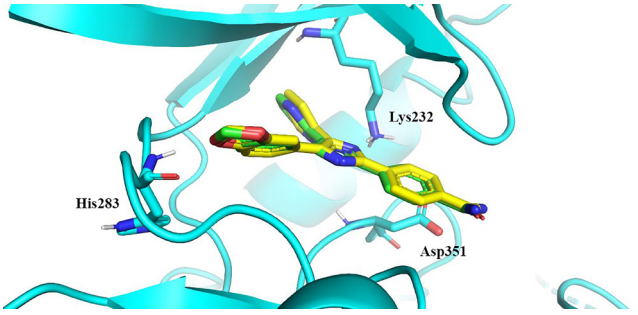

Figure 2: Magnified ribbon representation of superimposed co-crystal structure of ALK5-SB431542 complex and the redocked ligand. Green: co-crystalized ligand; Yellow: redocked ligand.

After molecular docking, all the ligands were ranked from the lowest to highest binding energies based on their best binding poses. This is because low binding energy indicates good binding affinity between the ligand and the protein. As references, several other known inhibitors of ALK5 were also included in the molecular docking analysis. The best binding energies for those ALK5 inhibitors ranged from -9.5 to -11.2 kcal/mol (Table I & Fig. 3). SB431542 was ranked the first among the others, with the highest binding affinity of -11.2 kcal/mol. Since the macromolecule structure

Table I: Lowest binding energy and amino acid interactions of known ALK5 inhibitors. Bolded amino acid residues are commonly interacting with known ALK5 inhibitors

Known Inhibitor	Lowest binding energy (kcal/mol)	Interaction with amino acid residues		
		Hydrogen bonding	Hydrophobic contact	ϖ interaction
SB431542	-11.2	**Lys232**, Asp351	**Val219**, **Ala230**, **Lys232**, Leu260, **Leu340**	Lys232
Galunisertib	-11.1	**Lys232**	Ile211, **Val219**, **Ala230**, **Lys232**, **Leu340**	
GW788388	-10.8	**Lys232**, Arg294	Ile211, **Val219**, **Ala230**, **Lys232**, Leu260, Gly286, **Leu340**	Lys232
Vactosertib	-10.6	**Lys232**	**Val219**, **Ala230**, **Lys232**, Lys337, **Leu340**	
SB505124	-10.3	**Lys232**	**Ala230**, **Lys232**, Leu260, **Leu340**	
SD208	-10.3	**Lys232**	**Lys232**	
SB525334	-10.2	**Lys232**	**Ala230**, **Lys232**, Leu260, **Leu340**	Lys232
R268712	-9.5	Ile211	Ile211, **Val219**, **Lys232**, Gly286, **Leu340**	

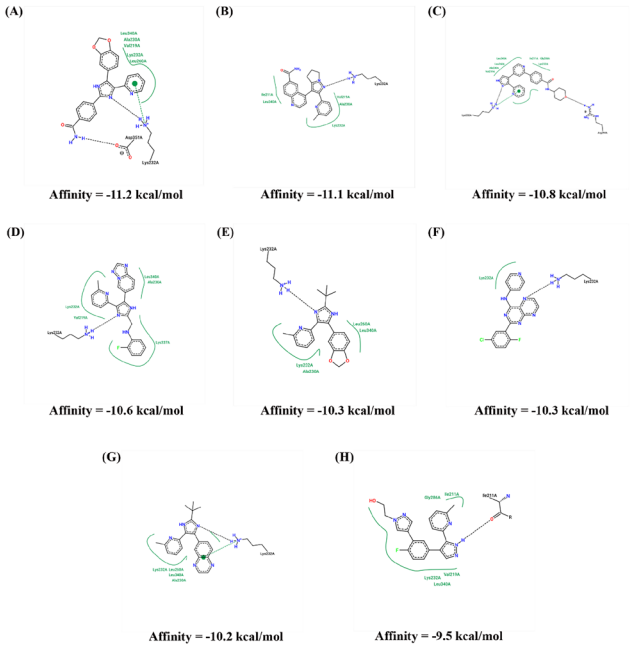

Figure 3: Poseview 2D interaction diagram of known ALK5 inhibitors. Black dashed line: hydrogen bond; Green dashed line: ϖ interaction; Green line: hydrophobic contact. (A) SB431542. (B) Galunisertib. (C) GW788388. (D) Vactosertib. (E) SB505124. (F) SD208. (G) SB525334. (H) R268712.

