Identification of a Novel Antagonist of the PDIA1 Reductase Activity Using Virtual Screening

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Abstract

Protein Disulfide Isomerase (PDI) protein family is known for assisting newly synthesized proteins to fold in the endoplasmic reticulum (ER) of most cell types. Meanwhile, extracellular functions of PDIs have been reported and associated to several processes including cell adhesion, thrombosis, cancer, and pathogenesis. In addition to their biological significance, the expression profile, localization and suitability for high-throughput screening are in favor of PDIs being good targets in the development of drug against several diseases. Indeed, few PDI inhibitors are available and show decreased specificity, potency and druggability.

In an attempt to identify more potent and specific PDIA1 inhibitors, we have carried out virtual screening of a large chemical compound database (10^7 compounds). Following this screening, we used an experimental plate-based PDIA1 reductase activity inhibition assay screening to identify and validate a lead compound for further optimization using combinatorial synthesis and computational drug design. Indeed, we identified a molecule that could be used as a lead candidate for the development of anti-inflammatory drugs.

Keywords: Protein Disulfide Isomerase, Thrombosis, Virtual Screening, Molecular docking, Inflammation.

Introduction

Fifty years ago, Protein Disulfide Isomerase (PDI) was discovered as the first folding catalyst and founding member of a large family of more than twenty thiol oxidoreductases that vary in length and domain arrangement. It was shown that PDIA1 catalyzes reduction, oxidation and isomerization of disulfide bonds and plays arole of chaperone *in vitro* (Cai et al., 1994) and in vivo (McLaughlin & Bulleid, 1998) independently of its redox activity. PDIA1 consists of four thioredoxin-like domains, a, a', b and b', plus a linker domain; x (Kozlov et al., 2010). Catalytic CGHC motifs reacting with thiol groups in substrate proteins are contained in catalytic domains a and a' , whereas non-catalytic domains b and b' are involved in substrate recognition and recruitment (Kozlov et al., 2010).

Although majority of cell's PDI is assisting folding of newly synthesized proteins in the endoplasmic reticulum (ER) (Vaux et al., 1990), extracellular PDI roles have been reported in previous studies. The role of protein disulfide isomerase (PDI) expressed on the surface leukocytes was investigated and PDI was shown

to play a role in integrin's ligand-induced conformational change which is essential for cellular adhesion and recruitment to the inflammatory site (Hahm et al., 2013). PDI was also reported to be upregulated in several cancers (Xu et al., 2014) , and this upregulation correlates with cancer metastasis and invasion. In addition, PDI was also associated with several virulence functions like HIV virus attachment(Ou & Silver, 2006), parasite virulence (Ben Khalaf et al., 2012). All of this data points towards PDI as a valuable and emerging drug target for inflammation, cancer and infectious diseases (Ben Khalaf, 2018).

In the present study, we established a virtual screening approach of a large dataset of small chemical compounds (10^7) in an attempt to identify more potent, soluble and specific PDI inhibitors,. The approach allowed the identification of one active compound that forms a specific and stable complex with PDIA1 b' domain and inhibited PDIA1 reductase activity *in vitro*. The identified hit constitutes a promising candidate for lead optimization in the development of anti-inflammatory and anti-thrombotic drugs.

Material and Methods

Ligand library preparation

A subset of 10,000,000 drug-like compounds was obtained in SDF format from the ZINC database curated subset (Drugs now) in its 2012 version (http://zinc.docking.org/) (Sterling & Irwin, 2015). Compounds were filtered according to Lipinski's rule of five : $150 \leq MW \leq 500$, H_donors ≤ 5 and H_acceptors ≤ 10, xlogP ≤ 5, Rb ≤ 7 and PSA < 150 (Lipinski, 2000). Ligands were prepared by adding Gasteiger charges if necessary, ensuring that atoms are assigned the correct AutoDock atom types, merging non-polar hydrogens, identifying aromatic carbons if any, and setting up torsion angles using AutodockTools.

Target Preparation

RCSB database (http://www.rcsb.org/) was used to obtain human Protein Disulfide Isomerase (PDI) crystal structure (PDB ID: 4EKZ (Wang et al., 2013), in its reduced form. Input structure was prepared using Autodock tools (Morris et al., 2009) by adding and merging non polar Hydrogen, removing water molecules, and computing Gasteiger charges. Finally, structures were converted to PDBQT format for docking with Autodock Vina (Morris et al., 2009).

