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The effect of vitamin D and paricalcitol on protein disulfide isomerase

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ABSTRACT: Protein disulfide isomerase (PDI), a multifunctional protein plays an important role as oxidoreductase, isomerase and chaperone in the cell. Prior studies have identified PDI is highly expressed in many different cancer types and presented as a new potential target for cancer treatment. Here, we investigated vitamin D and its analogue paricalcitol in silico interaction of the human PDI and inhibition of PDI reductase activity in vitro. We observed a non-covalent mechanism where the main skeleton of the vitamin D3 and paricalcitol structure is located at the hydrophobic site in the b′ domain of PDI and forms a hydrogen bond with a residue (His138) in this domain. They also form multiple weak hydrophobic interactions with various chemical groups of the b′ subunit. For the first time, we demonstrate that 1,25-dihydroxyvitamin D3 (1α,25(OH)2 vitamin D3) and paricalcitol inhibit the PDI reductase activity in vitro and their IC50 values are 20.79±1.43 nmol/L and 32.82±3.15 nmol/L respectively. The two compounds can also block the denitrosation activity of PDI.

KEYWORDS: Protein disulfide isomerase; paricalcitol; vitamin D3; molecular docking; activity of enzymes.

1. INTRODUCTION

1,25-dihydroxyvitamin D3 (1α,25$(OH)_2$ vitamin D3), a member of the secosteroid hormone family, plays an important role in calcium homeostasis and bone metabolism, as well as immune responses, hormonal and metabolic processes [1]. Multiple clinical and meta-analysis studies have associated plasma levels of vitamin D with cancer risk [2]. Vitamin D produces its effects in cells through vitamin D receptors (VDR) [3]. It has been shown that vitamin D can exert nongenomic effects in relation to membrane proteins other than VDR receptors. One of these proteins is the so-called 1,25D3-MARRS (membrane-associated, fast- responsive steroid binding) protein [4, 5]. The 1,25 D3-MARRS protein is also known as protein disulfide isomerase a3, (PDIa3) [6]. PDI is a member of the PDI protein family, which all contain thioredoxin-like domains. PDI is an enzyme with multifunctional catalytic activity, including thiol-disulfide oxidoreductase, disulfide isomerase, and redox-mediated protein folding (chaperone effect) [7]. It is found in all organisms (bacteria, yeast, humans, plants). It is localized in the endoplasmic reticulum (ER) of eukaryotic cells, where it contributes to maintenance of the oxidant environment and ER homeostasis. Apart from the ER, PDI has been localized on the cell surface, cytosol, mitochondria, and extracellular matrix [8]. The PDI protein, a polypeptide with a molecular weight of 57 kDa encoded by the P4HB gene, has catalytic active sites containing the Cysteine-Glycine-Histidine-Cysteine (CGHC) motif [9]. The common structural similarity between all members of PDI proteins is the presence of at least one thioredoxin (trx)-like domain. This sequence has been found to catalyze the reduction, isomerization of disulfide bonds and oxidation of thiols [10]. While PDI is considered essential to maintain healthy cells and tissues, recent studies suggest that this protein is involved in both the physiology and pathophysiology of the cell [8]. In particular, it has been reported to have a protective effect in neurodegenerative diseases. However, PDI may also play a role in mediating the entry of pathogens during infectious diseases [11]. PDI has been shown to be highly expressed in many different types of cancer, including brain, kidney, ovarian, prostate, lung, and male germ cell tumors [12]. Similar findings were observed in cytosolic and membrane-localized PDI. [13]. There

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is also PDI in platelet and endothelial cells. It has been shown that PDI secreted by these cells can play an important role in thrombus formation. Inhibition of extracellular PDI is presented as a molecular target in antithrombotic therapy showed in their study that there is a binding interaction between PDI and estradiol and that PDI has a role in the regulation of intracellular estradiol levels [14, 15]. It has been shown that the estradiol identified binding site on the PDI is different from the known peptide binding sites. The identification of the estradiol-binding domain on PDI may facilitate the development of novel PDI inhibitors for cancer and HIV therapies. In our study, we aimed to investigate the interaction of vitamin D and its analogue paricalcitol due to their structural similarity with estradiol with PDI protein. Moreover, we investigated the effect of this interaction on the reductase activity of PDI in vitro.