used in docking was a structure co-crystallized with SB431542, there has already been an induced-fit conformational change at the active site. Thus, it is not surprising to see the docking of SB431542 demonstrated the greatest binding affinity as compared to the other known inhibitors. Most of the known ALK5 inhibitors formed hydrogen bonding with Lys232 except for R268712, which has the lowest binding affinity of -9.5 kcal/mol among the inhibitors. However, all of the known inhibitors displayed hydrophobic contact with Lys232, while a few of them (SB431542, GW788388, SB525334) also formed ϖ interaction with Lys232. This makes Lys232 a very crucial amino acid residue for compound interaction in order to have a good binding affinity. In a study by Araujo, Lys232 was also used as a selection criterion for structure-based virtual screening for novel ALK5 inhibitors (16). In addition to Lys232, there are also several amino acid residues that were commonly forming hydrophobic contact with the known inhibitors, which include Val219, Ala230, and Leu340. Hydrophobic interaction with Leu340 is the second most common among the known inhibitors, with only SD208 not having this interaction. Six out of eight known inhibitors showed hydrophobic interaction with Ala230, while five known inhibitors had hydrophobic interaction with Val219. These amino acid residues would also be essential references for subsequent molecular docking analysis of the drugs. In fact, the top four known inhibitors (in terms of binding energies) had displayed interactions with all of these amino acid residues, while the other known inhibitors with lower binding affinity had lesser interactions.

The top five ligands with the best binding affinity were ergotamine, telmisartan, saquinavir, indinavir and nelfinavir (Table II & Fig. 4). Ergotamine has the best binding affinity of -10.8 kcal/mol. Similar to most known inhibitors, the best binding pose of ergotamine formed hydrogen bonding with Lys232. Pi interaction with Lys335 residue was also observed. There are also seven amino acid residues found to have hydrophobic contacts with the best binding pose of ergotamine. Three of the essential amino acid residues were among the hydrophobic interactions, namely Ala230, Lys232 and Leu340. Similar to ergotamine, telmisartan also has the highest binding affinity among the other drugs, with the binding energy of -10.8 kcal/mol. Its best binding pose also had one hydrogen bonding with Lys232. It also had the most number of hydrophobic interactions (nine in total) compared to the other drugs. Moreover, it displayed interactions with all four essential amino acid residues, which makes telmisartan the most promising candidate.

Saquinavir has the second highest binding affinity of -10.6 kcal/mol, which is only slightly inferior as compared to ergotamine and telmisartan. Two hydrogen bonding

Table II: Lowest binding energy and amino acid interactions of top five drugs. Bolded amino acid residues are commonly interacting with known ALK5 inhibitors

Ligand ID	Known Inhibitor	Lowest binding energy (kcal/mol)	Interaction with amino acid residues		
			Hydrogen bonding	Hydrophobic contact	ϖ interaction
ERM	Ergotamine	-10.8	**Lys232**	Ile211, Arg215, **Ala230**, **Lys232**, Leu260, Lys337 **Leu340**	Lys335
TLS	Telmisartan	-10.8	**Lys232**	Ile211, **Val219**, **Ala230**, **Lys232**, Leu260, Leu278, Lys335, Lys337, **Leu340**	
ROC	Saquinavir	-10.6	**Lys232**, Asp351	Ile211, **Ala230**, **Lys232**, Leu260, Leu278, Gly286, **Leu340**	
MK1	Indinavir	-10	Asp290, Lys337	Ile211, **Val219**, **Lys232**, Leu260, Leu278, Gly286, Lys337, **Leu340**	
1UN	Nelfinavir	-10	Leu278	Gly212, **Val219**, **Lys232**, Lys335	