Pocket analysis

Binding sites on PDIA1 were analyzed using "Pockets" plugin for Vegazz software (Pedretti et al., 2004). Parameters were set as follows: minimum apolar neighboring = 3, radius of a-sphere ranging from 0.3 to 0.6, maximum distance between spheres = 4.5, maximum distance for clustering = 1.73, minimum number of a-sphere = 30, maximum distance for single linkage = 2.5, and 2500 iterations for volume calculation. We calculated a score for each pocket by using a scoring function of several pocket descriptors including; Density of the cavity, Number of alpha spheres, Polarity Score, Proportion of apolar alpha spheres, Mean local hydrophobic density, Maximum distance between two alpha sphere, Composition of amino acids, Hydrophobicity, Volume, Charge, and B-factor scores. Output pdb files were visualized and analyzed by Pymol (Seeliger & de Groot, 2010).

Virtual Screening

A first round of docking of library compounds was performed to target PDIA1 b' domain using Autodock Vina (Trott & Olson, 2010) with an exhaustiveness of 12. Grid boxes were centered on PDIA1 binding pocket at the b'x domains junction and box's size was set to a total of 13824 $Å^3$. Docking simulation was performed on 20 stations equipped with Intel Core i7-3770 8 CPU @ 3.40 GHz of DELL origin, with 8 GB RAM and Windows 10 operating system. All re-docking simulations for selected compounds were performed using Autodock Vina using the same parameters with an exhaustiveness of 48.

Protein Disulfide Isomerase reductase activity assay

PDI can reduce insulin interchain disulfide bonds, creating free A and B chains. For the screening of selected compounds, human PDI activity was measured in a microplate PDI inhibitor screening assay kit (Abcam ab139480, USA). The reduction of insulin, catalyzed by PDI, results in the formation of insulin aggregates that bind to the red-emitting fluorogenic PDI detection reagent, in the presence of Dithiothreitol. Fluorescence was measured at Excitation/Emission = 490/580 nm on a "Fluorostar Omega" plate reader (BMG Labtech, Germany). Bacitracin at 1 mM was used as a control for a 50% reduction of human recombinant PDI activity as outlined by the kit protocol. In the first assay, compounds selected from the virtual screening were assayed at 20 µM. In the confirmation assay, selected compounds were assayed at concentrations ranging from 0.8 to 100 µM in 5-fold dilutions for dose-response determination. All experiments were performed in triplicate. Percentage of inhibition was calculated according to the below formulae:

$$\% \ Inhibition = [1 - [F_{compound} - F_{control})/(F_{vehicle} - F_{control})]] \times 100$$

where $F_{compound}$, $F_{control}$ and $F_{vehicle}$ are Fluorescence values for wells containing tested compounds, hexachlorophene and DMSO, respectively. IC_{50} values were calculated using nonlinear fitting for dose response by GraphPad Prism software, version 9 (IBM, USA).

Results

Docking Analysis

Reduced human Protein Disulfide Isomerase (PDI) crystal structure (PDB ID: 4EKZ) was used for docking with Vina (Morris et al., 2009). A total of 30 potential binding sites on PDIA1 were identified. A pocket within the b' domain, with a total volume of 1526.7041 $Å^3$, recorded the highest score of 34.5714 (fpocket scoring function). Figure 1A shows the domain structure of PDI and 1B the selected pocket surface representing a hydrophobic cavity surrounded by polar (N298 and T428) and charged (E239 and D297) residues. This pocket was selected for the virtual screening.