2. RESULTS

2.1 Molecular Docking

Redocking was performed to test the accuracy of our molecular docking method. The ligand ((4S,5S)-1,2- DITHIANE-4,5-DIOL) belonging to the 3UEM coded protein structure was docked (Figure1) and the RMSD value was calculated as 1.023 according to the reference structure. Our method is valid since the RMSD value is below 1.5 Angstrom In this study, we investigated the binding interactions of Vitamin D3 and paricalcitol with protein disulfide isomerase (PDI) using molecular docking techniques. The binding energies obtained from the docking studies were -7.4 kcal/mol for Juniferdin, -7.5 kcal/mol for Vitamin D3, and -8.5 kcal/mol for Paricalcitol (Table 1). The protein-ligand interactions, including the binding structures and types, are visually represented in Figure 1 and detailed in Table 2. Upon examining the data in Figure 1 and Table 2, the following results were obtained.

Table 1. Binding energies of ligands.

Ligand Name	**Juniferdin (positivecontrol)**	**Paricalcitol**	**Vitamin D_3**
Binding energy (kcal/mol)	-7,4	-8,5	-7,5

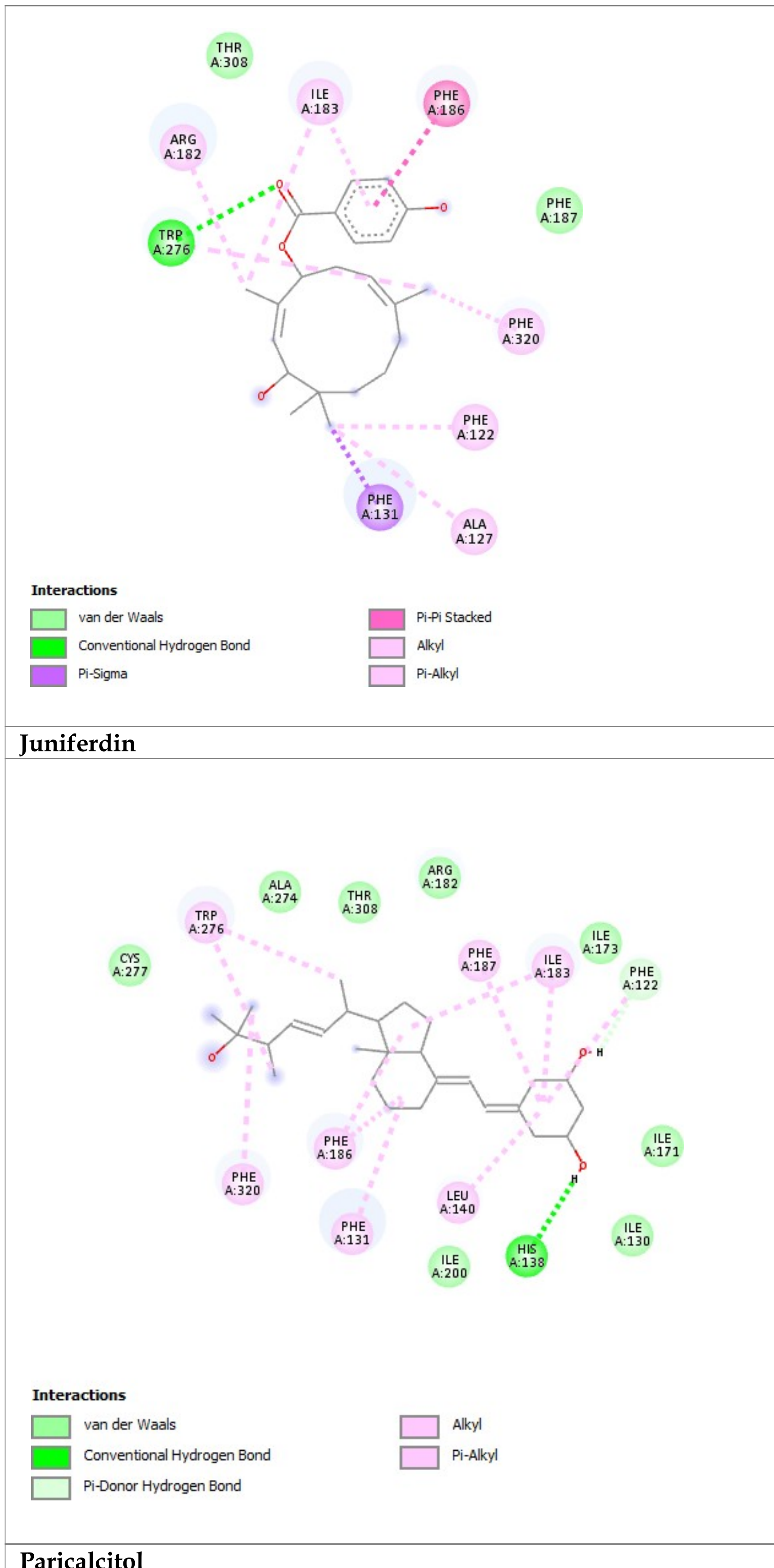
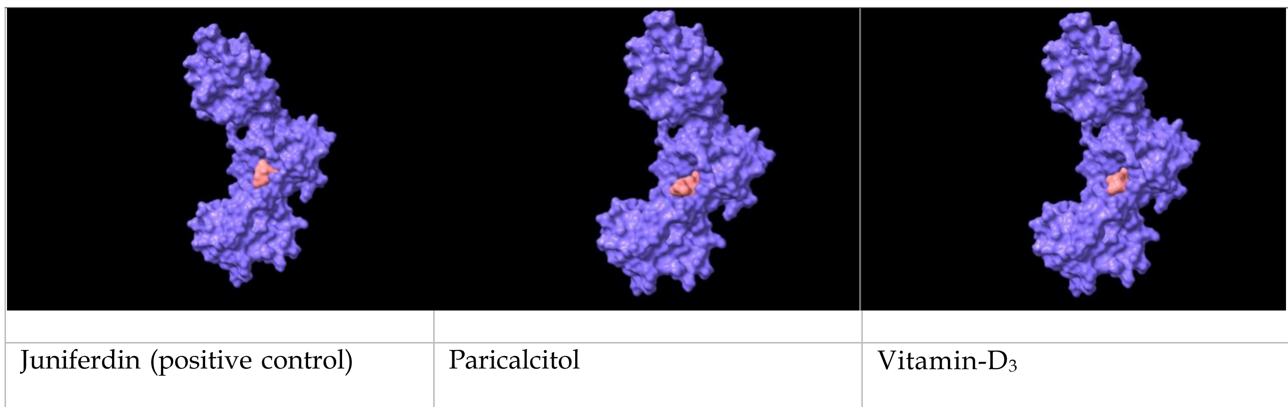


