Identification of afzelin potential targets in inhibiting triple-negative breast cancer cell migration using reverse docking

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Abstract

Background: Triple-negative breast cancer (TNBC) tends to be aggressive and metastatic, characteristics attributable to its cellular migration capabilities. Afzelin is a chemical compound with anti-metastatic potentials. This study aimed to predict proteins involved in TNBC cell migration which could be inhibited by afzelin.

Methods: The protein database was constructed from the Kyoto Encyclopedia of Genes and Genomes pathways collection which related to cell motility, then screened for druggability using SuperTarget and Therapeutic Target Database. The involvement of druggable proteins in the TNBC metastasis process was investigated through existing publications in The National Center for Biotechnology Information PubMed database. Inhibitory potential of afzelin toward target proteins was compared to the proteins' known-inhibitor, using the reverse docking method.

Results: Ten proteins identified as potential targets of afzelin, with the top 3 being ERK2, KRas, and FAK, respectively. Afzelin's 3-O-rhamnoside group played a dominant role in forming hydrogen bonds with the target proteins. Further analysis with STRING suggested that afzelin might be able to inhibit chemotaxis and haptotaxis of TNBC cells.

Conclusions: Afzelin was predicted to inhibit TNBC cell motility, by targeting ERK2, KRas, and FAK activation.

Keywords: afzelin, cell migration, reverse docking, TNBC

Introduction

Triple-negative breast cancer (TNBC) is one of breast cancer subtypes characterized by a lack of estrogen receptors, progesterone receptors, and human epidermal growth factor receptor 2 (HER2) expression. About 10% to 20% of breast cancers are categorized as TNBC subtypes.[1] Although based on California study, TNBC was often found in women of African descendant,[2] its was also found frequently in other ethnicities according to the survey of several countries with the largest population in the world such as China (25.5%),[3] India (27.9%–31%),[4,5] Indonesia (12%–25.5%),[6,7] and Pakistan (18%).[4]

TNBC is marked by its aggressive pathological behavior and poor prognosis. The TNBC mortality and recurrence rate is the highest within 3 years after diagnosis.[8] Distant metastases are found in 94.1% of TNBC patients. The lowest overall survival occurs when TNBC metastasizes to the brain, liver, and pleura.[9] Therefore, in addition to primary tumors treatment, the management of TNBC also needs to target the inhibition of metastasis.

Metastasis is the result of a series of cellular and biological events, each of which has different characteristics and requirements. On the other hand, almost all events have a similar requirement, which is the migration ability of cancer cells.[10] The cell migration in cancer occurs through hijacking physiological mechanisms, which involve several types of pathways that deliver extracellular stimuli to intracellular and from intracellular to the effector response and finally lead to cancer cell motility.[11]

Afzelin or kaempferol 3-O-rhamnoside belong to the flavonol glycoside group. Afzelin has been identified in 56 plants, making it readily available. Some of these plants are known to be edible, for example Annona purpurea, Piper umbellatum, Zingiber zerumbet, Nymphaea odorata, and Ginkgo biloba.[12] Afzelin is distributed in all plant parts, mainly plays a role in photosynthesis, similar to flavonoids in general.[13] The addition of the rhamnoside group makes afzelin structure different and unique than kaempferol, which might contribute to its ability to inhibit different signaling proteins and better selectivity.[14] A previous study has suggested the potential of afzelin as an inhibitor of TNBC cell migration. Although it was proven that afzelin reduced focal adhesion kinase (FAK) expression and inhibits Rac1-GTPase activation, the target proteins of afzelin have not been identified.[15] Given its potential to inhibit TNBC metastasis, further exploration is needed to identify target proteins of afzelin, as part of developing targeted therapy.

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Currently, virtual screening is extensively used to predict the binding of massive databases of ligands to a specific target, to identify the most promising compounds from the database for further study. Reverse docking is the opposite of the virtual screening method, in which clinically relevant proteins are screened against one active compound through docking method. Hence, reverse docking is also known as "one ligand many targets approach". The result of reverse docking is a list of target proteins ranked based on 'a score' that approximates free binding energy.[16]

This study aimed to identify potential target proteins (PTPs) of afzelin that are associated with TNBC cell migration. A previous study showed that afzelin decreased MDA-MB-231 cell motility. In this study, afzelin was docked to some proteins associated with various signaling pathways that regulate TNBC cancer cell migration and which were considered druggable targets. Afzelin interaction with the target proteins was compared with known inhibitors based on its binding energy. Prediction of afzelin PTPs in TNBC cell migration identified through reverse docking has never been conducted previously.