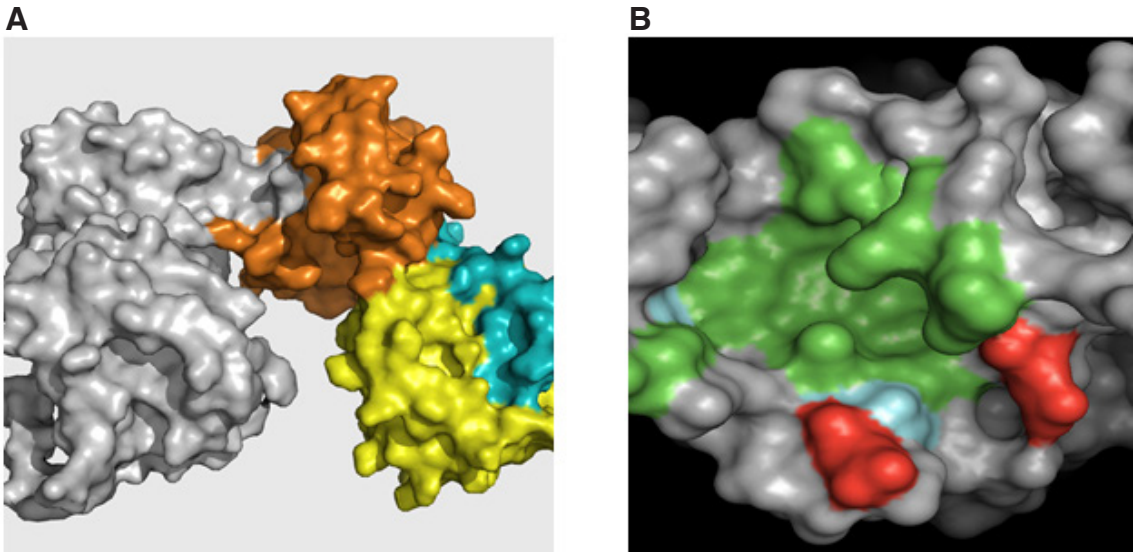
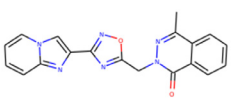
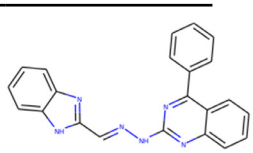


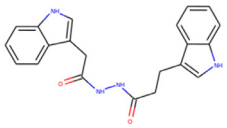
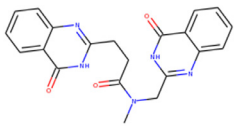
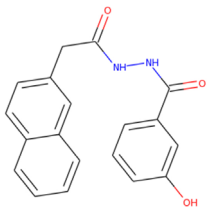
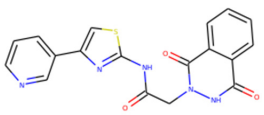
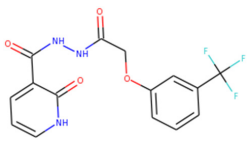
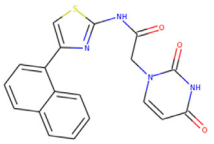
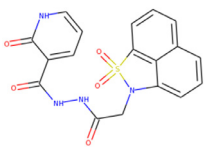
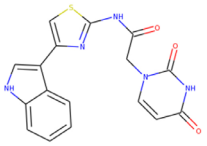
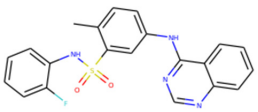
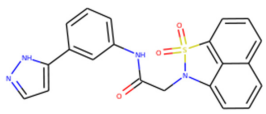
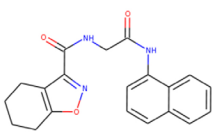
Figure 1. (A) Surface representation of PDI, b' domain is represented in orange, a' domain in yellow and x linker in blue. (B) Surface representation of the target pocket in b' domain. The pocket is organized in a hydrophobic cavity (Green) surrounded by charged residues (Red) and polar residues (Light Blue).

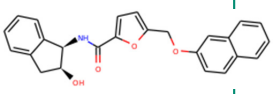
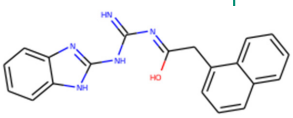
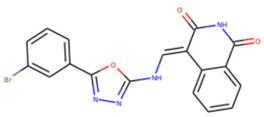
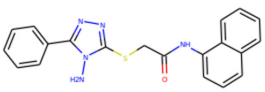
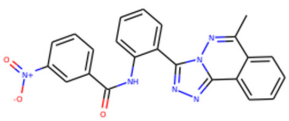
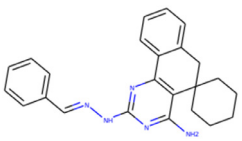
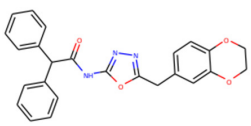
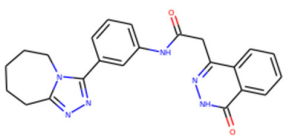
Virtual Screening

