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Original Article

Inhibition of adipocyte senescence by ferulic acid present in pigmented rice through the PPAR-γ and NF-κB protein signaling pathways

[Inhibición de la senescencia de los adipocitos por el ácido ferúlico presente en el arroz pigmentado a través de las vías de señalización de las proteínas PPAR-γ y NF-κB]

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Abstract

Context: Senescence-associated secretory phenotype (SASP) marks the progression of cell senescence, where the cell produces a high level of inflammatory mediators and differentiation regulators. Targeting those mechanisms becomes a clear strategy to slow cellular senescence. Ferulic acid (FA) present in pigmented rice is well-known for its broad biological activity, including anti-inflammation and antioxidants. However, the role of FA as an anti-aging agent and its mechanism is limited.

Aims: To evaluate the potential target of FA in modulating cellular aging using computational and *in vitro* analysis.

Methods: Binding of FA to the target proteins analyzed by molecular docking analysis using PyRx and AutoDock Vina software. Lipid accumulation of mouse preadipocytes 3T3-L1 cells was analyzed using oil red O staining. Immunofluorescence staining was performed to analyze the level of peroxisome proliferator-activated receptor-gamma (PPAR-γ) and nuclear factor-*kappa* B (NF-κB) proteins.

Results: Several transcription factors and inflammatory mediators were used as targets for molecular docking, including CCAAT-enhancer-binding proteins-alpha (C/EBP-α), PPAR-γ, NF-κB, I*kappa*B kinase-beta (IKK-β), interleukin-6 (IL-6), IL-8, and their respective receptors. Although other protein targets have excellent binding energy and suitable FA-residues interactions, the molecular dynamics analysis showed that PPAR-γ was the most suitable target for FA in controlling the SASP process with high structural integrity and outperformed ligand-protein interaction stability. FA reduces the accumulation of lipids in differentiated preadipocyte cells. Importantly, FA treatment showed increases in the level of PPAR-γ while reducing the NF-κB proteins *in vitro*.

Conclusions: Consequently, the FA could perform the anti-aging activity in preadipocyte cells by reducing SASP progression through activation of PPAR-γ signaling.

Keywords: cellular aging; ferulic acid; pigmented rice; PPAR-γ; senescence-associated secretory phenotype.

Resumen

Contexto: El fenotipo secretor asociado a la senescencia (SASP) marca la progresión de la senescencia celular, donde la célula produce un alto nivel de mediadores inflamatorios y reguladores de la diferenciación. Dirigirse a esos mecanismos se convierte en una estrategia clara para frenar la senescencia celular. El ácido ferúlico (FA) presente en el arroz pigmentado es bien conocido por su amplia actividad biológica, que incluye antiinflamatorios y antioxidantes. Sin embargo, el papel del FA como agente antienvejecimiento y su mecanismo es limitado.

Objetivos: Evaluar el potencial del FA en la modulación del envejecimiento celular mediante análisis computacional e *in vitro*.

Métodos: La unión del FA a las proteínas objetivo se analizó mediante análisis de acoplamiento molecular utilizando PyRx y el software AutoDock Vina. La acumulación de lípidos de las células preadipocitos de ratón 3T3-L1 se analizó mediante tinción con rojo O. Se realizó tinción de inmunofluorescencia para analizar el nivel de proteínas del proliferador de peroxisomas gamma (PPAR-γ) y el factor nuclear *kappa* B (NF-κB).

Resultados: Se utilizaron varios factores de transcripción y mediadores inflamatorios como objetivos para el acoplamiento molecular, incluidas las proteínas de unión al potenciador CCAAT alfa (C/EBP-α), activado por PPAR-γ, NF-κB, la cinasa I*kappa*B beta (IKK-β), la interleucina-6 (IL-6), la IL-8 y sus respectivos receptores. Aunque otros objetivos proteicos tienen una excelente energía de unión e interacciones adecuadas con residuos de AG, el análisis de dinámica molecular mostró que PPAR-γ era el objetivo más adecuado para FA para controlar el proceso SASP con una alta integridad estructural y una estabilidad de interacción proteína-ligando superior. FA reduce la acumulación de lípidos en células preadipocíticas diferenciadas. El tratamiento con FA mostró aumentos en PPAR-γ mientras reducía las proteínas NF-κB *in vitro*.

Conclusiones: En consecuencia, el FA podría realizar la actividad antienvejecimiento en las células preadipocíticas al reducir la progresión de SASP a través de la activación de la señalización PPAR-γ.

Palabras Clave: ácido ferúlico; arroz pigmentado; envejecimiento celular; fenotipo secretor asociado a la senescencia; PPAR-γ.

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Abbreviation: 3D: Three-dimension; ADME: Absorption, distribution, metabolism, and excretion; ALA: Alanine; ARG: Arginine 288; ASN: Asparagine; ASP: Aspartate; C/EBP-α: CCAAT-enhancer-binding proteins-alpha; CID: Compound identity; DMEM: Dulbecco's modified eagle medium; FA: Ferulic acid; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; GLU: Glutamate; gp: P-glycoprotein; HIS: Histidine; IBMX: 3-isobutyl-1-methylxanthine; IgG: Immunoglobulin G; IKK-β: I*kappa*B kinase-beta; IKK-β: Inhibitory *kappa* B kinase beta; IL-6: Interleukin-6; IL-6Rβ: Interleukin-6 receptor beta; IL-8: Interleukin-8; IL-8Rβ: Interleukin-8 receptor beta; ILE: Isoleucine; LEU: Leucine; LYS: Lysine; NF-κB: Nuclear factor-*kappa* B; PDB: Protein data bank; PHE: Phenylalanine; PID: Post-induction day; PPAR-γ: Peroxisome proliferator-activated receptor-gamma; RMSD: Root-mean-square deviation; RMSF: Root-mean-square fluctuation; SASP: Senescence-associated secretory phenotype; SD: Standard deviation; SEM: Standard error of mean; SER: Serine; THR: Threonine; TRP: Tryptophan; TYR: Tyrosine; VAL: Valine.

INTRODUCTION

The development of the senescence-associated secretory phenotype (SASP), which is a significant source of inflammatory and tissue-remodeling signals, is a primary aspect of cellular senescence (Fitsiou et al., 2021; Kumari and Jat, 2021). Among several tissues, adipocytes are the most vulnerable tissue undergoing the aging process (Ou et al., 2022). As the primary energy storage and endocrine organ, dysregulation of adipocyte metabolism is often associated with numerous physiological disorders, including inflammation, insulin resistance, wound healing, and glucose metabolism (Lafontan, 2014). Adipocytes change at multiple levels, highlighting the biomechanism of age-related conditions.