Figure 1. 2D visualization of protein ligand interactions

Table 2. Binding interactions of ligands with PDI

Ligand Name	Interaction Type	Amino Acids	Distance (Å)
Juniferdin	Hydrophobic Interactions	PHE122, PHE131, PHE131, ARG182, ILE183, PHE186, PHE320	3.59, 3.48, 3.45, 3.20, 3.22, 3.82, 3.95
Juniferdin	Salt Bridges	ARG182	5.31
Juniferdin	pi-Stacking	PHE186	4.21
Paricalcitol	Hydrophobic Interactions	PHE122, PHE131, PHE131, LEU140, ILE183, ILE183, PHE186, PHE186, PHE186, PHE187, TRP276, THR308, PHE320	3.65, 3.39, 3.58, 3.51, 3.47, 3.36, 3.77, 3.52, 3.65, 3.66, 3.83, 2.93, 3.77
Paricalcitol	Hydrogen Bonds	HIS138	1.90
Vitamin D3	Hydrophobic Interactions	PHE131, PHE131, PHE131, LEU140, ARG182, PHE186, PHE186, PHE186, PHE187, ILE200, THR308, PHE320	3.00, 3.30, 3.32, 3.36, 3.44, 3.46, 3.49, 3.81, 3.56,

Juniferdin, serving as the positive control, exhibited a binding energy of -7.4 kcal/mol. It formed multiple hydrophobic interactions with the residues PHE122, PHE131, ARG182, ILE183, PHE186, and PHE320, with distances ranging from 3.20 Å to 3.95 Å. Additionally, it formed a salt bridge with ARG182 at a distance of 5.31 Å and a pi-stacking interaction with PHE186 at a distance of 4.21 Å. Paricalcitol demonstrated the strongest binding affinity among the tested compounds, with a binding energy of -8.5 kcal/mol. It formed 13 distinct hydrophobic interactions with residues PHE122, PHE131, LEU140, ILE183, PHE186, PHE187, TRP276, THR308, and PHE320. The hydrophobic interactions had distances ranging from 2.93 Å to 3.83 Å. Additionally, Paricalcitol formed a hydrogen bond with HIS138 at a distance of 1.90 Å. The extensive network of hydrophobic interactions and the hydrogen bond suggest a robust and stable binding within the hydrophobic pocket of the PDI b-b′ domains (Figure 2, Figure 3). Additionally, all three tested molecules were observed to bind to the same pocket of the protein (Figure 2). 1α,25(OH)$_2$ vitamin D$_3$ exhibited a binding energy of -7.5 kcal/mol. It formed hydrophobic interactions with residues PHE131, LEU140, ARG182, PHE186, PHE187, ILE200, THR308, and PHE320, with distances ranging from 3.00 Å to 3.92Å. Vitamin D$_3$ also formed a hydrogen bond with HIS138 at a distance of 2.35 Å. Although Vitamin D$_3$ showed a significant number of hydrophobic interactions and a hydrogen bond, its binding affinity was slightly lower than that of Paricalcitol.



Juniferdin (positive control)	Paricalcitol	Vitamin-D_3

Figure 2: The poses of the structures with the best scores in which the ligands bind to the protein structure

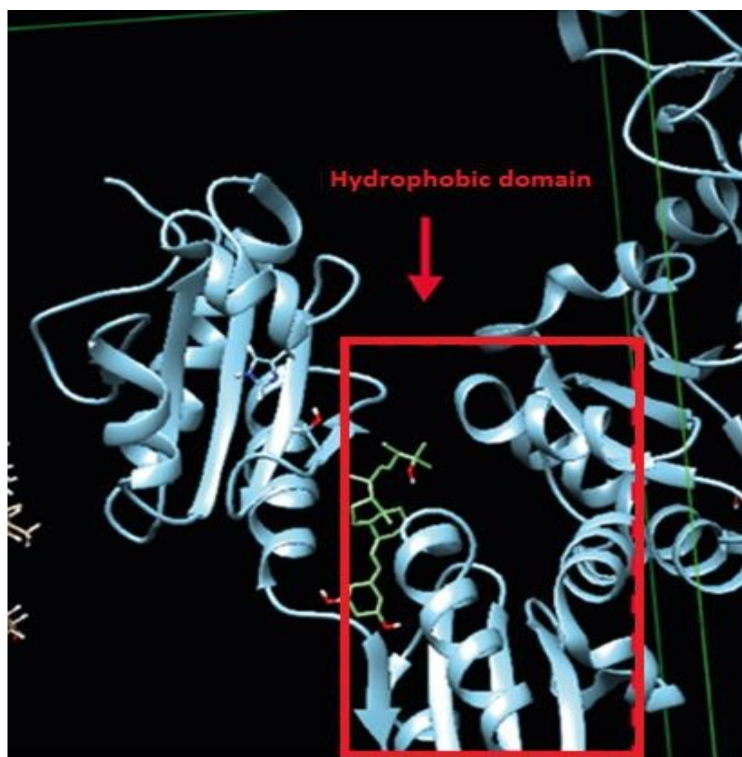


Figure 3. The placement of paricalcitol (PDI b-b′ domain)