A subset of 10,000,000 Drug-like compounds from the ZINC database were subject to a first round docking to PDIA1 b' domain and used a total of 158,400 CPU-hour. The overall average docking score was -5.92 KJ/mol with a standard deviation of 0.68 KJ/mol. Compounds were ranked according to the docking scores and a total of 40 compounds were selected for the Drug-likeness filtering using DruLito (Bickerton et al., 2012). A total of 21 molecules with no major concern were identified and selected for further steps. Selected compounds were then subject to a second docking to PDIA1 b' domain. For protein-ligand interactions study, we used best conformations in order to generate protein-ligand complexes. Table 1 summarizes the structure and docking scores of selected compounds.

Table 1. Structure and Docking scores of selected compounds from the first screening round

Molecule ID	Smiles	Docking Score (Kcal/mol)	Zinc ID	Structure
1	Cc1nn(Cc2nc(-c3cn4ccccc4n3)no2)c(=O)c2ccccc12	-9.6	ZINC000095391719	
2	C(=N/Nc1nc(-c2ccccc2)c2ccccc2n1)\c1nc2ccccc2[nH]1	-9.6	ZINC000003897988	

3	O=C(CCc1c[nH]c2ccccc12)NNC(=O)Cc1c[nH]c2ccccc12	-9.3	ZINC000006787786	
4	CN(Cc1nc2ccccc2c(=O)[nH]1)C(=O)CCc1nc2ccccc2c(=O)[nH]1	-9.1	ZINC000006586991	
5	O=C(Cc1ccc2ccccc2c1)NNC(=O)c1cccc(O)c1	-9.2	ZINC000009954506	
6	O=C(Cn1[nH]c(=O)c2ccccc2c1=O)Nc1nc(-c2cccnc2)cs1	-9.1	ZINC000007138561	
7	O=C(COc1cccc(C(F)(F)F)c1)NNC(=O)c1ccc[nH]c1=O	-9.6	ZINC000012940942	
8	O=C(Cn1ccc(=O)[nH]c1=O)Nc1nc(-c2cccc3ccccc23)cs1	-9.3	ZINC000012794965	
9	O=C(CN1c2cccc3cccc(c23)S1(=O)=O)NNC(=O)c1ccc[nH]c1=O	-9	ZINC000012891277	
10	O=C(Cn1ccc(=O)[nH]c1=O)Nc1nc(-c2c[nH]c3ccccc23)cs1	-9.1	ZINC000009726218	
11	Cc1ccc(Nc2ncnc3ccccc23)cc1S(=O)(=O)Nc1ccccc1F	-9.1	ZINC000024059026	
12	O=C(CN1c2cccc3cccc(c23)S1(=O)=O)Nc1cccc(-c2ccn[nH]2)c1	-9	ZINC000024887647	
13	O=C(CNC(=O)c1noc2c1CCCC2)Nc1cccc2ccccc12	-10.1	ZINC000065626355	

14	O=C(N[C@@H]1c2ccccc2C[C@@H]1O)c1ccc(COc2ccc3ccccc3c2)o1	-9.8	ZINC000084606006	
15	N=C(/N=C(\O)Cc1cccc2ccccc12)Nc1nc2ccccc2[nH]1	-9.1	ZINC000006909626	
16	O=C1NC(=O)c2ccccc2/C1=C\Nc1nnc(-c2cccc(Br)c2)o1	-9.4	ZINC000016721439	
17	Nn1c(SCC(=O)Nc2cccc3ccccc23)nnc1-c1ccccc1	-9.2	ZINC000002415420	
18	Cc1nn2c(-c3ccccc3NC(=O)c3cccc([N+](=O)[O-])c3)nnc2c2ccccc12	-9.8	ZINC000009187177	
19	Nc1nc(N/N=C/c2ccccc2)nc2c1C1(CCCCC1)Cc1ccccc1-2	-9.1	ZINC000004733241	
20	O=C(Nc1nnc(Cc2ccc3c(c2)OCCO3)o1)C(c1ccccc1)c1ccccc1	-9.4	ZINC000009812433	
21	O=C(Cc1n[nH]c(=O)c2ccccc12)Nc1cccc(-c2nnc3n2CCCCC3)c1	-9.8	ZINC000008763069	