Materials and methods

Construction of target proteins database

In this study, the afzelin targets were signal transduction proteins involved in TNBC cell migration. First, protein exploration was carried out using pathways identified in Kyoto Encyclopedia of Genes and Genomes (KEGG).[17] Of the 530 pathway maps in the KEGG, 8 pathway maps related to cellular motility were established, which were part of Environmental Information Processing, Cellular Processes, and Human Diseases network (Table 1). Of these pathways, 160 proteins were identified, which were then examined for potential druggability through Supertarget and Therapeutic Target Database.[18,19] Afterward, the druggable protein's involvement in TNBC metastasis was evaluated through National Center for Biotechnology Information (NCBI) database using keywords overexpressed and/or metastasis and TNBC. The final result was a database of druggable TNBC migration proteins (subsequently will be referred to as target protein). FAK still being considered a candidate of the afzelin' target protein, that was explored in catalytic domain.

Preparation of afzelin ligand, known inhibitors and target proteins structure

Afzelin structure was prepared using known 3-dimensional structure presented in PubChem. The structure of target proteins was chosen and downloaded from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RSCB PDB), which have co-crystal complexes with known inhibitor and high resolution (<3 Å). Protein and inhibitor of each complex were separated using PyMol, and each was saved as a .pdb extension. Target proteins that did not have inhibitor—protein complex structure in RSCB PDB database, were obtained through existing publications. In these cases, the target proteins were downloaded from RSCB PDB while the known inhibitors were obtained from PubChem or generated using ChemSpider. Missing residues and atoms of each protein structure were repaired using Molsoft-ICM Pro. Water molecules and co-factors, which did not affect the binding site, were removed. Hydrogen atoms were added.

Reverse docking using PyRx

Reverse dockings in this study were performed with AutoDock Vina, which were integrated into PyRx–Virtual Screening Tool version 0.8.[20] PyRx predicted possible binding modes of ligand-protein complexes and corresponding binding energy (kcal/mol). The negative value of binding energy indicated that the ligand was predicted to be bound to a target macromolecule. A more negative the numerical values of the binding energy, indicated a better prediction of binding between ligands and macromolecules.

The reverse docking procedure was performed as follows: (1) each co-crystal inhibitor and corresponding protein were re-docked, to validate the docking position and binding energy. For each protein and associated inhibitor that was identified based on publication, docking was done at its important binding site residues as stated in the publications. AutoGrid was used for the preparation of the grid map using a grid size $25 \times 25 \times 25$ xyz point. (2) Afzelin was docked to each target proteins with the same grid box used for re-docking protein and corresponding known inhibitor. (3) Docking results were sorted according to the docking score differences between afzelin and known inhibitor and tabulated for further analysis. PoseView (http://proteinsplus.zbh.uni-hamburg.de/) was used to compare between protein-afzelin and protein-known inhibitor interactions, complemented with 3-dimensional illustrations using Pymol version 1.7.5.0 (Schrodinger, LLC.).

Results

Reverse docking results

The search process for target protein candidates identified 16.88% (27 of 160 proteins) in 8 KEGG pathways involved in cell migration that was classified as druggable proteins. Exploration of NCBI PubMed database demonstrated that 74% (20 target proteins) were overexpressed and involved in TNBC migration and metastasis (Table 2). Re-docking of each known inhibitor to its target protein exhibited varying binding energy, with the highest on protease-activated receptor-1 (PAR1) and the lowest on Na$^+$/H$^+$ exchangers isoform 1 (NHE1). Reverse docking of afzelin to target protein resulted in binding energy ranging from -4.7 to -11 kcal/mol, with the average binding energy of -8 kcal/mol. Further study on the potential of afzelin in inhibiting TNBC cell migration proteins activity was carried out based on the calculation of binding energy differences between afzelin and known inhibitors.

