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 potentialities. The purpose 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 fluorescence 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 BHI3 mimetic mode of action. Reverse docking in BHI3-hydrophobic groove showed that afzelin interact with Bcl-XXL, Bcl-B, and MCL1, in the order from the highest to lower binding energy. Afzelin and corresponding BHI3 only proteins formed hydrogen bonds with the same amino acid residues when interacted with Bcl-XXL, Bcl-B, and MCL1. The outcomes predicted that afzelin induced apoptosis in MDA-MB-231 breast cancer cells through BHI3 mimetic effect, particularly on Bcl-XXL.

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 survival2-3.

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 clones might have intrinsic resistance or become resistant during chemotherapy exposure. These events are related to early recurrence and metastasis in patients with TNBC4-5. 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 metastasis5. 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 apoptosis5. Many TNBC had high frequency of TP53 mutations6, which makes direct targeting of apoptotic pathway modulators, downstream of TP53, might increase sensitivity to apoptosis in TNBC with mutant TP53.

Afzelin is a secondary metabolite of the flavonol flavonoside group that plays an important role in plant photosynthesis7. Afzelin can be found in more than 50 types of plants8. 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 activation9,11. 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. Aflazin 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 10^6 cells were grown in 24-well plate until 80% confluent, in the incubator with 5% CO_2, 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 aflazin 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 (Bfl-1) [12-14]. Potential drugubility of each proteome was confirmed through Research Collaboratory for Structural Bioinformatics Protein Data Bank (RSCB PDB) or existing publication in Pumbed NCBH.

Preparation of aflazin ligand and target proteins structure:
Aflazin 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, MCL-1, and Bfl-1). 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 [43]. 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 Molohso-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 Å. Aflazin 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 aflazin interactions with each target protein and tabulated for further analysis. Pose View (https://proteins.pus) was used for comparing amino acid that interacted with aflazin and reference-based BH3-only proteins, completed with a two-dimensional illustration. Subsequently, the amino acid of target proteins that were interacted with aflazin or with BH3-only protein will be referred to as amino acid residue (AAR).

RESULTS:

Induction of apoptosis in MDA-MB-231 by aflazin:
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 aflazin in MDA-MB-231 cell. Aflazin 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%) aflazin 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 aflazin treatment occurred primarily through apoptosis.

Comparison of binding energies among all of aflazin and target protein interactions:
Aflazin interacted with all Bcl-2 family proteins that were targeted in this study. The strongest binding energy between aflazin and the Bcl-2 protein family was found in its interaction with Bcl-W followed by Bcl-W and Bcl-B. Interaction was mediated by the presence of three types of contacts which were hydrogen bonds, hydrophobic contacts and π-π stacking (Table 1). The rhinomyside group of aflazin contributed to forming hydrogen bonds with all target proteins, except with Bcl-W.

Analysis of amino acid residues that were interacted with aflazin:
Representative BH3 only protein or key AARs of each target proteins were used as a guideline for setting docking site, thus it can be confirmed whether aflazin interaction in the hydrophobic pocket of target proteins 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 aflazin formed a hydrogen bond with Bcl-XL and Bcl-B, at the same AAR as their corresponding BH3-only protein (PUMA and BIM, respectively). Aflazin interacted with MCL-1 at the same AAR as its three corresponding BH3-only proteins (PUMA, BIM, and NOXA). Moreover, aflazin formed two hydrogen bonds with MCL-1, at the same key AARs as Bim. Aflazin and BH3-only proteins also interacted with BFL1 and Bcl-W at the AAR residues, but the interactions were in the form of hydrophobic contact that had weaker binding energy than hydrogen bond (Table 1).

<table>
<thead>
<tr>
<th>BH3 pro-survival Protein (PDB ID)</th>
<th>BH3-2 Activator/Sensitizer Protein's Key Residues</th>
<th>Aflazin</th>
<th>Amino Acid Residue Interactions</th>
<th>Bcl (kcal/mol)</th>
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<tbody>
<tr>
<td></td>
<td>BAX; Arg196, Arg207, Arg216, Arg198[15]</td>
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<tr>
<td>BAG-1; Asp35, Lys35, Lys95, Val169, Lys130, Val141, Ala162, Lys164[13]</td>
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<td>Bcl-W (1DDL)</td>
<td>BAX; Thr72, Lys179, Ala179, Val178, Ala179 and Lys160[15]</td>
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<td>Bcl-B (2B38)</td>
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<tr>
<td></td>
<td>Ser86, Lys97, Lys98[19]</td>
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<tr>
<td>MCL-1 (Q5M)</td>
<td>PUMA: Arg241, Arg196, His205, Arg237, Asn240, Arg244, Phe258[22]</td>
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<td></td>
<td>BAX; Asp214, Asp219, Gln221, Asp224, Phe256, Gln258[23] NOXA: Asp215, Arg238, Cys268[24]</td>
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<td>BH1 (1UL)</td>
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<td>Bcl-2 (2MD0)</td>
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<td>BAX: Leu97, Thr95, Arg492[26]</td>
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Table 1. Analysis of Bcl-2 family proteins interaction with aflazin.
DISCUSSION:
Flavonol has been confirmed to be effective in promoting apoptosis in TNBC cancer cells. Azelfin is a secondary metabolite of the flavonoid subgroup. It is kaempferol with the addition of 3-O-α-L-rhamnoside, which might cause the interaction of azelfin with sialic acid residues to be more selective. In this study, azelfin increased apoptosis significantly in MDA-MB-231 at doses 400 and 800 μg/mL (Figure 1). Azelfin-induced apoptosis in TNBC cell demonstrates previous reports that azelfin enhances apoptosis in estrogen receptor-positive breast cancer. Based on these in vitro results, we predicted the underlying mechanism of azelfin-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. The intrinsic or mitochondrial pathway of apoptosis is triggered by stimuli mediated by pro-receptors, which produce intracellular signals mediated by mitochondria. The Bcl-2 family has an important role in the mitochondrial pathway. Their deregulation, through amplification or overexpression, also occurs in TNBC and is associated with poor prognosis, making them attractive targets for anticancer therapies. 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 groove. Subsequently, Bax and Bak will be translocated which allows them to form multimers, permeabilize the mitochondrial outer membrane and execute apoptosis cascades.

Through reverse docking, we found similarities between BH3-only proteins and azelfin interacting with AAs, which supported predictions that azelfin could have the same BH3-mimetic effect as anti-apoptotic Bcl-2 family activators and sensitizers. In accordance with its affinity and AAs similarity with BH3-only protein, azelfin was more likely to interact with Bcl-XL than the other target proteins. Azelfin also interacted with Bcl-I and MCL-1, even though its binding energy was lower than that of Bcl-XL. Azelfin was predicted to be able to mimic three BH3-only proteins in inhibiting Bcl-XL, Bcl-I, and MCL-1. Intriguingly, both Bcl-XL had two conserved cysteines with high hydrophobicity. Antagonism of apoptosis in MDA-MB-231 might be the combined result of interaction of azelfin with Bcl-XL, Bcl-I, and MCL-1. These results were in line with the previous study which showed azelfin exposure increase caspases in MCF-7 which are downstream of Bax and Bak activation.

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 35 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. Therefore, if azelfin 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 azelfin effect as BH3-mimetics on anti-apoptotic Bcl-2 family proteins identified from our investigation. Prediction of azelfin interaction with other pro-survival proteins, particularly inhibitors of apoptosis (IAPs) family, will complement the understanding of azelfin mechanism in enhancing apoptosis. The study of azelfin 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 anti-apoptotic Bcl-2 protein family. Thus, the use of azelfin 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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