The C/EBP-α and PPAR-γ served a significant role in cell senescent (Cartwright et al., 2007; Madsen et al., 2014). The C/EBP-α is a key regulator in SASP progression, and inhibition of C/EBP-α diminishes the occurrence of SASP (Guan et al., 2020). On the other hand, low expression of PPAR-γ takes place during cell senescence (Xu et al., 2018), which the agonistic activity of PPAR-γ could reverse into longevity extension through repairing glucose metabolism and mitochondrial functionality (Xu et al., 2020). Further, activation of PPAR-γ possesses an anti-inflammatory mechanism (Wang et al., 2017). The anti-inflammatory through PPAR-γ will positively regulate cell senescence because a low inflammatory state is also associated with aging cells and developing a physiological activity called "inflammaging" (Franceschi et al., 2000). Upon activation, PPAR-γ exerts anti-inflammatory activity by inhibiting several pro-inflammatory mediators such as IL-6 and IL-8 (Heming et al., 2018; Wang et al., 2009). Thus, targeting those two cytokines is critical to amplifying the inhibitory activity of SASPs.

As a part of SASP factors and the inflammaging (Koelman et al., 2019; Ou et al., 2022), IL-6 and IL-8 contribute to the reinforcement of cell growth arrest and prolong the inflammation (Barajas-Gómez et al., 2017; Kuilman et al., 2008; Moran et al., 2008). Since IL-6 and IL-8 were also involved in senescent-associated phenotypes (Ortiz-Montero et al., 2017), the inhibition of those two cytokines signaling may promote cellular longevity. Either blocking IL-6 and IL-8 interaction with their respective receptors or regulating IL-6 and IL-8 signaling could be achieved by repressing NF-κB activity because of its role as the main transcription cascade in inflammasome formation (Rea et al., 2018). Thus, regulating of those key mediators may be important to inhibit cell senescent and prevent aging. Senescence is a state of stable cell cycle termination in the fight against cell proliferation. The senescence cycle can prevent physiological responses to genomic instability due to the accumulation of DNA damage, which plays a physiological role in maintaining tissue stability during normal development (Sun et al., 2024).

Understanding the mechanisms of age-related impaired immune regulation is important in the approach between nutritional factors, nutrient intake of bioactive compounds, and the immune system, which demonstrates the key role of dietary strategies as regulators of immune responses, inflammation, and endocrine disorders. These factors can stimulate immunomodulators of immune senescence at the cellular level (Aiello et al., 2019). Ferulic acid (FA) belongs to a phenolic group and is the most common cinnamic acid compound found in plants, including pigmented rice (Samyor et al., 2017; Stompor-Gorący and Machaczka, 2021; Zduńska et al., 2018). FA present in pigmented rice is a secondary metabolite that has many beneficial properties in the pharmaceutical, health, and food sciences (Callcott et al., 2018; Srinivasan et al., 2007). It is reported that FA possesses various biological activities, including antioxidant (Srinivasan et al., 2007), anti-tumor (Damasceno et al., 2017), anti-inflammation (Wijayanti et al., 2021; 2022), anti-microbial (Wijayanti et al., 2022), and anti-diabetic (Wijayanti et al., 2021). Since the FA from pigmented rice has antioxidant and anti-inflammation properties, FA may be useful in modulating SASP development by controlling oxidative stress and inflammation. This research aimed to explore the potential senescence-regulatory activity of FA through the modulation of the SASP signaling pathway using *in silico* and *in vitro* approaches.

MATERIAL AND METHODS

Structure retrieval

The three-dimensional (3D) structure of FA was downloaded from the PubChem database with compound identity (CID): 445858. The 3D structure of the protein target was retrieved from protein data bank (PDB) with the following identities: NF-κB (1SVC), IKK-β (3BRT), C/EBP-α (6DC0), IL-6 (1ALU), IL-6 receptor beta (IL-6Rβ) (1I1R), IL-8Rα (2LNL), IL-8 and IL-8Rβ (6LFM), and PPAR-γ (1FM6). The protein structure was downloaded in .pdb format and prepared by deleting water, pre-attached ligands, and unwanted chain structure before the docking analysis.

Molecular docking

The FA structure was inserted into PyRx software and converted into a .pdbqt ligand using the Open-Babel plugin (Dallakyan and Olson, 2015). Energy minimization was performed with the following parameters: Universal Force Field (uff) as the force field (Rappe et al., 1992), conjugate gradients as the optimization algorithm, and the total number of steps was 200 with 1 of the number of steps for the update. AutoDock Vina was employed to analyze the molecular docking with the proteins treated as rigid macromolecules and FA structure as flexible molecules (Trott and Olson, 2010). Specific docking was used to calculate the binding energy and binding pose of the FA. The grid settings and binding sites for every target protein are shown in Table 1. FA's binding poses with the lowest root-mean-square deviation (RMSD) value was selected for subsequent analysis.

Docking analysis and visualization

The binding energy from the lowest RMSD binding pose was determined as the binding energy for each complex. FA binding pose was complexed with each protein structure using PyMOL before ligand-amino acid interaction analysis. The interaction of the ligand with the amino acid of each protein target and structural visualization was performed using Discovery Studio 2019. FA and protein complexes with the strongest interactions at the active or binding sites of the target proteins were then subjected to molecular dynamics analysis for further evaluation.

Molecular dynamics

YASARA 20.12.24 software (Krieger and Vriend, 2015) was used to perform the molecular dynamics analysis with the following parameters: AMBER14 force field (Maier et al., 2015), pH 7.4, 0.9% NaCl concentration, 310°K temperature, 0.997 water density, cuboidal cell shape, and save interval every 25 ps. For membrane-bound protein targets, simulation was done by md_runmembrane.mcr macros with the default membrane parameter settings and simulation above parameters. Structural integrity and stability were then estimated by the value of the RMSD of the protein backbone and the RMSD of the ligand structure. Ligand binding stability was measured according to the RMSD of ligand movement. In addition, root-mean-square fluctuation (RMSF) measured the fluctuation of each residue in the proteins.

Table 1. Amino acid target and grid settings for each protein target in molecular docking analysis.