Identification of potential target proteins

Ten target proteins had higher binding energy with afzelin than known inhibitors (marked with an "*" in the "affinity of afzelin"

Table 1

Pathways related to cellular motility in KEGG database

Pathways	KEGG code	KEGG Network
RAP1 signaling	04015	Signal transduction
Focal adhesion	04510	Cellular Processes
Adherents junction	04520	Cellular Processes
Tight junction	04530	Cellular Processes
Actin cytoskeleton regulation	04810	Cellular Processes
ECM-receptor interactions	04512	Environmental Information Processing
Pathway to cancer	05200	Human Diseases
Proteoglycan in cancer	05205	Human Diseases

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Table 2

Results of reverse docking of target protein with known inhibitor and afzelin

Target name[*]	PDB ID	Known inhibitor	Affinity of known inhibitor	Affinity of afzelin	Reference of target protein-known inhibitor complex
B-catenin[21]	1JPW	MSAB	−7,0	−6,2	[22]
Cdk4[23]	1GIH	1PU	−11,3	−9,0	[24]
CK-2[25]	3BE9	PO4	−12,8	−11	[24]
	3MB7	14I	−11,2	−9,6	
	3PE1	3NG	−11,1	−10,1	
	4RLL	E9I	−10,3	−9,3	
	4KWP	EXX	−7,6	−8,6[†]	
c-Src[26]	2O1Q	STI	−12,4	−9,2	[24]
	4MX0	DB8	−7,2	−8,1[†]	
	2BDF	24A	−8,1	−7,9	
	5J5S	6G3	−12,6	−6,7	
	3G5D	1N1	−10,1	−9,3	
EGFR[27]	3POZ	O3P	−10,5	−9,5	[24]
	3W33	W19	−11,5	−7,7	
	4G5J	0WM	−7,3	−8,5[†]	
	5FED	5X4	−9,8	−7,7	
	4ZAU	YY3	−7,3	−7,3	
ERK-2[28]	4ZZN	CQ8	−7,4	−8,2[†]	[24]
	4QTA	38Z	−14,1	−9,4	
	4XP0	42A	−5,2	−8[†]	
	3QYW	6PB	−6,4	−7,8[†]	
FAK[29]	4EBV	007	−11,1	−6,3	[24]
	414E	1BQ	−9,8	−8,0	
	3BZ3	YAM	−11,2	−8,8	
	4K8A	K8A	−6,0	−7,6[†]	
Integrin alfa5 beta3[30]	1L5G	IPS-02001	−6,7	−7,1[†]	[31]
KRas[32]	5v9o	91G	−11,4	−7,5	[24]
	5KYK	6ZD	−8,3	−8,1	
	6FA3	D1Z	−6,4	−7,6[†]	
	4NMM	Y9Z	−10,5	−8,4	
	5OCG	9R5	−5,1	−7,2[†]	
MAPKK[33]	3E8N	VRA	−8,9	−8,4	[24]
	3EQB	LUG	−8,4	−6,3	
	3VVH	4BM	−9,7	−7,6	
	4AN3	5Y0	−9,0	−8,1	
NHE1[34]	2YGG	KR 33028	−5,0	−4,7	[35]
N-WASP[36]	1T84	WSK	−7,7	−8,5[†]	[24]
p130Cas[37]	3T6G	1IT6	−5,7	−6,0[†]	[38]
PAR1[39]	3VW7	VPX	−15,1	−8,1	[24]
PI3K[40]	1E7U	KWT	−9,2	−7,9	[24]
	4XE0	40L	−8,8	−5,6	
	4FHJ	0TZ	−8,1	−8,9[†]	
	4GB9	0WR	−9,1	−8,7	
	3L54	LXX	−8,7	−8,6	
PKC[41]	3IW4	LW4	−11,5	−9,1	[24]
	4RA4	3KZ	−10,1	−8,1	
Rac[42]	1MH1	EHop	−6,3	−5	[43]
RhoA[44]	5JHH	RA0	−7,2	−5,8	[24]
ROCK1[45]	3V8S	0HD	−8,8	−6,2	[24]
	5wnf	B4V	−10,8	−7,7	
	3TV7	EDO	−8,7	−8,1	
	4W7P	37J	−9,6	−7,3	
SHP-2[46]	1PXH	SNA	−9,2	−5,9	[47]

CDK4 = cyclin-dependent kinase 4, CK2 = casein kinase-2, EGFR = epidermal growth factor receptor, ERK-2 = extracellular signal-regulated kinase, FAK = focal adhesion kinase, MAPKK = mitogen-activated protein kinase kinase, NHE-1 = Na(+)/H(+) exchanger 4, NWASP = neural-Wiskott–Aldrich Syndrome protein, PAR1 = proteinase-activated receptor-1, PI3K = phosphatidyl inositol-3 kinase, PKC = protein kinase C, ROCK1 = Rho-associated protein kinase 1, SHP-2 = Src homology region 2 domain-containing phosphatase-2.

