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In silico prediction of Anti-apoptotic BCL-2 proteins Modulation by Afzelin in MDA-MB-231 Breast cancer cell

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ABSTRACT:
Triple-negative breast cancer (TNBC) has aggressive characteristics, and lower overall- and disease-free survival compared to other breast cancer subtypes. TNBC tends to be apoptotic resistant, which allegedly related to dysregulation of anti-apoptotic Bcl-2 family proteins. Afzelin is a chemical compound that has anti-cancer potentials. The purposes of the study were analysing the effect of afzelin on apoptosis of MDA-MB-231 *in vitro*, and the interaction between afzelin and anti-apoptotic Bcl-2 family proteins through in silico approach. Apoptosis induced by afzelin was analysed by fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit with propidium iodide (PI) through flow cytometry, with subsequent ANOVA analysis. Identification of pro-survival Bcl-2 family proteins and its key amino acid residues was based on literature reviews, followed with protein structures mining from Protein Data Bank (PDB). Afzelin chemical structure was obtained from PubChem. Reverse docking performed by Autodock Vina. Afzelin significantly increased apoptosis on MDA-MB-231 in a dose-dependent manner. The interactions of afzelin and anti-apoptotic Bcl-2 family proteins were based on BH3-mimetic mode of action. Reverse docking in BH3-hydrophobic groove showed that afzelin interact with Bcl-XL, Bcl-B, and MCL1, in the order from the highest to lower binding energy. Afzelin and corresponding BH3-only proteins formed hydrogen bonds with the same amino acid residues when interacted with Bcl-XL, Bcl-B, and MCL1. The outcomes predicted that afzelin induced apoptosis in MDA-MB-231 breast cancer cells through BH3 mimetic effect, particularly on Bcl-XL.

KEYWORDS: Afzelin, apoptosis, TNBC, docking, Bcl-2 family.

INTRODUCTION:

Breast cancer has the second-highest incident and ranked fourth in terms of deaths among various types of cancer in the world[1]. Triple-negative breast cancer (TNBC) subtype, which is not expressing the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), has the most aggressive characteristics and lowest overall- and disease-free survival[2,3].

Adjuvant therapy modalities for TNBC are still dependent on chemotherapy because there are no specific molecular mutations that can be targeted. On the other hand, heterogeneity of tumor cells in a patient leads to different responses to chemotherapy among various clonal cancer cells, in which some clonal might have intrinsic resistance or become resistant during chemotherapy exposure. These events are related to early recurrence and metastasis in patients with TNBC[4]. Exploration of new chemotherapy agents is needed, in order to improve therapeutic choices that are more precise and supporting personalized therapy.

Hallmark of TNBC that can be targeted by therapy is apoptosis resistance, which can be caused by overexpression of anti-apoptotic proteins. Apoptosis is programmed cell death that ends with phagocytosis, thus does not trigger inflammation and subsequently does not lead to inducing primary tumor growth and metastasis[5]. The family of anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins facilitates apoptosis resistance of TNBC. Exposure to anti-apoptotic proteins competitive antagonists causes cancer cells more sensitive to apoptosis[5]. Many TNBC had high frequency of TP53 mutations[6], which makes direct targeting of apoptotic pathway mediators, downstream of TP53, might increase sensitivity to apoptosis in TNBC with mutant TP53.

Afzelin is a secondary metabolite of the flavonol rhamnoside group that plays an important role in plant photosynthesis[7]. Afzelin can be found in more than 50 types of plants[8]. Previous research has shown that afzelin reduced breast cancer cell viability that was sensitive to estrogen and progesterone (MCF-7)[9], and prostate cancer cells that androgen-sensitive (LNCaP) and androgen-independent (PC-3)[10]. Decreased viability of cancer cells is thought to be related to caspase cascade activation[9,11]. However, it is not yet known whether afzelin can increase apoptosis in TNBC and whether the effect of afzelin to cancer cells apoptosis is related to its interactions with anti-apoptotic Bcl-2 family proteins. This will become valuable information in overcoming apoptotic resistance in TNBC caused by increased expression of anti-apoptotic Bcl-2 family proteins or TP53 mutation. Through this study, the potential of afzelin in apoptosis modulation was

investigated in vitro using MDA-MB-231 breast cancer cell. Afzelin interaction with anti-apoptotic Bcl-2 family protein was explored through reverse docking, which is a powerful approach for bioactive compounds target fishing.