2.2 PDI Reductase Activity Inhibition

In *in vitro* PDI activity inhibition studies with vitamin D and its analog paricalcitol, triple studies were performed for all concentrations and the averages were taken. 1α,25$(OH)_2$ vitamin D3 at a concentration of 100 nmol/L inhibited recombinant PDI reductase activity by 96% and paricalcitol by 94% (Figure 4). The IC50 value for paricalcitol was determined as 32.82±3.15 nmol/L, and the IC50 value for vitamin D was determined as 20.79±1.43 nmol/L.

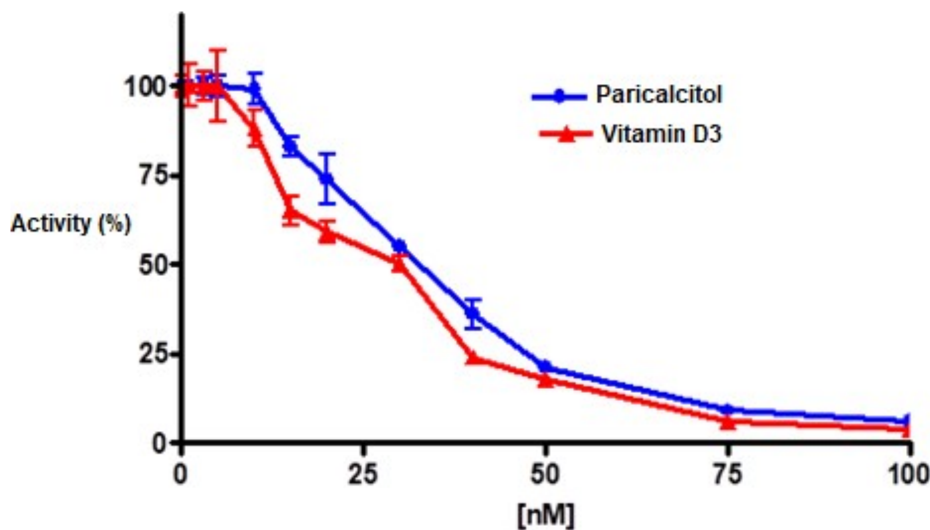


Figure 4. The concentration dependent effects of 1α,25$(OH)_2$ vitamin D3 and paricalcitol on the reductase activity of PDI.

2.3 PDI Denitrosation Activity Inhibition

We examined the effect of Vitamin D and paricalcitol at 100 nmol/L concentration on the denitrosation activity of PDI. While paricalcitol inhibited enzyme activity by 91% within 15 min, vitamin D caused inhibition by 94% (Figure 5). The inhibition rate of PDI with 1α,25$(OH)_2$ vitamin D_3 and paricalcitol were close to bacitracin (1000 µM) inhibition rate.