Protein	Grid center position (Å)			Grid dimension (Å)			Reference
	X	Y	Z	X	Y	Z	
NF-κB	31.6846	24.0996	34.8308	30.3024	26.2166	31.7568	(El-Ashmawy et al., 2018)
IKK	24.941	24.9897	17.0372	28.9363	27.776	25	(Müller et al., 1995)
C/EBP-α	Maximum	Maximum	Maximum	Maximum	Maximum	Maximum	(Briot et al., 2018)
IL-6	6.0326	-23.6139	18.9767	18.4543	26.2166	22.591	(Vishvakarma et al., 2022)
IL-6Rβ	12.9018	-17.8512	28.9193	23.6793	28.0097	33.3681	(Briot et al., 2018)
IL-8	Maximum	Maximum	Maximum	Maximum	Maximum	Maximum	(Lampiasi and Montana, 2016)
IL-8Rα	14.0751	-34.9288	-12.2215	21.3117	22.0333	19.3103	(Briot et al., 2018)
IL-8Rβ	134.0885	138.4223	92.4262	21.3098	20.6211	22.8539	(Lampiasi and Montana, 2016)
PPAR-γ	17.3327	-16.7362	8.6492	22.6201	21.5358	19.7022	(Namgaladze and Brüne, 2016)

Ferulic acid extraction, cell culture, and differentiation of 3T3-L1 cells

FA was isolated from endogenous black rice from Indonesia, according to earlier studies (Maier et al., 2015; Wijayanti et al., 2021). Mouse preadipocytes 3T3-L1 cells were cultured in DMEM containing 10% FBS and 1% antibiotic. The culture medium was changed every two days. Two days post-confluence, 20 mg/mL insulin, 0.5 mM IBMX (3-isobutyl-1-methylxanthine), and 1 mM dexamethasone were added to the culture medium for three days to induce adipogenesis and accumulation of lipids of the 3T3-L1 cells (Lan et al., 2020; Stavova et al., 2017; Zoico et al., 2010). Post-induction day (PID) 0 of preadipocytes was started just before adipogenesis stimulation. The cells were then incubated with the culture medium containing 20 mg/mL insulin for an additional two days. After that, the cells were cultured in DMEM until PID 20. FA was added to the culture media at concentrations of 75 mg/mL and 150 mg/mL, respectively, and incubated for six hours prior to oil red O staining or immunofluorescence analysis.

Oil red O staining

Lipid accumulation was analyzed using oil red O staining (ScyTek Laboratories Inc., UT, USA) using a standard method. Cells were fixed in 4% paraformaldehyde for 1 hour at room temperature. After that, cells were washed using distilled water and subsequently incubated with propylene glycol for 5 minutes at room temperature. Oil red O solution was applied overnight in a humidity chamber. Finally, cells were incubated with 85% propylene glycol for 1 minute and washed with distilled water.

Immunofluorescence analysis

Immunofluorescence staining was performed to analyze the level of PPAR-γ and NF-κB proteins in 3T3-L1 cells based on previous studies with a minor modification (Lestari et al., 2015). Cells were fixed with 4% paraformaldehyde for 20 min, incubated in methanol for 5 min, and washed with PBS. Cells were incubated with bovine serum albumin 1% to block non-specific protein binding sites. After that, cells were incubated with rabbit anti-PPARα mouse monoclonal antibody (dilution range 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-NF-κB p50 rabbit polyclonal (dilution range 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for 1 h under the dark conditions. Cells were washed and incubated with secondary antibodies goat anti-rabbit IgG rhodamine (dilution range 1:1500; Santa Cruz) and goat anti-mouse IgG-FITC (dilution range 1:1500; Santa Cruz) for 1 h under the dark conditions. The cells were visualized using Olympus confocal laser scanning microscopy (CLSM; Olympus Corporation, Tokyo, Japan) and Olympus FluoView software version 1.7a (Olympus Corporation, Tokyo, Japan).

In silico data analysis

Ligand conformations with the highest rankings and lowest binding affinities from the docking process were selected for complex formation. Amino acid-ligand interactions were analyzed and visualized using Discovery Studio 2019, with 2D interaction maps and 3D models used to depict protein-ligand interactions. Molecular dynamics simulations were conducted to assess structural flexibility and integrity by calculating the root-mean-square deviation (RMSD) of atomic positions and the root-mean-square fluctuation (RMSF) of residue positions (Rohman et al., 2023). The RMSD of backbone atoms and RMSF were used to evaluate the complex's stability, while the RMSD and movements of ligand conformations were analyzed to assess the stability of ligand structure and protein-ligand interactions (Meylani et al., 2024).

RESULTS

Binding of rice-pigmented ferulic acid to the target proteins

The binding of FA to the target proteins occurred at relatively low energy. Interaction of FA with several pro-inflammatory mediators, mainly IL-8, occurred at the lowest energy binding compared to the other possible targets. However, FA appeared to have a low probability of acting as an inhibitor of nuclear receptors such as IKK-β, C/EBPα, and NF-κB (Table 2).

Amino acid and ligand structure interaction also defined the structural integrity of the protein-ligand complex. Thus, the binding site interaction was also evaluated in this study (Fig. 1). Among several interaction chemistries, the hydrogen bond is the most robust interaction. Complexes with a more significant number of hydrogen bonds will have more stable interactions (Chen et al., 2016; Kubinyi, 2021). FA interaction with NF-κB and FA-IL-6 only had a hydrogen bond. Several interactions, such as hydrophobic interaction, electrostatics, and van der Waals, also occurred. This suggests a less stable interaction between FA and both proteins. FA interaction with IL-6Rβ comprised six hydrogen bonds among all target proteins and became the most extensive number of others. Importantly, FA interaction with PPAR-γ had a more significant number of interactions with the target residues, i.e., ARG288, SER289, HIS 323, and HIS449. FA-IL-6 and FA-IL-6Rβ also have more interaction with target residues. ASP140, THR143,

Table 2. Cellular location and binding affinity of each protein target in molecular docking analysis against ferulic acid (FA).