[*] Drug-able target proteins that were overexpressed and contributed to TNBC cell metastasis as supported by existing publications.

[†] Binding energy between target protein and afzelin that was higher than with the known inhibitor.

column in Table 2). Subsequently, this target proteins would be referred to as PTP. Afzelin demonstrated greater binding energy with ERK2/MAPK1 compared to 9 other proteins (KRas, FAK, EGFR, CK2, PI3K, NWASP, c-Src, ITGAB3, p130cas). This result was supported by stronger afzelin affinity with 3 known inhibitors of ERK2/MAPK1, compared to KRas with 2 known inhibitors and 8 other PTPs with only 1 known inhibitor (Table 2). The difference in affinity of afzelin with ERK2/MAPK1

Table 3

Potential target proteins of afzelin in TNBC cells migration, according to interacting residues

Target name	Gene	PDB ID	Interacting residues of known inhibitor	Interacting residues of afzelin
ERK2	MAPK1	4XP0	HB: Asp104; Met106	HB: Lys52; Gln103; Met106
			HI: Ala50,	HI: Ile29; Val37
KRas	KRAS	5OCG	HB: Ser39; Asp54A	HB: Glu37; Gln70; Leu6
			HI: Leu56	HI: Leu56; Thr74
FAK	PTK2	4K8A	HB: Cys502	HB: Glu506; Lys454
			HI: Leu553	HI: ILE428; Leu553; Val436; Gly505
EGFR	EGFR	4G5J	HB: Met793	HB: Thr854; Asp855; Lys745
			HI: Leu718; Ala743; Leu792; Cys797: Leu844	HI: Leu718
CK2	CSNK2A1	4KWP	HB: Asn118	HB: Leu45; Asn118; Val116
			HI: Leu45; Met163	HI: Met163; Val66; Ile174
c-Src	SRC	4MX0	HB: Ser342	HB: Ala390; Ala293
			HI: Leu393; Gly344; Leu273	HI: Leu273; Leu393; Val281
PI3K	PIK3CA	4FHJ	HB: Val882	HB: Asp841; Asn951; Ser806
			HI:Met804; Ile879; Ile963	HI:Ile831; Ile879; Ile963; Met953
NWASP	WASL	1T84	HB: His8	HB: Gly10; Asp18
			HI: Leu59; Gly10; Val9; Gly58; Ile53	HI: Leu59; Gly10; Val9; Gly58; Ile53
ITG α5β3	ITGA5 ITGB3	1L5G	HB:Ser121; Asn215; Ser123	HB: Arg216; Glu220; Asp217; Ser123; Tyr122; Tyr166
p130cas	BCAR1	3T6G	HB: Lys783	HB:Val779; His790
			HI: Ile786; Val827; Leu823;	HI: Ile786; Val827

HB = hydrogen bond, HI = hydrophobic interaction, underline = the same interacting residues between afzelin and known inhibitor.

and known inhibitors with ERK2/MAPK1 was also greater (−2.9 kcal/mol) than in other PTPs (Table 2). KRas, FAK, and EGFR binding energy with afzelin was slightly stronger than −1 kcal/mol compared to known inhibitors. Prediction of afzelin PTPs in TNBC cell migration which was tested by reverse docking has never been proven through any publications.

Further analysis with PoseView showed that afzelin and known inhibitors interacted with target proteins at the same residue. This similarity was found in one residue (MAPK1, KRas, FAK, EGFR, and ITG α5β3), 2 residues (CK2, PI3K, c-Src, p130cas) and 5 residues (NWASP) (Table 3). This result confirmed that each known inhibitor and afzelin interacted with PTPs in the same pocket. The binding energy between afzelin and all 10 PTPs was higher than the known inhibitor. PoseView analysis identified more hydrogen bonds and/or hydrophobic interactions in the afzelin-PTP interaction compared to known inhibitor-PTP interaction.