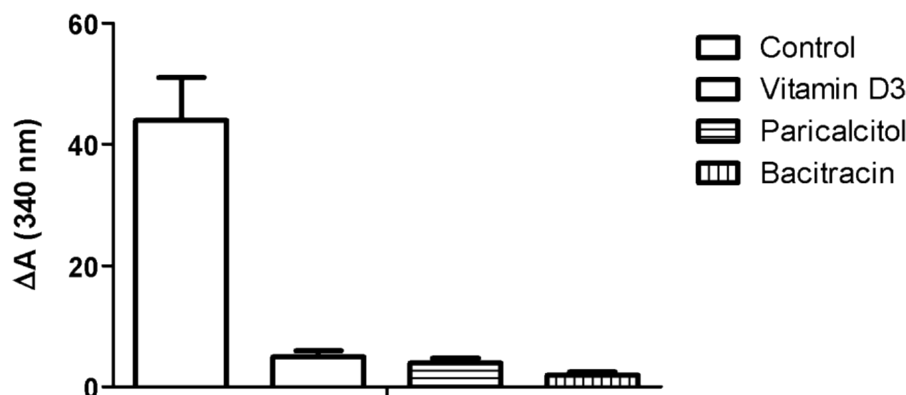


Figure 5. The effect of 1α,25$(OH)_2$ vitamin D3 and paricalcitol on the denitrosylation activity of PDI

3. DISCUSSION

With the determination of the pathophysiological roles of PDI, targeted studies have been carried out by many researchers in recent years to examine the inhibition mechanisms in vitro and in silico and to discover potent inhibitors. The PDI protein has four domains, a, b, b′, and a′. The a and a′ domains are functionally thioredoxin-like subunits and each contains catalytic Cys-X-Cys motifs that react with thiols of newly synthesized proteins to exhibit disulfide oxidoreductase activity. On the other hand, the b and b' subunits, although structurally similar to thioredoxin, do not contain catalytically active cysteines. Instead, the b and b' domains are responsible for regulating the binding of the substrate to the a and a' regions [21]. The links between fields a-b and b-b′ in the PDI are short. The polypeptide chain consisting of 17 amino acid residues provides the connection between the b′ and a′ regions, and the short region is called the X segment. The C-terminal, on the other hand, is located opposite the active site in the a′ region, and although it does not directly participate in the catalytic activity of PDI, the C-terminal interacts with the a′ subunit and regulates the

substrate interaction [22]. Subunit b′ recognizes unfolded proteins. The b subunit regulates the substrate binding capacity of the b' subunit together with the x segment [23]. In the study examining the interaction between PDI and estradiol, it was shown that the binding site defined for estradiol on PDI is different from the known peptide binding sites. It has been observed that the binding site is mainly located in a hydrophobic pocket consisting of the b' domain of PDI and partially the b domain [15]. Our *in silico* studies showed that paricalcitol and vitamin D3 bind to the hydrophobic domain where estradiol interacts with PDI. It was observed that it interacts with the b' subunit in this area. This result was not surprising given the chemical structural similarity of both molecules to estradiol. Notably, there appears to be no literature exploring the structure of protein disulfide isomerase (PDI) with PDB code 3UEM. However, limited studies suggest that the PDI enzyme forms hydrogen bonds with the cysteine amino acid of ligands. Additionally, studies on the b-b' region of the protein have observed Hydrogen bonding with the histidine amino acid. Our findings align with the literature regarding b-b', as both paricalcitol and vitamin D3 form hydrogen bonds with Histidine amino acids in this region [15, 24] The chaperone effect of PDI is regulated by the redox level of the enzyme. When the GSH/GSSG ratio is 2- 3:1 in the ER, it acts as oxidase; Since the GSH/GSSG ratio outside the ER is 30-100:1, it acts more as a reductase. While PDI oxidase has an effect at pKa 4.5 level; It shows more reductase effect at pKa 7.1 level PDI is also located in the extracellular part of the cell membrane. Membrane PDI mainly acts as a reductase and acts as an isomerase on the cell membrane surface [25]. Therefore, we specifically examined its effect on inhibition of PDI reductase activity.