Protein	Cellular location	Binding affinity (kcal/mol)
NF-κB	Cytoplasm-nucleus	-5.4
IKK beta	Cytoplasm-nucleus	-4.2
C/EBP-alpha	Nucleus	-4.5
IL-6	Secreted	-5.0
IL-6R beta	Plasma membrane	-4.9
IL-8	Secreted	-6.1
IL-8R alpha	Plasma membrane	-6.9
IL-8R beta	Plasma membrane	-6.1
PPAR-γ	Cytoplasm	-5.8

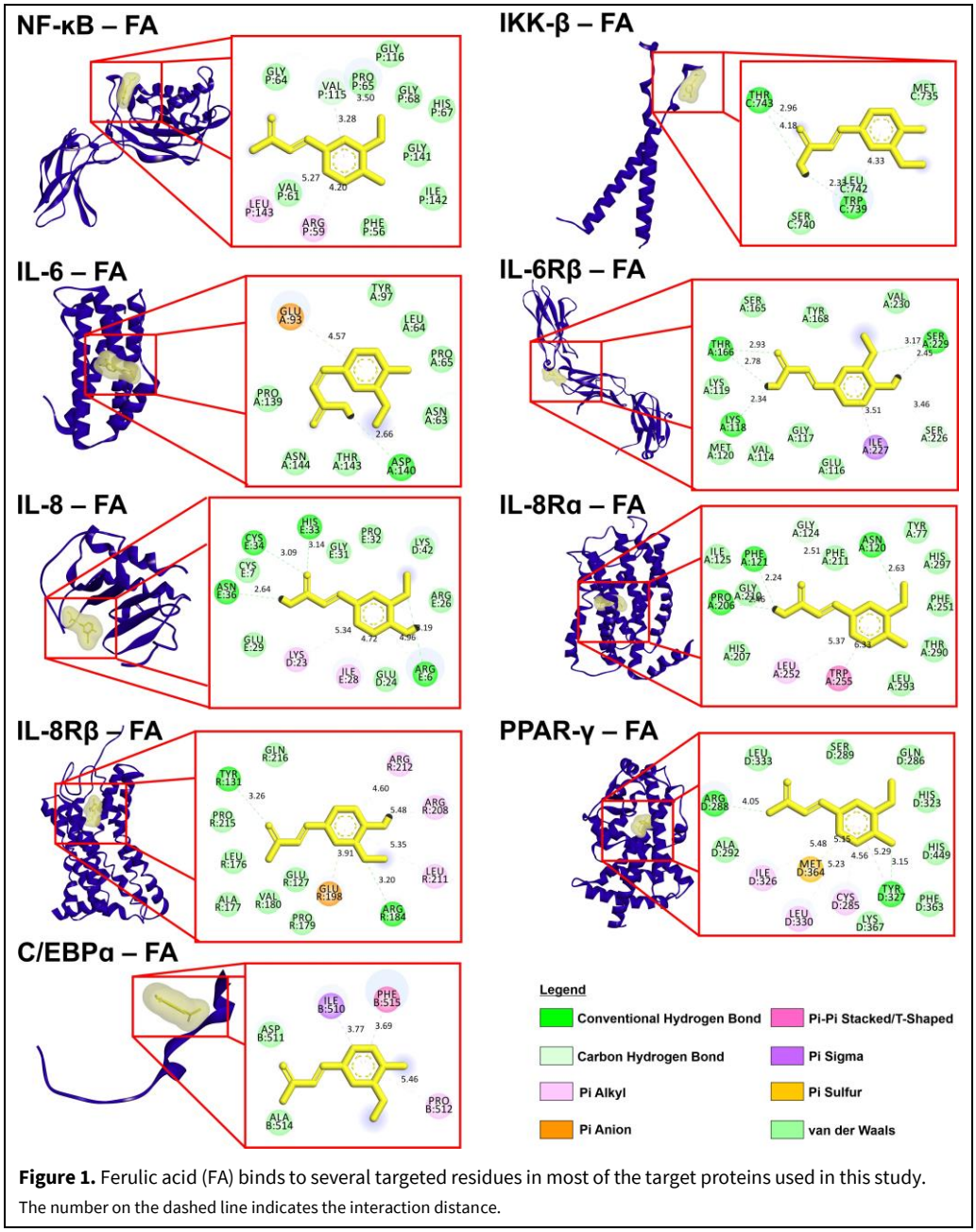

Figure 1. Ferulic acid (FA) binds to several targeted residues in most of the target proteins used in this study.
The number on the dashed line indicates the interaction distance.

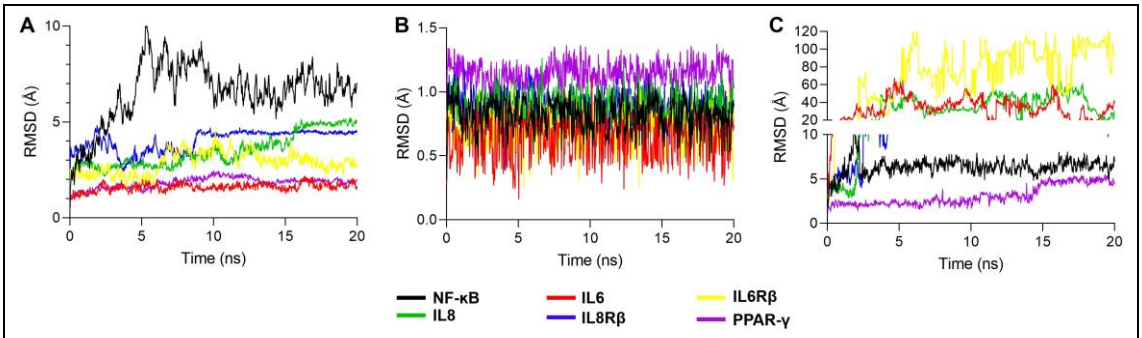

Figure 2. Structural dynamics of selected complexes on molecular dynamic simulation under physiological conditions.
(A) Structural stability of the proteins as the receptor, **(B)** structural stability of FA's structure after interacting with each target protein, and **(C)** ligand movement describing the distance of ferulic acid (FA) from the initial site along the simulated time.

and ASN144 of FA-IL-6 interact with FA through the van der Waals interactions and hydrogen bonds, while the SER165, TYR168, and VAL230 of FA-IL-6Rβ interact with FA via van der Waals interactions. FA formed interactions with DNA binding sites of NF-κB at ARG59 and HIS67, while TRP739 was the only target residue in IKK-β that interacted with FA by a hydrogen bond. FA-IL-8 also formed a hydrogen bond at ARG6 among several target residues. Fortunately, two Pi-Alkyl bonds formed at ARG 208 and ARG212 in FA-IL-8Rβ. We found that only FA-IL-8Rα and FA-C/EBPα did not interact with target residues. In addition, the interaction between FA and IKK-β only contained one hydrogen bond and the highest binding energy. Thus, the complexes of FA with FA-IL-8Rβ, C/EBPα, and IKK-β were not considered for molecular dynamics analysis.