The interaction of afzelin with all PTPs showed that hydroxyl of ring B frequently acts as a hydrogen donor (60% PTPs), particularly the rhamnose moiety (90% PTPs) which has 3 potential hydrogen donors (Table 4). The interaction of afzelin with all PTPs showed that hydroxyl of ring B frequently acts as a hydrogen donor (60% PTPs), especially the moiety rhamnoside (90% PTPs) which has 3 potential hydrogen donors (Table 4). The same case occurred to SL0101, which had similar structure to afzelin, which was capable of specifically inhibiting p90 ribosomal S6 kinase (SSR).[48]

In the final analysis, we predicted interactions among all identified PTPs using the STRING version 10.5 database (https://string-db.org/). The results were used to confirm whether PTPs were related to cell migration and which processes were involved in biological functions and cellular components. Eleven proteins were included in the STRING analysis because integrin α5β3 was encoded by 2 genes (ITGa5 and ITGb3). The represented edges were more than expected edges (37 vs 12), which indicated that the relationship between proteins was not random and at least had partial biological connection. In line with the results, the average interaction of nodes (node degree) was 6.3, which
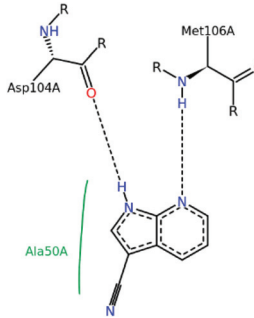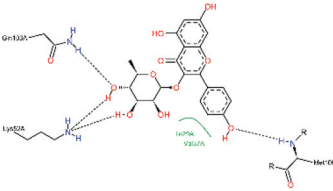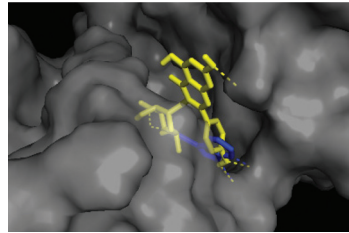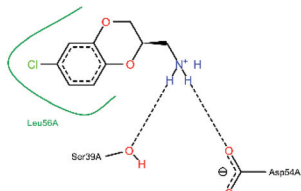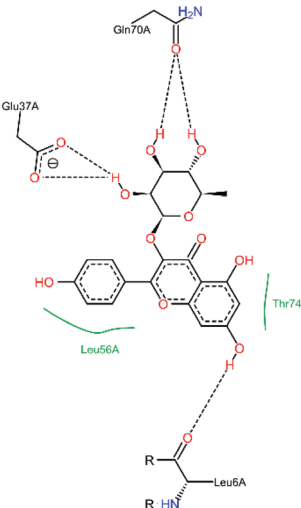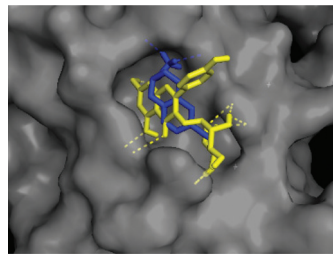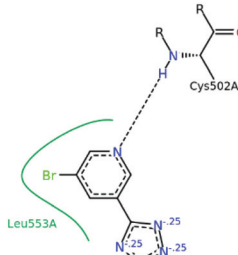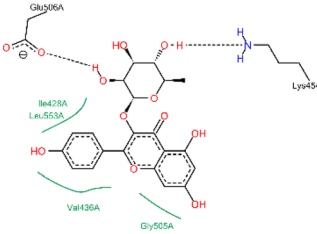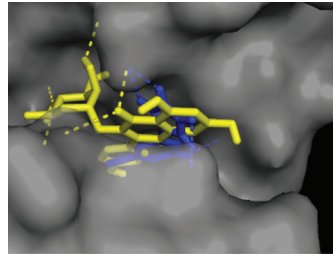
signified that each node was associated with at least 6 other proteins (Fig. 1). The highest number of node degree has belonged to c-Src with 10 nodes.

Target proteins have been verified through published literature to ensure that they were overexpressed and/or involved in TNBC metastasis. Thus, the STRING analysis results could represent cell migration and metastasis in TNBC. Consistent with the research hypothesis, 6 PTPs (integrin α5β3, BCAR1, c-Src, PIK3CA, KRAS, and EGFR) of afzelin were part of Biological Function Gene Ontology (GO) for cell migration.