MATERIAL AND METHODS:

Cell culture:

The human TNBC cell line (MDA-MB-231) was obtained from ATCC® (HTB-26™). A total of 5 x 10^4/ml cells were grown in 24-well plate until 80% confluent, in the incubator with 5% CO2, at 37°C. MDA-MB-231 was cultured in DMEM High Glucose (ATCC) supplemented with 10% (v/v) fetal bovine serum, 100U/ml of penicillin, 100µg/ml streptomycin and 1% (v/v) non-essential amino acids (all from Gibco, Invitrogen).

Cell apoptosis assay:

MDA-MB-231 in 24-well plate treated with afzelin at concentration 100, 200, 400, and 800µg/ml. After 24 hours, the cells were harvested and stained with fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit with propidium iodide (PI) (BioLegend) according to the manufacturer's protocol. In brief, 5 µl FITC-annexin V and 10µl propidium iodide was added to cell suspension and incubated for 15 minutes in the dark, at room temperature. The cells were analyzed through flow cytometry (FACS Calibur, BD Biosciences). The early and late apoptosis were evaluated on fluorescence 3 (FL3 for PI) versus fluorescence 1 (FL1 for annexin V) plots. Percentage of apoptotic cells was the sum of the percentage of cells stained with only annexin V (early apoptosis) and cells stained with both annexin V and PI (late apoptosis). Percentage of necrotic cells was all of cells stained with PI only.

Statistics:

All results were expressed as mean ± SEM. Analysis of variance (ANOVA) followed post hoc analysis - Least Significant Difference (LSD) was used to explore possible pair-wise comparisons of means between different treatments. A P-value of <0.05 was considered statistically significant.

Identification of anti-apoptotic Bcl-2 family proteins as potential drug targets:

The drug targets were pro-survival Bcl-2 family proteins. Proteins searching was carried out based on literature reviews, which identified six Bcl-2-family proteins: Bcl-2, Bcl-extralarge (Bcl-XL), Bcl-2-like protein-2 (Bcl-W), myeloid cell leukemia-1 (MCL-1), Bcl-2-related protein-A1 (BFL-1/A1) and Bcl-2-like protein-10 (Bcl-B) [12–14]. Potential druggability of each proteome confirmed through Research Collaboratory for Structural Bioinformatics Protein Data Bank (RSCB PDB) or existing publication in Pubmed NCBI.

Preparation of afzelin ligand and target proteins structure:

Afzelin structure was prepared from PubChem. The Bcl-2 family protein structure was chosen from RSCB PDB. To guide the determination of docking locations, the selected proteins were those that interact with BH3-only Bcl-2 protein, PUMA (Bcl-2, Bcl-XL, MCL1, and Bfl1). For target proteins which its complex structure with BH3-only-protein was not found in RSCB PDB (Bcl-W and Bcl-B), the docking site was determined based on key amino acid residues in existing publications[14,15]. Proteins prepared through PyMol version 1.7.5.0 (Schrodinger, LLC.) and each saved as .pdb extension. Protein structure with missing residues and atoms were repaired using Molsoft-ICM Pro.

Docking using PyRx:

Docking in this study was performed with Autodock Vina integrated into PyRx – Virtual Screening Tool version 0.8 16, which predicts possible binding modes of ligand-protein complexes and corresponding binding energy (kcal/mol). For grid map preparation, each target proteins and corresponding BH3-only protein were uploaded and the grid box was centered at BH3-only protein. If docking sites were guided by key residues from existing publications, the grid box was centered in the area occupied by the residues. The grid map used a grid size of 25 x 25 x 25 XYZ point. Afzelin was docked to each target protein with the determined grid box, with three repetitions. The docking results were sorted by docking scores differences of the afzelin interactions with each target protein and tabulated for further analysis. Pose View (https://proteins.plus) was used for comparing amino acid that interacted with afzelin and reference-based BH3-only proteins, completed with a two-dimensional illustration. Subsequently, the amino acid of target proteins that were interacted with afzelin or with BH3-only protein will be referred to as amino acid residue (AAR).