Structural and binding stability of ferulic acid to the selected target proteins

The chosen protein complexes underwent molecular dynamics simulations under physiological conditions to evaluate their structural integrity and stability. The parameters measured included the RMSD, RMSF, and the number of hydrogen bonds formed throughout the simulation (Vishvakarma et al., 2022). RMSD of the protein backbone describes the protein's stability as the FA receptor. NF-κB exhibited a significantly elevated RMSD value (Fig. 2A), indicating that its structure was unstable. Other complexes remained stable during simulation, with the RMSD value lower than 4 Angstrom (Å). Nevertheless, FA complexes with IL-8 and IL-8Rβ tend to be less stable over time. Among all the simulated complexes, IL-6 and PPAR-γ performed better than the other target proteins with low fluctuations of RMSD value (Fig. 2A).

The structure of FA did not differ in all the simulated complexes. The fluctuations occurred in the low range of RMSD (Fig. 2B). Therefore, the FA structure remained stable after binding with the target proteins. Unfortunately, this structural stability was not always matched by stable interactions with the simulated proteins. Almost all the simulated complexes have dramatic fluctuation of RMSD, indicating that the binding of FA might be detached or displaced during the simulations. Only FA-NF-κB and FA-PPAR-γ had stable interaction, described by the slight fluctuation of RMSD values (Fig. 2C). Unfortunately, FA-NF-κB showed more significant fluctuations in the backbone proteins (Fig. 2A). Hence, PPAR-γ became the best target candidate for FA to regulate SASP progression.

The value of RMSF was also evaluated to determine the stability of protein structure per residue (Fig. 3). Among all, NF-κB had the most fluctuated residues with the highest value up to 7 Å. Other simulated proteins showed a lower fluctuation at the amino acid levels. However, several residues that displayed a fluctuated state, such as GLU51 and ALA135 of IL-6; LEU3, PHE86, and GLU253 of IL-6Rβ; ALA2 and ARG6 of IL-8; LEU29 and ILE282 of IL-8Rβ; and THR241 and LYS244 of PPAR-γ. Notably, none of the FA binding sites displayed structural instability during the simulation. However, ARG6 in IL-8 appeared unstable during the simulation, and that residue was a part of the FA binding site. PPAR-γ consistently showed a stable conformation during the simulation. Thus, the data consistently supported that FA might bind and activate PPAR-γ to suppress the SASP development.

Pharmacokinetics properties of ferulic acid

The FA demonstrates high gastrointestinal (GI) absorption, indicating good oral bioavailability. Additionally, its ability to penetrate the blood-brain barrier (BBB) suggests potential central nervous system (CNS) activity. In terms of distribution, FA is not a substrate for P-glycoprotein (P-gp), a transporter that typically reduces drug absorption and bioavailability. This is advantageous, as it implies the molecule is less likely to be effluxed, potentially leading to higher

drug levels and improved efficacy. Regarding metabolism, FA does not inhibit cytochrome P450 (CYP) enzymes, which are crucial for drug metabolism. This reduces the risk of drug-drug interactions or altered drug clearance. As for excretion, the molecule has a low skin permeation rate, which is desirable for orally administered drugs, minimizing the risk of skin irritation or unintended systemic absorption through the skin. Moreover, FA meets several drug-likeness criteria, such as Lipinski's Rule of Five, Ghose's, and Veber's criteria. However, it violates Muegge's and Lead-likeness criteria, which may be due to its molecular weight, indicating some potential limitations for drug development. Overall, FA shows promising ADME properties, including high GI absorption, good BBB permeability, and minimal metabolic interactions, although the violations of Muegge's and Lead-likeness criteria could pose some challenges for further development (Fig. 4).

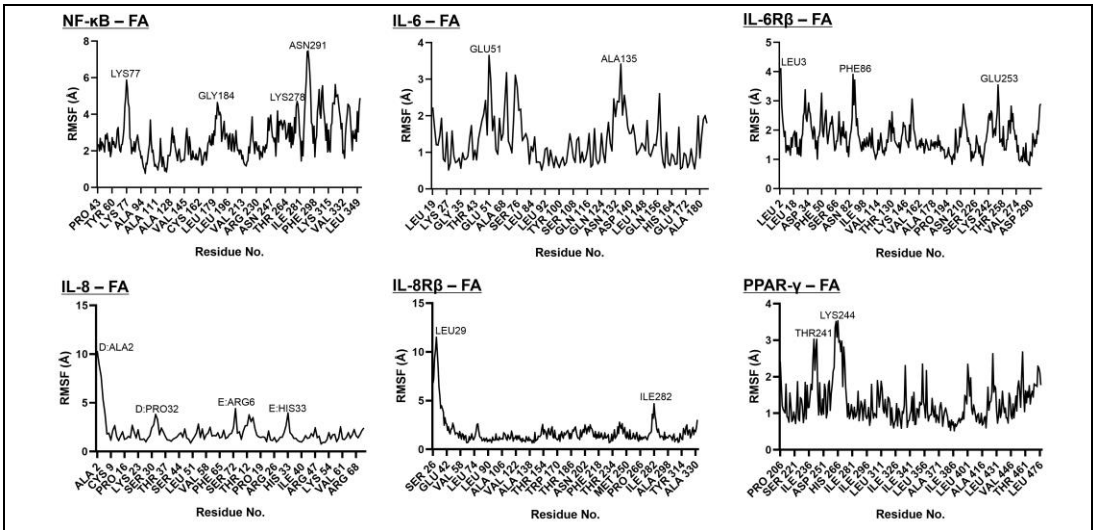

Figure 3. Molecular dynamics simulation of the fluctuations for each target protein residue after binding to ferulic acid.

Figure 4. The absorption, distribution, metabolism, and excretion (ADME) properties of ferulic acid **(A)** were predicted using SwissADME, while organ toxicity **(B)** and toxicity endpoints **(C)** were predicted using Pro-Tox 3.0. In the toxicity results, negative values indicate inactive toxicity properties, while positive values suggest potential active toxicity.

The toxicity prediction results suggest that FA is likely inactive for most organ toxicity endpoints, including hepatotoxicity, neurotoxicity, respiratory toxicity, and cardiotoxicity. However, there are concerns about its potential for nephrotoxicity, as it is predicted to be active for this endpoint. Additionally, FA was predicted to be active for immunotoxicity and clinical toxicity, indicating possible risks to the immune system and general clinical health. While FA shows promise due to its predicted low activity for many toxicity endpoints, the potential risks for nephrotoxicity, immunotoxicity, and clinical toxicity should be carefully evaluated in future development and testing (Fig. 4).