Discussion

The interesting result of this study was that all of afzelin's PTPs were involved in cell chemotaxis. Based on the Biological Function GO tree, cell chemotaxis is a subset of cell migration. Migration refers to cell transfer from one place to another, whereas chemotaxis is more specific in terms of directed-movement of motile cell that is guided by a specific chemical concentration gradient. For the success of TNBC metastasis, cancer cells should have the ability to migrate to a micro-environment that is beneficial for cell survival and proliferation. Various chemical stimuli can influence the direction of TNBC cell migration such as EGF, insulin-like growth factor-1 (IGF-1), C-X-C motif chemokine 12 (CXCL12), and chemokine (C-C motif) ligand-18 (CCL18).[47,48] In line with these results, 7 afzelin's PTPs were part of the cell surface receptor signaling pathway, especially EGFR signaling pathway (BCAR1, c-Src, PIK3CA, PTK2, MAPK1, KRAS, and EGFR) and integrin-mediated signaling pathway (BCAR1, c-Src, PTK2, and integrin α5β3) with overlapping proteins involvement between both pathways. While EGFR signaling pathway is activated by chemokines (EGF, TGF-α, amphiregulin, epigen), integrin α5β3-mediated pathway is activated by integrin-extracellular matrix (ECM) ligand interaction (vitronectin and fibronectin),[49,50] which lead to special cell migration type termed as haptotaxis. This indicated that afzelin was not only likely able to inhibit TNBC cell chemotaxis but also haptotaxis.

Table 4

PoseView analysis of top 3 potential target proteins of afzelin in TNBC cells migration

Target name	Known inhibitor*	Afzelin*	Interaction of afzelin and known inhibitors with PTP in the same pocket[†]
ERK2			
KRas			
FAK			

*Black dash line: hydrogen bond; green line: hydrophobic interaction.

[†] Interaction illustrations using Pymol version 1.7.5.0. Yellow molecule: afzelin; blue molecule: known inhibitor.

Epithelial to mesenchymal transition of TNBC cells support mesenchymal motility mode at the early metastatic process. Mesenchymal movements occur in a cycle of polarization, protrusion, adhesion, translocation of the cell body, and retraction of rear cell.[51] Cell leading edges are the result of anterior–posterior cell polarity caused by epithelial to mesenchymal transition. In cell leading edges, lamellipodium and focal adhesion provide traction in forward migration.[52] STRING analysis based on Cellular Components GO showed that afzelin's PTPs were part of focal adhesion (ERK2, FAK, p130cas, and integrin α5β3), cell leading edge (N-WASP, c-Src, FAK, p130cas, PIK3, and integrin α5β3), and lamellipodia (N-WASP, p130cas, PIK3, and integrin α5β3). Therefore, afzelin inhibition of PTPs that contribute to lamellipodium formation and focal adhesion modulation at cell's leading-edge, was predicted to reduce cell

traction. This, in turn, will inhibit TNBC cells from moving forward.

In the following discussions, we will focus on the top 3 PTPs with the strongest binding energy and highest node degree. ERK2/MAPK1, KRas and FAK, which were PTPs with the greatest binding energy difference than known inhibitor, correlate with cell migration regulation. In general, ERK/Ras pathway is activated by ECM ligand and growth factor. Activation of EGFR by chemokines and integrins by ECM ligand will activate Ras, Raf, MEK1/2, and ERK, respectively. ERK activation leads to proline-leucine-serine/threonine-proline residue phosphorylation in protein kinase substrates, such as myosin light-chain kinase (MLCK), paxillin, FAK, and calpain. Interactions of activated paxillin, FAK, and calpain play an important role in the dynamics of cell adhesion,[53] while MLCK