The results of the in vitro PDI reductase activity inhibition test with vitamin D3 and paricalcitol are consistent with our in-silicone studies. Our in vitro inhibition studies have shown for the first time in the literature that paricalcitol, a vitamin D analogue, reduces the reductase activity of the PDI enzyme. A similar effect was determined for the first time with the active form of vitamin D3. When the PDI activity was examined against varying concentrations of insulin used as the substrate of the enzyme, it was observed that inhibition continued at a high substrate concentration. Our initial findings are that paricalcitol causes a non- competitive inhibition on the enzyme's activity. This finding is in the direction we expected considering the binding area of paricalcitol that we showed by in silico study. However, further studies are needed in this regard. Estrogens have been shown to inhibit the reductase activity of PDI at 1 μM concentration (substrate insulin was used) by 40-60% in samples containing human placenta cytosolic fractions, and 20-30% in membrane samples. It has been observed that estrogens significantly reduce the isomerase activity of the enzyme. Investigators also examined the effect of inhibition against the substrate concentration [26]. Similar to our findings, they observed that inhibition was not eliminated at high substrate concentration. Although it is not the same molecule, considering the structural similarity, our findings are consistent with the work of Tbris et al. (1989). It has been reported that molecules such as T3 and estrogen hormones, somatostatin, bacitracin, bisphenol A and phenyl arsenic oxide bind to PDI and inhibit its activity in vitro. However, only a few have been shown to inhibit PDI under experimental in vivo conditions [27, 28]. In a study using di-eosin-oxidized glutathione (DIE-GSSG) as a substrate and DTT as a co-substrate, it was found that 17β-estradiol, 17α-ethinylestradiol, and diethylstilbestrol exhibited inhibitory effects at concentrations as low as 10^{-8} M [29]. They observed that it exhibited a significant inhibitory effect on its activity. In our study, the IC50 value for $1\alpha,25(OH)_2$ vitamin D_3 was 20 nmol/L and the IC50 value for paricalcitol was observed as 32 nmol/L. It has been shown that 17b-estradiol can also inhibit the isomerase activity of the enzyme [30]. Isomerase activity of PDI was not studied in our study. However, we also investigated the effect of paricalcitol on the denitrosation activity of the enzyme. It has been observed that the denitrosation activity of the enzyme also decreases with paricalcitol and vitamin D (in the presence of nitroso glutathione 100 μM, GSH 1.2 mM, 3 μU PDI). PDI denitrosation activity is also carried out through reductase activity (Khan and Mutus, 2014). We have also demonstrated the inhibition of the reductase activity of PDI through denitrosation. NO has been reported to interact with PDI in several ways. PDI can form S-nitrosylated proteins by adding NO to proteins, or it can release NO from S-nitrosylated proteins and substrates by denitrosation. PDI on the cell surface can act as a transnitrosylase that

delivers NO to cells [31]. Inhibition of PDI reductase activity by 1α,25$(OH)_2$ vitamin D3 and paricalcitol may have a role in their anticancer [15] and antithrombotic effects [32-34]. More research is needed to clearly understand the mechanism.

4. CONCLUSION

Our in-silico study showed that Vitamin D and Paricalcitol have interactions with b′ domain of PDI protein (with very good docking scores). Moreover, in in vitro studies, we also observed that 1α,25$(OH)_2$ vitamin D_3 and paricalcitol inhibited the reductase activity of the PDI enzyme.

5. MATERIALS AND METHODS

5.1 Molecular Docking

The human protein-disulfide isomerase enzyme has a resolution of 2.29 Å, belongs to the human class, has no mutation and has one bound ligand, with its single-chain structure, the file with the code name 3UEM, obtained by X-ray diffraction method, was downloaded from the Protein Data Bank. Docking operations were performed using a 20 Angstrom grid box with 5,397 -25,01 2,80 X,Y,Z coordinates. Ligands were built using Chimera 1.15 visualization software by downloading smiles codes from Pubchem database. Ligands were prepared for docking by calculating Gasteiger charges with Chimera software tools and recorded in mol2 format. After the protein structure was prepared for docking (charge determination, hydrogen addition, deletion of nonstandard bound groups), docking scores were obtained using Autodock Vina 4.2.6 embedded in Chimera 1.15 software. The structures with the best scores were then searched for bond types and structures using the Protein-Ligand Interaction Profiler server [16]. BIOVIA Discovery Studio Visualization software was used for 2D visualizations.