Ferulic acid inhibits fat accumulation in differentiated preadipocyte cells by regulating PPAR-γ and NF-κB protein level

In this study, we use 3T3-L1 mouse preadipocyte cells to examine the effect of FA treatment on cellular senescence. In 3T3-L1 cells, the senescence cells can be observed within a few days following the induction of differentiation (PID). Compared with control cells, the senescence-induced 3T3-L1 cells showed a significant increase in lipid accumulation, as determined by oil-red O staining after 15 and 20 PID (Fig. 5). Importantly, we observed that treatment with 75 mg/mL or 150 mg/mL FA could reduce lipid accumulation in senescence cells (Fig. 5). This result indicates that FA acts as a negative regulator of cell senescence in 3T3-L1 preadipocyte cells.

In silico analysis showed that FA binds to PPAR-γ with an excellent affinity and ability (Figs. 1 and 2A-C). To gain an insight into the mechanism by which FA suppresses fat accumulation *in vitro*, we measured the expression of PPAR-γ and NF-κB in FA-treated senescence cells at 15 PID (Fig. 6). Similar to the previous study, the expression of PPAR-γ was peaked during adipocytes differentiation and maturation, and subsequently decreased along with cell aging, starting from 15 PID (Stavova et al., 2017). We observed that treatment with 75 mg/mL FA increases the level of PPAR-γ protein in preadipocyte cells that undergo senescence. However, cells treated with 150 mg/mL FA slightly enhanced the expression of PPAR-γ compared to senescence cells treated with 75 mg/mL FA (Fig. 6). A reduction of NF-κB protein was detected in senescence cells treated with both 75 mg/mL and 150 mg/mL FA (Fig. 6). These findings indicate that FA inhibits cell senescence in 3T3-L1 cells likely by regulating the PPAR-γ and NF-κB signaling pathways.

DISCUSSION

The concept of inflammaging was introduced more than two decades ago, relying on inflammation-driven cellular senescence (Franceschi et al., 2000). Cells that undergo senescence will produce a secretory phenotype and develop a phenotypic characteristic called SASP (Ou et al., 2022). The inflammation factor drives SASP progression, and IL-6 along with IL-8 play primary inflammatory mediators during senescence (Barajas-Gómez et al., 2017; Koelman et al., 2019; Ortiz-Montero et al., 2017). Thus, blocking those inflammatory mediators may alleviate the SASP development. IL-6 and IL-8 signaling could be inhibited by blocking the IL-6 and IL-8 ligands or their respective receptors. A previous study reported that blocking IL-6 can be achieved by interacting with the inhibitor through several residues involved in the binding sites (Malik et al., 2021). Unfortunately, FA only bound three of those target sites with relatively high binding energy (Fig. 1 and Table 2). The structure's stability and binding integrity also showed a weak interaction, suggesting it may not be a suitable candidate for FA to delay the senescence process. It is reported that IL-6 interacts with greater affinity with IL-6Rβ than IL-6Rα (Ward et al., 1994); therefore, in this study, we only conducted a molecular docking of FA with IL-6Rβ and not with IL-6Rα. Still, FA only interacted with three among several targeted residues to perform an inhibitory activity. In addition, according to the molecular dynamics analysis, the complex's stability and integrity are also low. The results were in line with previous studies that reported the anti-inflammatory effect of FA through inhibition of those cytokines at transcriptional levels (Lampiasi and Montana, 2016; Liu et al., 2022). Thus, the regulation of the cellular senescence of FA may not be achieved by the inhibition of IL-6 signaling by blocking the receptor-ligand complex.

Besides IL-6, IL-8 signaling was also used as a target in this study. Similar to the IL-6 pathway, FA appeared to have a low probability of blocking IL-8 signaling by interacting with IL-8 ligand or IL-8R. Although the binding energy of FA-IL-8Rα showed a promising result, the residues involved in its interaction were away from the targeted residues. In addition, molecular dynamics analysis also revealed the instability of FA-bound IL-8 and IL-8Rβ, represented by the value of the RMSD backbone. The detachment of FA with IL-8 and IL-8Rβ revealed low stability of the complexes.