RESULTS:

Induction of apoptosis in MDA-MB-231 by afzelin

Flow cytometry analysis of Annexin V-FITC/PI dual staining was used to examining changes of phosphatidylserine exposure, the apoptotic marker, due to inducing capacity of afzelin in MDA-MB-231 cell. Afzelin induced apoptosis in MDA-MB-231 in a dose-dependent manner. The percentage apoptotic cells were significantly increased compared with untreated cells (4.3%), following 400 µg/ml (7.3%) and 800 µg/ml (32%) afzelin treatment for 24 hours. The same trend found on the percentage of necrotic cells although the amount was quite low compared with apoptotic cells (Figure 1). These results indicated that cell death caused by afzelin treatment occurred primarily through apoptosis.

Comparison of binding energies among all of afzelin and target protein interactions:

Afzelin interacted with all Bcl-2 family proteins that were targeted in this study. The strongest binding energy between afzelin and the Bcl-2 protein family was found in its interaction with Bcl-XL followed by Bcl-W and Bcl-B. Their interaction was mediated by the presence of three types of contacts which were hydrogen bonds, hydrophobic contacts and π-π stacking (Table 1). The rhamnoside group of afzelin contributed to forming hydrogen bonds with all target proteins, except with Bcl-W.

Analysis of amino acid residues that were interacted with afzelin:

Representative BH3 only protein or key AARs of each target protein were used as a guideline for setting docking site, thus it can be confirmed whether afzelin interaction in the hydrophobic pocket of target protein involved the same key AARs as BH3 only proteins (Figure 2). Key AARs of one to three BH3-only proteins per target protein were obtained from the literature. Reverse docking simulations showed that afzelin formed a hydrogen bond with Bcl-XL and Bcl-B, at the same AAR as their corresponding BH3-only protein (PUMA and BIM, respectively). Afzelin interacted with MCL1 at the same AAR as its three corresponding BH3-only proteins (PUMA, BIM, and NOXA). Moreover, afzelin formed two hydrogen bonds with MCL1, at the same key AARs as Bim. Afzelin and BH3-only proteins also interacted with BFL1 and Bcl-W at the AAR residue, but the interactions were in the form of hydrophobic contact that had weaker binding energy than hydrogen bond (Table 1).

Table 1. Analysis of Bcl-2 family proteins interaction with afzelin

Bcl-2 pro-survival Protein (PDB ID)	Bcl-2 Activator/Sensitizer Protein's Key Residues	Afzelin	
		Amino Acid Residues Interactions	BE (kcal/mol)
Bcl-XL (2M04)	PUMA: ***His113***[17]		-7.5
	BIM : Ser106, Asp107, Asp136, Arg139 [18]		
	BAD : A93, Phe105, Leu108, **Val126**, Leu130, Val141, Ala142, **Phe146, Leu150** [19]		
Bcl-W (1O0L)	BIM : Val173, Leu174, Ala177, **Val178**, Ala179 and Leu180[15]		-7.4
Bcl-B (4B4S)	BIM : ***Ser40***, Phe54, Met71, Val75, Phe83, ***Arg85***, Ser86, Leu89, Leu90 [14]		-7.3
MCl1 6QFM	PUMA: ***Arg263*** [20,21] His205, Asp237, Asn241, Arg244, Phe251 [22] BIM: Arg214, Asp218, Gln221, ***Asp256, Arg263***[20,23] NOXA: Lys215, ***Arg263***, Cys286 [21]		-7.0
Bfl1 5UUL	PUMA: Cys55, Arg92 [24] BIM: **Val74**, Lys77, Glu80, Phe95, Ile148, Leu152, Arg153 [25] NOXA: Val44/Val48, Glu80/Asp81, Arg88, Val40/Val90, Phe95, Lys147 [24]		-6.4
Bcl-2 (6QG8)	PUMA : Arg146 [20] BIM: Leu92, Ile95, Arg146[26]		-6.3

BE binding energy; Dashed lines representing hydrogen bonds. Green residue and spline segments showing hydrophobic contact residue and contacts. Greenline with circle end showing π-π stacking. Bold amino acid residues representing similarity with hydrophobic contacts or π–π stacking of afzelin interaction. Combined italic and bold amino acid residues representing similarity with hydrogen bonds of afzelin interaction.