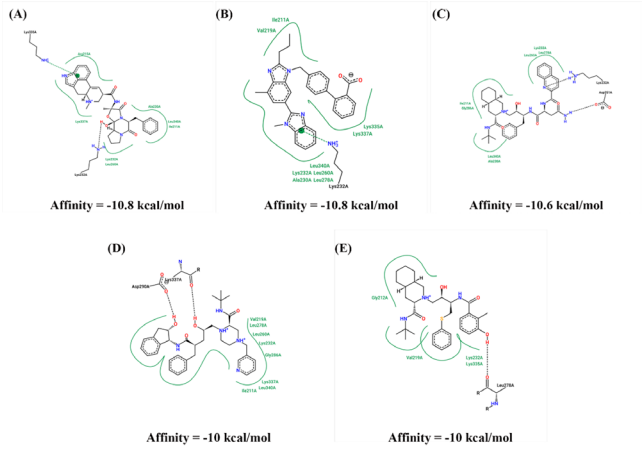

Figure 4: Poseview 2D interaction diagram of top five drugs. Black dashed line: hydrogen bond; Green dashed line: ϖ interaction; Green line: hydrophobic contact. (A) Ergotamine. (B) Telmisartan. (C) Saquinavir. (D) Indinavir. (E) Nelfinavir.

interactions with Lys232 and Asp351 were demonstrated by the best binding pose of saquinavir, which is similar to SB431542. It had hydrophobic contacts with several amino acid residues, which also include three of the essential amino acid residues, Ala230, Lys232 and Leu340. Although its binding energy is slightly inferior, the amount of amino acid interactions was quite similar to ergotamine. Next, indinavir and nelfinavir both had the best binding energy of -10 kcal/mol. Both of them did not display hydrogen bonding with Lys232. However, hydrogen bonding with other amino acid residues was observed. There are two hydrogen bonding interactions between the best binding pose of indinavir with Asp290 and Lys337; while the best binding pose of nelfinavir had only one hydrogen bonding with Leu278. Although indinavir did not demonstrate hydrogen bonding with Lys232, it still showed a good amount of hydrophobic interaction with three of the essential amino acid residues, namely Val219, Lys232, and Leu340. Nelfinavir had the least amount of amino acid interactions compared to the above four drugs. There were only four hydrophobic contacts observed, which include only two of the essential amino acid residues Val219 and Lys232. Based on the binding affinity and amount of amino acid interactions, this makes nelfinavir the least promising candidate among the other four drugs.

Ergotamine is an agonist at α-adrenoceptor, 5-hydroxytryptamine receptor and dopamine D2 receptor. It serves as a vasoconstrictor and is commonly used for the treatment of migraine (17). No previous study has investigated its ability to bind ALK5 and inhibit EMT to date. However, Felber & Bencheqroun reported that long-term treatment with ergotamine can cause severe fibrotic valvulopathy (18). It was postulated that activation of 5-hydroxytryptamine receptor upregulates TGF-β, which resulted in fibroblast and smooth muscle cell proliferation. This makes ergotamine unsuitable for inhibiting EMT even though our molecular docking result suggested that ergotamine is a strong ALK5 inhibitor. On the other hand, telmisartan is an inhibitor of angiotensin II receptor that is commonly used for treating hypertension (19). Unlike ergotamine, there were previous studies that investigated the effect of telmisartan on EMT. Treatment with telmisartan in renal proximal tubular cells were found to reduce EMT via inhibition of NAPDH oxidase/ERK1/2/ET-1 pathway and agonistic activation of PPAR-γ (20-21). Whether ALK5 inhibition played a part in the ability of telmisartan in EMT inhibition remains to be elucidated. Investigation of the inhibitory effect of telmisartan on the other type of cell lines would also be needed in future studies.