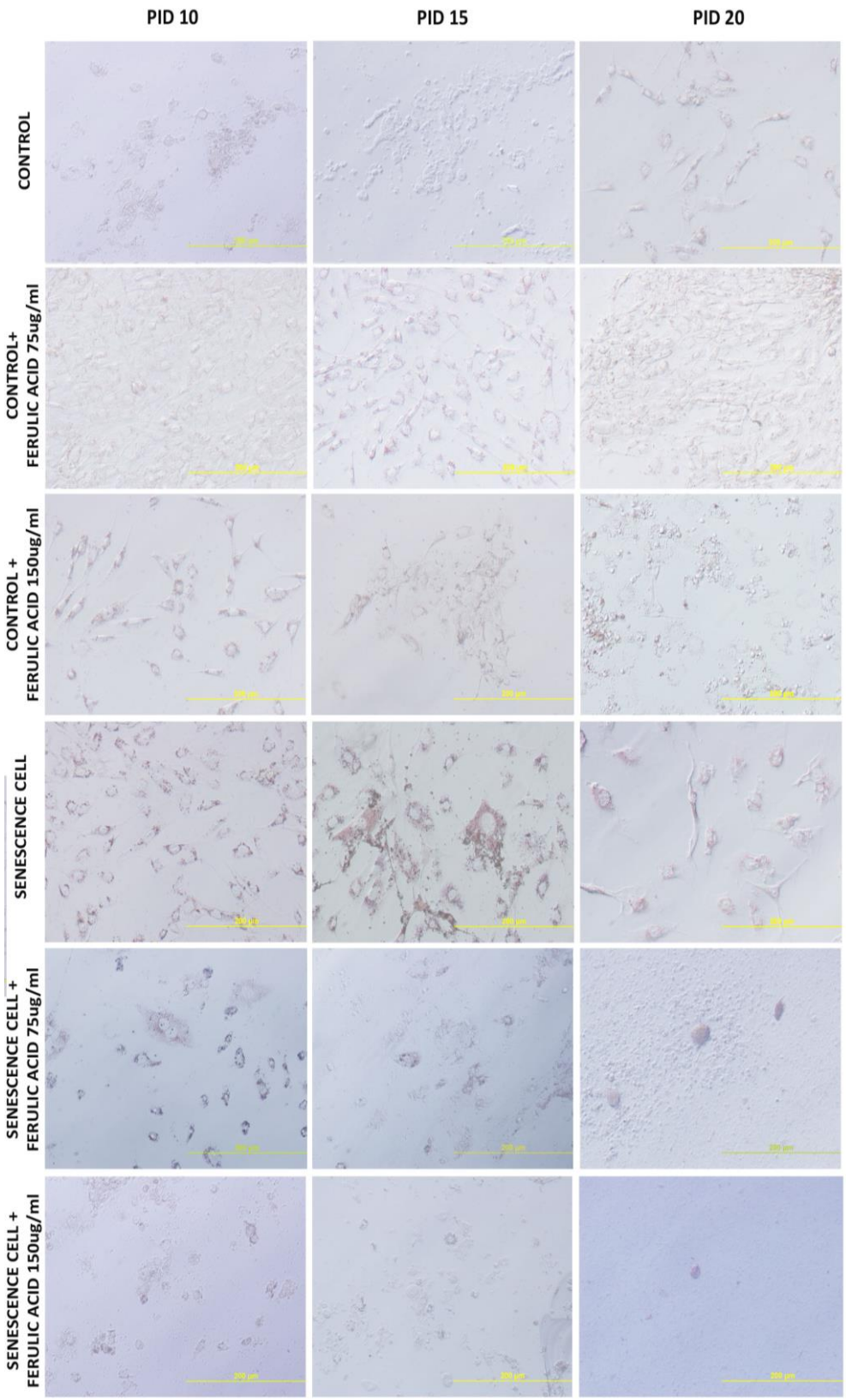

Figure 5. Lipid accumulation of senescence 3T3-L1 cells in control and ferulic acid-treated groups.

The lipid deposits were indicated with red stain. The analysis was performed at 10, 15, and 20 of post-induction day (PID). Scale: 200 µm.

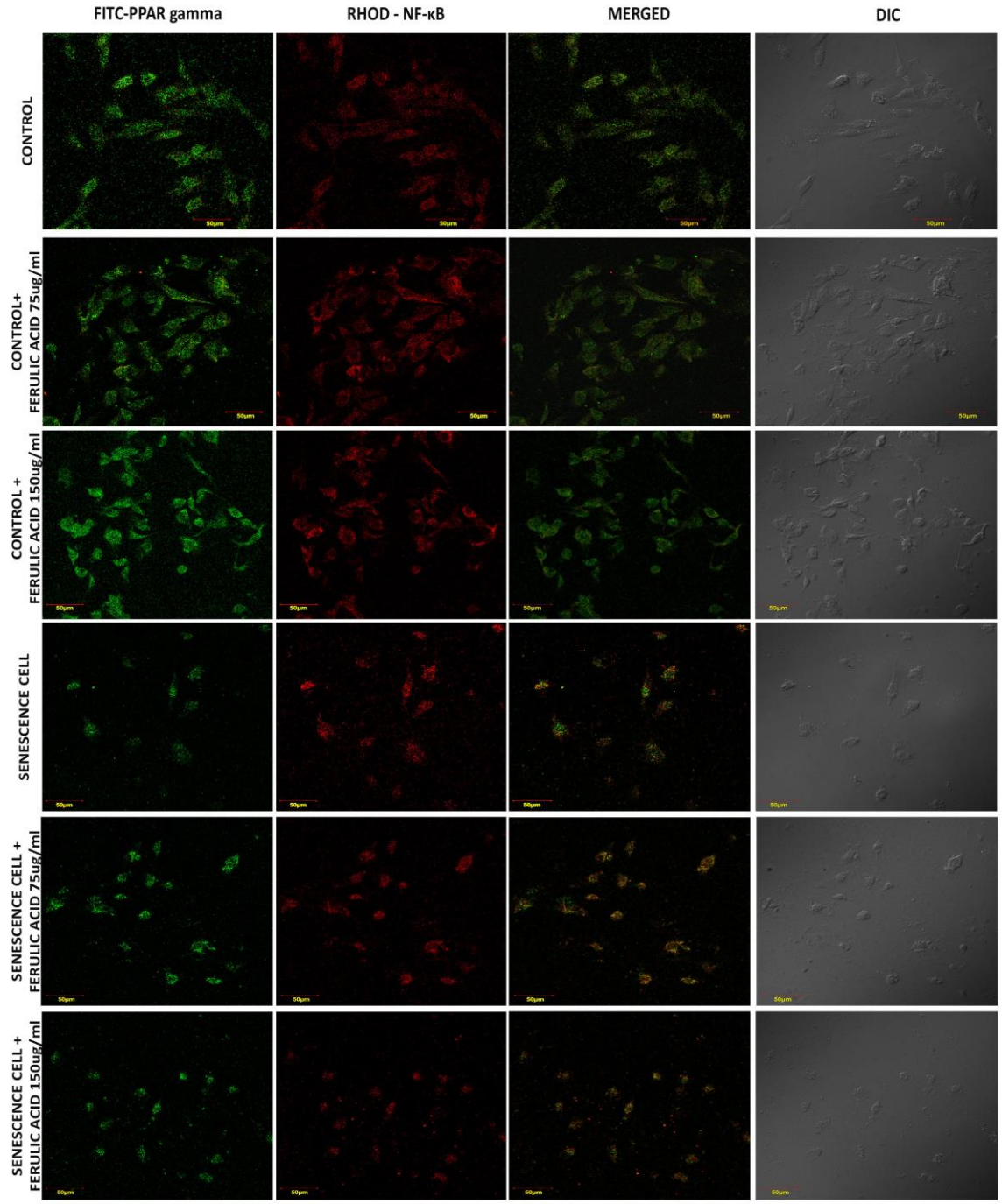

Figure 6. The level of PPAR- γ and NF-κB proteins of differentiated 3T3-L1 cells in control and ferulic acid-treated groups.

Immunofluorescence of PPAR- γ was labeled with FITC staining (green), NF-κB was labeled with rhodamine (red, RHOD). Differential interference contrast (DIC) panels showed the optical contrast of cell structure observed under laser confocal scanning microscopy (CLSM). Scale: 50 µm.

Treatment of FA causes a reduction in the level of NF-κB protein *in vitro* (Fig. 5). In agreement with this result, ferulic acid reduces the expression and inhibits nuclear translocation of NF-κB in human oral cancer cells (Manoharan et al., 2014; Muthusamy et al., 2019).