5.1.1 Validation of Molecular Docking Method

To understand the accuracy of our molecular docking method, redocking was performed. After the original ligand was docked back to the protein structure, it was compared with the original crystal structure. The DOCKRMSD web server was used for this [17]. For the best exposure, the RMSD value is expected to be less than 1.5 Angstroms [18].

5.2 The In vitro Inhibition of PDI Reductase Activity

Protein disulfide isomerase inhibitory activities of 1α,25$(OH)_2$ vitamin D_3 and paricalcitol were analyzed using PDI Inhibitor Screening Assay Kit (Abcam, ab139480). In the assay, insulin was used as the substrate, and recombinant human PDI as the enzyme. The principle of the fluorometric method is accomplished by monitoring the enzyme-catalyzed reduction of insulin in the presence of dithiothreitol (DTT). Dose-response assays were performed with 0 to 100 nM 1α,25$(OH)_2$ vitamin D_3 (active form, Sigma-Aldrich) or paricalcitol (Zemplar, AbbVie) added 15 min before the initiation of enzymatic reaction. Reactions were performed as described in an kit assay protocol. Bacitracin (1000 μM) was used as an inhibitor control in the assay [19]. The fluorescence of samples was measured using a fluorescence microplate reader (Epoch™ Take3 Plate, Ex/Em 500/603nm). The results were expressed relative fluorescence unit (RFU). The IC_{50} values were calculated that indicate the concentration required to inhibit 50% PDI activity.

5.3 The Inhibition of PDI Denitrosation Activity

The inhibition of PDI denitrosation activity was determined by monitoring the changes in absorption of nitroso glutathione (GSNO) at 340 nm after incubation of 1α,25$(OH)_2$ vitamin D_3 and paricalcitol. A fresh solution of GSNO was prepared from GSH (Sigma-Aldrich) and $NaNO_2$ (Sigma-Aldrich) before the experiment (Hart, 1985). 620 μl of concentrated HCl was taken and diluted with 5.9 ml of deionized water. GSH was dissolved in this solution. In another tube, $NaNO_2$ was dissolved in 1 ml of deionized water. Then, sodium nitrite solution was slowly added to the acidic glutathione solution and mixed carefully for 5 minutes, protected from light. The pH was

adjusted to 6.0 by the addition of 1 N NaOH. 1 mM GSNO solution was prepared. The concentration of GSNO was checked in the spectrophotometer. Absorption of GSNO at 335 nm was measured. The absorbance of 1 mM solution at 335 nm should be 0.92. Free thiols (such as DTT or GSH) are required for the thiol-disulfide exchange activity of PDI. GSH is required in the denitrosation reaction of PDI. In their study on the denitrosation activity of PDI by [20]. They reported that the optimal GSNO concentration for denitrosation activity was 100 mM, and the GSH level was 1.2 mM. We used these concentrations in our study. The reaction was performed in PDI assay buffer (0.1 M potassium phosphate buffer, pH 7.0, and 2 mM EDTA) containing 100 mM GSNO and 3 mU PDI. $1\alpha,25(OH)_2$ vitamin D_3 and paricalcitol (100 nmol/L) was added. All measurements were performed using a microplate reader spectrophotometer (Bio-tek Instruments ELX 808ru) at 25 °C. The inhibition of PDI denitrosation was monitored by absorbance change after GSH (1.2 mM) addition. Percent inhibition was calculated from the following equation:

$$\%\text{inhibition} = \frac{(\text{A340 control- A340 sample})}{(\text{A340 control})} \text{X100}$$

5.4 Statistical Analysis

The data was expressed as mean ± SD (standard deviation). The experiments were performed in triplicate. IC_{50} values of PDI reductase inhibition were calculated using the nonlinear regression analysis (Graphpad Prism 5, Graphpad Software, San Diego, USA).

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Conflict of interest statement: None

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