Saquinavir, indinavir and nelfinavir are the first generation HIV protease inhibitors. Emerging of HIV resistance to these antiretroviral agents have become a problem in the treatment of acquired immunodeficiency syndrome (AIDS) patients (22). Therefore, it would be beneficial to explore other potentials of these agents and repurpose them for the treatment of other diseases. In a recent study by Sanchez, nelfinavir was reported to decrease mesenchymal markers like type 1 collagen and α-sma in TGF-β1-induced normal human fibroblast culture (23). Phosphorylation of Smad2/3 and Akt were also significantly reduced by nelfinavir treatment,

showing that both canonical and non-canonical signaling pathway of TGF-β were inhibited by nelfinavir. However, it is still unclear whether the inhibition occurred via ALK5 inhibition. In the same study, an in silico proteome-wide screening was performed to predict the off-target interactions of nelfinavir. Interestingly, the data predicted several possible targets, which include ALK5 as the higher ranking candidate. This suggest that the inhibition of ALK5 could be one of the mechanism of actions that inhibited TGF-β signaling in the in vitro results of that study.

The changes in the expression of epithelial and mesenchymal proteins are the criteria of EMT process. After TGF-β1 induction, BEAS-2B cells showed a significant decrease in expression of E-cadherin and increased expression of α-sma and vimentin as compared to the normal group, where the cells received no treatment. These changes were prevented in the positive control group, where the cells were treated with a known ALK5 inhibitor, SB431542 (Fig. 5). Both α-sma and vimentin expression were significantly reduced while E-cadherin expression showed significant increase in the TGF-β1-induced BEAS-2B cells treated with SB431542. This indicates that inhibition on ALK5 was able to inhibit the EMT process in BEAS-2B in this experiment. However, all selected drug candidates were not able to prevent the changes in EMT markers similar to SB431542.

DISCUSSION

The experimental results of the drug candidates failed to correlate with the result in molecular docking in this study. This suggests that the prediction of ALK5 inhibition by the drug candidates in molecular docking was only false positive. There were many past studies that reported successful experimental validation using drug compounds with strong binding affinity to the therapeutic targets in molecular docking studies. Liu and colleagues reported a computational screening study of traditional chinese medicine to target neuraminidase from influenza A H1N1 virus. The study identified quercetin and chlorogenic acid with the highest binding affinity to neuraminidase, and later in vitro and in vivo experiments demonstrated that the compounds protected the influenza-infected cells from cytopathic effects and improved the survival rate in infected mice (23). In another study, molecular docking was used to identify a benzofuran derivative as strong inhibitor of STAT3, and the compound was able to dose-dependently decrease the phosphorylation of STAT3 in IL-6-induced HepG2 cells (24). Despite those above-mentioned successful examples, there are also studies that reported unsuccessful experimental results of hit compounds selected from molecular docking screening. In a virtual screening study of natural compounds to inhibit Akt signalling protein, STL1 and AC1 demonstrated favourable binding energy similar to IQO, a known inhibitor of Akt. However, only STL1 (40 μM) inhibited the phosphorylation of Akt in the HG3 cellular model. AC1 did not show any inhibitory effect towards Akt phosphorylation, even with increased concentration to 100 μM (25). Huang and colleagues also used molecular docking to identify 22 compounds as histone deacetylase inhibitors, but in the end there