The virtual analysis showed that the binding of FA with NF-κB have less interaction in targeted residues and low stability in molecular dynamic simulations (Figs. 1 and 3). This indicates that NF-κB is not a direct target of FA to inhibit cell senescence. Alternatively, FA might inhibit NF-κB signaling indirectly, possibly through the regulation of other NF-κB signaling components. It was reported that FA could regulate inflammation by alleviating the expression of phosphorylated IKK in macrophages (Lampiasi and Montana, 2016). However, the detailed mechanism of how FA downregulated the level of phosphorylated IKK is still unclear. Since FA is unlikely to bind directly to IKK-β (Fig. 1), we hypothesized that FA may

modulate kinases or phosphatases of IKK protein. The reduction of phosphorylated IKK will prevent NF-κB nuclear translocation and downregulate the expression of pro-inflammatory cytokines, including IL-6 and IL-8 (Hirabayashi, 1995; Liu et al., 2022). Thus, the anti-aging activity of FA might be achieved through indirect inhibition of NF-κB, IL-6, and IL8 signaling pathways.

FA is capable of reducing the accumulation of lipid droplets in differentiated 3T3-L1 cells (Fig. 4). Moreover, the level of PPAR-γ was increased in FA-treated senescence cells (Fig. 5). As the central mediator of the senescence-associated pathological pathway, PPAR-γ was widely targeted to reach cellular longevity (Erol, 2007). PPAR-γ was repressed in senescence cells (Xu et al., 2018), and upregulating PPAR-γ was reported to have a beneficial role in delaying age-associated diseases (Xu et al., 2020). Upregulation of PPAR-γ repaired the fatty acid metabolism and reversed the aging progression (Wang et al., 2021). Saturated fatty acids could activate a Toll-like receptor and induce inflammaging (Rocha et al., 2016). Inversely, catabolism of fatty acids induced anti-inflammatory activity (Namgaladze and Brüne, 2016), thus preventing inflammaging and reversing the aging process. In this study, an *in silico* analysis has demonstrated that FA interacted with several agonistic residues of PPAR-γ (Al Sharif et al., 2019) with stable structural conformation and protein-ligand interaction upon molecular simulations (Figs. 1 and 2A-C). The interaction of FA with PPAR-γ might induce agonistic activity, resulting in the activation of PPAR-γ signaling. Alternatively, those interactions may increase the level of PPAR-γ in senescence cells by enhancing protein stability and/or inhibiting degradation of PPAR-γ protein. Interestingly, FA was reported to weaken inflammation through the agonist activity of PPAR-γ (El-Ashmawy et al., 2018; Mahmoud et al., 2019). Thus, activation of PPAR-γ by FA becomes the most possible pathway to decrease the inflammaging and reverse the aging progression in adipocytes.

We emphasized in a summary in the graphical abstract (Fig. 7) that healthy adipocyte cells undergo cellular senescence through the pathway of C/EBP-α-induced PPAR-γ activation and the inhibition of several SASP factors. FA directly interacts with PPAR-γ agonist residues with stable structural conformations and protein-ligand interactions in molecular simulations. This interaction induced an increase in PPAR-γ expression to reduce the accumulation of lipid droplets in the differentiated mouse 3T3-L1 pre-adipocyte cell line. Furthermore, FA can indirectly reduce NF-κB, IL-6, and IL-8 signaling. The results of this study suggest that regulation of PPAR-γ signaling by FA may generate beneficial effects in preventing cell senescence of adipocyte aging progression. Some pre-adipocyte cells, such as mouse cell lines 3T3-L1 and 3T3-F442A, undergo one or two rounds of cell division before differentiating. There is a transcriptional cascade between adipocyte genes, including PPAR-γ and C/EBPα, that are regulators of nuclear adipocyte differentiation. In the early stages of adipocyte differentiation, the expression of C/EBPβ and C/EBPδ increases, leading to upregulation of C/EBPα and activation of PPAR-γ, thereby promoting adipogenesis (Müller, 1995; Zhang et al., 2020). PPARγ is regulated by lipid side-products that most likely play a role in endothelial response and adaptation to FA fluxes originating from blood and adipocytes. This results in the deregulation of endothelial PPARγ-dependent mechanisms and disrupts FA influx and release from AT, resulting in local and systemic dysfunction, and subsequently impacts aging on ligand-mediated PPARγ responses (Briot et al., 2018).

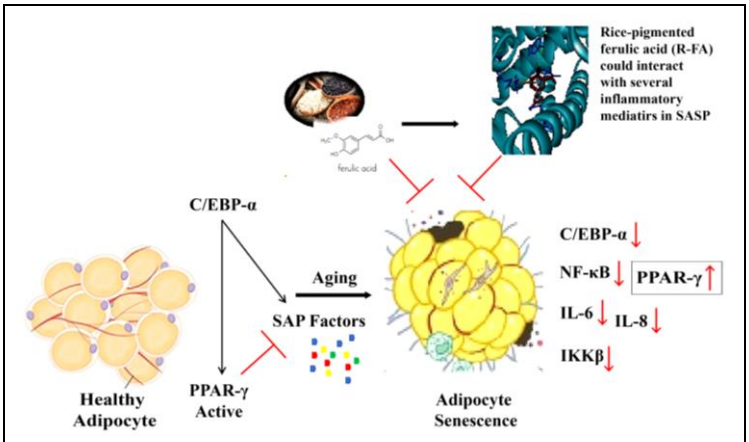

Figure 7. Graphical abstract of the proposed mechanism of ferulic acid inhibits inflammaging.

FA directly interacts with and activates PPAR-γ to prevent cellular senescence. FA might be capable of attenuating NF-κB, IL-6, and IL-8 signaling through an indirect mechanism.

CONCLUSION

FA exhibited strong interactions with several key inflammatory mediators, including NF-κB, IL-6, IL-6Rβ, IL-8, IL-8Rβ, and PPAR-γ. Notably, FA demonstrated high interaction stability with PPAR-γ, suggesting its potential anti-aging properties through modulation of the SASP process via PPAR-γ agonistic activity. Consistent with this, FA treatment was shown to increase PPAR-γ protein levels in adipocyte cells. Additionally, FA may indirectly regulate NF-κB, IL-6, and IL-8 signaling pathways to prevent cell senescence.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Fatchiyah F	Hermanto FE	Virgirinia RP	Rohmah RN	Triprisila LF	Suyanto E	Miyajima K
Concepts or ideas	x						
Design	x						
Definition of intellectual content	x					x	x
Literature search	x	x	x	x	x		
Experimental studies	x	x		x	x		
Data acquisition	x	x	x	x	x		
Data analysis	x	x	x	x	x		
Statistical analysis	x		x				
Manuscript preparation	x		x			x	x
Manuscript editing	x		x			x	x
Manuscript review	x	x	x	x	x	x	x

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