DISCUSSION:

Flavonol has been confirmed to be effective in promoting apoptosis in TNBC cancer cells [27–29]. Afzelin is a secondary metabolite of the flavonol subgroup. It is kaempferol with the addition of 3-O-α-L-rhamnoside, which might cause the interaction of afzelin with signal transduction proteins to be more selective [19]. In this study, afzelin increased apoptosis significantly in MDA-MB-231 at doses 400 and 800 µg/ml (Figure1). Afzelin-induced apoptosis in TNBC cell complements previous reports that afzelin enhances apoptosis in estrogen receptor-positive breast cancer [9,11]. Based on these in vitro results, we predicted the underlying mechanism of afzelin-induced apoptosis through reverse docking approach.

Antagonism of anti-apoptotic Bcl-2 family proteins is considered a promising therapeutic approach for apoptosis pathways activation in cancer [30,31]. The intrinsic or mitochondrial pathway of apoptosis is triggered by stimuli mediated by non-receptors, which produce intracellular signals mediated by mitochondria. The Bcl-2 family has an important role in the mitochondrial pathway [32]. Their deregulation, through amplification or overexpression, also occurs in TNBC and is associated with poor prognosis, making them attractive targets for anticancer therapies [33]. Most Bcl-2 family proteins inhibitors act as an agent that mimics the Bcl-2 homology-3 (BH3) domains of the pro-apoptotic Bcl-2 family members. These inhibitors neutralize Bcl-2 proteins by binding to their surface hydrophobic grooves. Subsequently, Bax and Bak will be displaced which allows them to form multimers, permeabilize the mitochondrial outer membrane and execute apoptotic cascade[34].

Through reverse docking, we found similarities between BH3-only proteins and afzelin on interacting AARs, which supported predictions that afzelin could have the same BH3-mimetic effect as anti-apoptotic Bcl-2 family activators and sensitizers. In accordance with its affinity and AARs similarity with BH3-only protein, afzelin was more likely to interact with Bcl-XL than the other target proteins. Afzelin also interacted with Bcl-B and MCL1, even though its binding energy was lower than Bcl-XL. Afzelin was predicted to be able to mimic three BH3-only proteins in inhibiting MCL1, especially BIM which had two similar residues in hydrogen bindings. Afzelin-induced apoptosis in MDA-MB-231 might be the result of combined interaction of afzelin with Bcl-XL, Bcl-B, and MCL1. These results were in line with the previous study which showed afzelin exposure increase caspases in MCF-7 which are downstream of Bax and Bak activation [11].

However, we should consider that this study was carried out on MDA-MB-231 cell that has specific characteristics of anti-apoptotic Bcl-2 family protein expression. MDA-MB-231 has upregulated Bcl-XL [35] and normal MCL1 [36] expressions. The expression of Bcl-2 proteins in TNBC patients was reported being varied for Bcl-2, MCL1, and Bcl-XL, while BFL1 expression was not up-regulated [35–37]. Therefore, if afzelin will be given to TNBC with different characteristics of anti-apoptotic Bcl-2 family protein expression than MDA-MB-231, it may produce a different apoptotic effect. Further studies, including in vitro and in vivo, are needed to confirm afzelin effect as BH3-mimetics on anti-apoptotic Bcl-2 family proteins identified from our investigation. Prediction of afzelin interaction with other pro-survival proteins, particularly inhibitors of apoptosis (IAPs) family, will complement the understanding of afzelin mechanism in enhancing apoptosis. The study of afzelin in combination with chemotherapy will be interesting to explore, particularly chemotherapy agents which the terminal response on TNBC cells is dictated by the intrinsic expression levels of the anti-apoptotic protein Bcl-2. Thus, the use of afzelin to induce apoptosis can be adjusted with each TNBC characteristics.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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