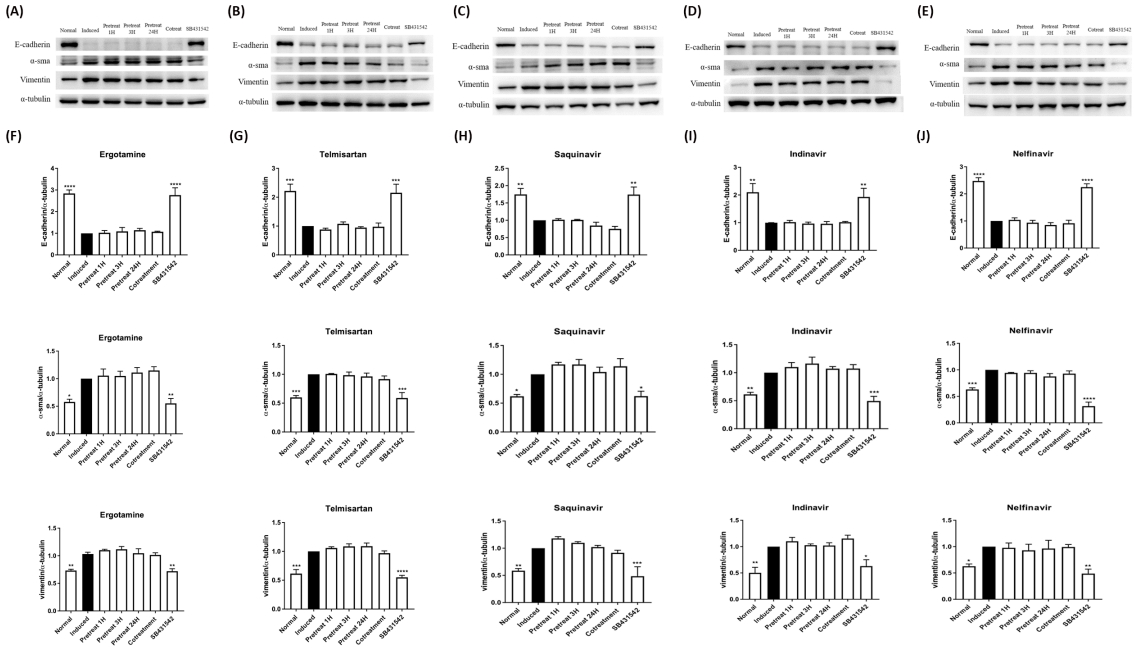

Figure 5: Effects of drug treatment on EMT markers in TGF-β1-induced BEAS-2B cells. Protein expression of E-cadherin, α-sma, and vimentin in cells treated with (A) Ergotamine, (B) Telmisartan, (C) Saquinavir, (D) Indinavir, and (E) Nelfinavir. Quantitative data were presented as mean ± S.E.M. of three independent experiments in densitometry analysis (F-J). Normal group represents cells that were remain untreated. Induced group represents cells that were induced with TGF-β1 only. SB431542 group represents cells that were treated with known ALK5 inhibitor and TGF-β1. * indicates significant different from induced group, P<0.05; ** indicates significant different from induced group, P<0.01; *** indicates significant different from induced group, P<0.001; **** indicates significant different from induced group, P<0.0001.

were only three of them showing inhibitory activity in the enzymatic assay (26). These studies have highlighted the issue of false positive result in molecular docking, which can hinder the process of virtual screening and drug discovery.

In hindsight, there are some improvements that can be made for the molecular docking method to reduce the false positives in this study. Most docking-based virtual screening assumes a rigid protein and flexible ligand model. In reality, the protein structure is supposed to be dynamic with lots of movement (27). If flexible protein model is employed, the search space for a large protein molecule would require an enormous amount of computational power. As a result, it is more practical to perform docking of flexible small molecule of ligand into a rigid large molecule of protein. Most of the protein structures are available in the PDB as three-dimensional X-ray crystallography structure. A single protein may have several crystal structures available in the PDB, and each of them can have differences in conformation. As mentioned before, protein-ligand binding is a dynamic event. Different ligand binding to the same protein can cause different induced-fit conformational changes in the ligand binding site of the protein. Therefore, the choice of crystal structure can affect the success of molecular docking (28). A cross-docking method would help in the selection of an appropriate crystal structure of a protein. In cross-docking, all the crystal structures of a protein can be retrieved. The bounded ligand in the crystallized protein-ligand complex will be used to dock into the other protein crystal structures. In other words, each crystal structure will undergo the docking of ligands that came from the other crystal structures. By doing this cross-docking, RMSD can be used to evaluate whether the binding pose of those ligands in the other crystal structures are different from its own crystallized state (experiment binding pose). Crystal structure that can reproduce ligands binding poses similar to the experimental structure would serve as the suitable protein structure for docking-based virtual screening (29). In a study by Bhojwani and Joshi, cross-docking was employed for the selection of the most suitable protein structure for VEGFR-2 kinase. From 36 crystal structures obtained from PDB, the authors utilised self-docking and cross-docking method to eliminate inappropriate structure, and eventually identify the protein with PDBID of 4ASE as the most crystal structure (30). This method could be referred as an example for future virtual screening of drug compounds using molecular docking.