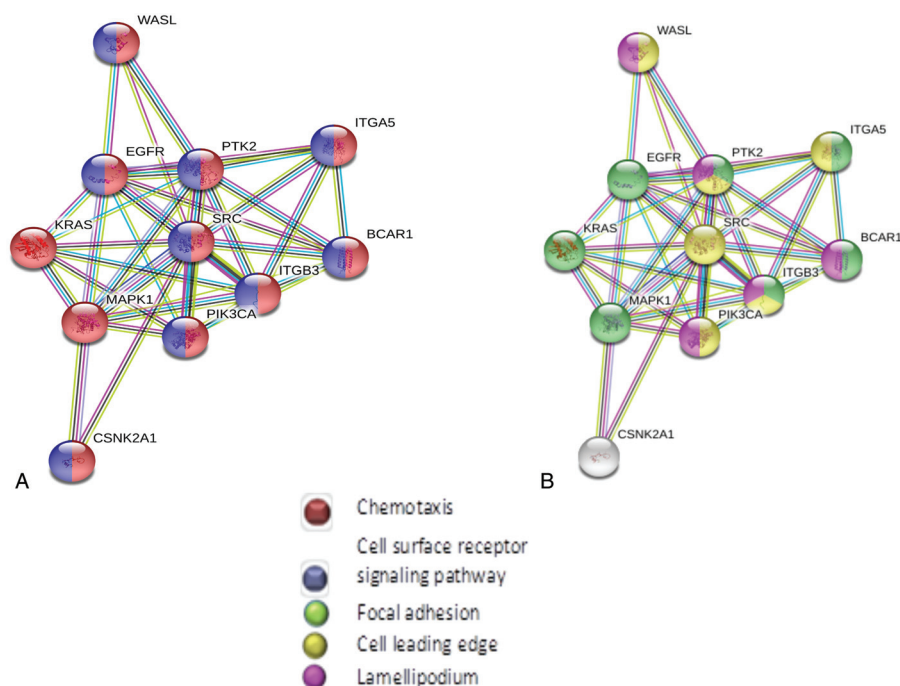Rachmi *et al* Porto Biomed. J. (2020) 5:6

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Figure 1. Protein-protein interaction (PPI) maps of the 10 PTPs of afzelin were represented by gene encoding. There were 11 nodes in the map instead of 10 because integrin has 2 subunit (alpha-5 and beta-3), that were expressed by different genes (ITGA5 and ITGB3). (A) Color coded based on biological function gene ontology (GO): cell chemotaxis and cell surface receptor signaling pathway. (B) Color-coded based on cellular component GO: focal adhesion, cell leading edge and lamellipodium.

activation contributes to the organization of membrane protrusion including lamellipodium. Directly, co-location of ERK with Wave2 regulatory complex (WRC) at the lamellipodial leading edge resulted in phosphorylation of 2 components of WRC, WAVE2, and Abi1. Phosphorylations is required for interactions with Arp2/3 and actin during cell protrusion formation.[51] If afzelin can inhibit PTPs as predicted in this study, afzelin may as well able to prevent TNBC cell migration through disruption of both assembly-disassembly of adhesion and actin polymerization, thus prevents productive leading-edge advancement during cell migration. This inhibition will likely occur in the context of chemotaxis and haptotaxis.

Top 3 PTPs with most interactions with other PTPs are c-Src (10 nodes), EGFR (9 nodes) and FAK (9 nodes). Src is an important downstream mediator of EGFR and integrin and upstream mediator of Ras that contributes to outside-in signaling. Src can be activated by cytoplasmic proteins such as FAK or Crk-associated substrate (CAS) which play an important role in integrin signaling inside-out.[54] Activated Src will interact with p130cas (BCAR1), which then together with CRK activates Rac1 and later PAK1. The result is cytoskeleton rearrangement, mainly in the form of lamellipodium at the cell leading edge.[55] The inhibition of Src will increase Rho activity and further reduce Rac activity.[46] This event will inhibit turn over and stabilization of focal adhesion, and in the end reduce cell motility. Therefore, the ability of afzelin to inhibit EGFR, Src, p130cas, and FAK at once may result in unique cellular response and more effective TNBC cell motility inhibition.

Further analysis of the PTPs indicated that afzelin might act by modulating EGFR signaling pathway (chemotaxis) and integrin-mediated signaling pathway (haptotaxis). At the cellular level, the inhibition of TNBC migration by afzelin was predicted to occur through disruption of focal adhesion and lamellipodium organization at cell leading edge that affected cell traction to move forward. Afzelin potency might also be influenced by inhibition of proteins that play a central role in the interaction between PTPs, such as c-Src, EGFR, and FAK. Further studies, including in vitro and in vivo studies, are needed to confirm PTPs of afzelin identified from our investigation. It is important to consider the characteristic of afzelin which has a rhamnose group that will be hydrolyzed by intestinal flora.[56] For this reason, parenteral administration or developing more stable bio-isosteric compounds with afzelin as the lead structure should be considered for in vivo research.

Conclusion

Our results indicated that afzelin is a potential inhibitor of TNBC cancer cell migration. Reverse docking method identified ten PTPs for afzelin, with the top 3 possible targets being ERK2/MAPK1, KRas, and FAK.

Acknowledgments

Conflicts of interest

The authors declare no conflicts of interest.

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