In addition to cross-docking, negative controls could be established to ensure that the protein structure do not mistakenly recognise inactive ligands as strong binders. While positive controls are used as a benchmark to identify ligands that are strong binders, the negative controls include a set of inactive decoy ligands that displayed no binding to the target experimentally. By combining both positive and negative controls in the benchmarking datasets, it could reduce biases in the assessment of molecular docking result (31). Early decoys selection would be difficult due to insufficient experimental data to select the inactive compounds. In a study by Bissantz and colleagues, the authors included 10 known active ligands in a random database of 990 ligands. The 990 random ligands were assumed to be decoys, and the docking was evaluated based on its ability to associate the 10 active ligands with the best docking score among the other ligands (32). However, it might be possible that unknown active ligands were included in the benchmarking dataset, which could result in false negative data. It is recommended to use known inactive compounds as decoys that were tested experimentally. Therefore, it is not entirely bad that selected drug candidates from virtual screening failed to demonstrate the expected effects experimentally, since they can be useful as decoys in the benchmark dataset. Mysinger and colleagues described experimental inactive decoys as compounds that showed no activity at concentration of 30 μM and above (33). This could be used as a reference for the decoys selection in the future studies involving molecular docking.

Another recommendation in future studies is to combine ligand-based approach together with structure-based method at the virtual screening stage. In ligand-based approach, ligands that are highly similar to known active compounds are predicted to be likely exhibiting similar activity against its protein target. Thus, compounds library will be screened based on their similarity level with the known active compound. However, the ligand-based approach has its own limitation due to the lack of data relative to inactive compound, which is useful for better calibration of ligand-based method in distinguishing between the active and inactive compounds (34). Since current study has identified several inactive drug ligands, they would be useful in ligand-based screening. The drug library could be screened and ranked based on their similarity to known active ligand and dissimularity to known inactive ligand. Subsequently, the hit compounds will be subjected to molecular docking to study the binding affinity and activity. This complementary approach could provide better outcome in identifying positive drug lead during experimental validation.

CONCLUSION

This study aims to utilise drug repurposing strategy to identify inhibitors on TGF-β receptor type 1 (also named ALK5). Initial computational screening had identified several drug compounds with strong binding affinity to ALK5, which include ergotamine, telmisartan, saquinavir, indinavir, and nelfinavir. In the subsequent in vitro experiments, the selected drug candidates did not exhibit the expected effects on EMT inhibition, while the known ALK5 inhibitor (SB431542) was able

to significantly inhibit EMT. As a result, it is concluded that the results from computational screening was only false positives. Future studies involving computational screening of drug compounds are recommended to further optimise and improve on the molecular docking method by having inclusion of negative controls and stringent selection of protein structure. As for the future research on EMT inhibition via targeting TGF-β, the intracellular signalling molecule of TGF-β such as Smad could be another ideal target of inhibition. In comparison to targeting the receptor, inhibition on only a subset of downstream signalling pathway may give new insight on the effects of different signalling pathways on the initiation of EMT process. Furthermore, different cellular model such as cancer cell line could also be employed to explore the effects of the drugs in cancer EMT.

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