Protein Disulfide Isomerase reductase activity assay

PDI activity assay was conducted using fluorescence-based assay measuring the insulin reduction catalyzed by PDI reductase activity. As expected, Bacitracin at 1 mM achieved an inhibition rate of 48% ±5% compared to the negative control. The 21 selected compounds were first assayed in a single dose of 20 µM and five compounds displayed an inhibition rate of more than 40%, as summarized in Table 2. A second dose response assay was conducted on the five compounds with concentrations ranging from 0.8 to 100 µM. Four compounds displayed inconsistent data for IC_{50} determination. A single molecule, Compound 10, was confirmed to be active with an IC_{50} of 90.38 ± 7 µM. Compound structure and Dose-response curve are shown by Figure 2 A and 2 B respectively, and drug-like properties are detailed in supplementary file 1.

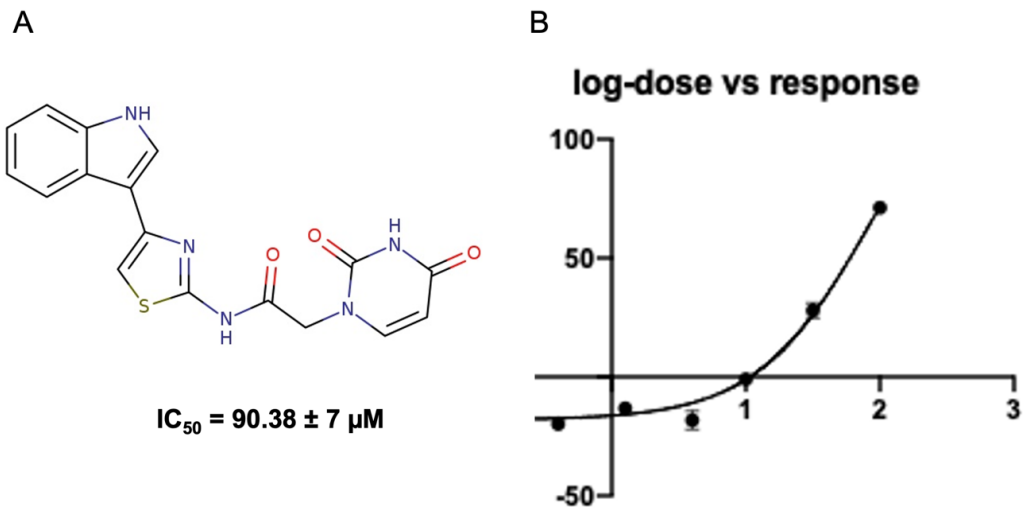


Figure 2. (A) Structural representation of 2-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-N-[4-(1H-indol-3-yl)-1,3-thiazol-2-yl]acetamide (Compound 10). (B) Logarithmic dose-response curve of compounds 10 inhibitory effect of PDI reductase activity, IC_{50} is predicted to be 90.38 µM.

Table 2. Inhibition percentage of selected compounds in first-round screening

Molecule ID	Zinc ID	Inhibition % (20 µM)	SD
1	ZINC000095391719	14.95	0.11
2	ZINC000003897988	52.98	0.06
3	ZINC000006787786	43.23	0.11
4	ZINC000006586991	6.15	0.00
5	ZINC000009954506	4.17	0.02
6	ZINC000007138561	10.36	0.10
7	ZINC000012940942	4.61	0.02
8	ZINC000012794965	0.22	0.03
9	ZINC000012891277	6.79	0.02
10	ZINC000009726218	41.36	0.03
11	ZINC000024059026	14.39	0.17
12	ZINC000024887647	42.35	0.03
13	ZINC000065626355	2.23	0.09
14	ZINC000084606006	0.00	0.01
15	ZINC000006909626	8.22	0.02
16	ZINC000016721439	48.26	0.08
17	ZINC000002415420	2.81	0.01
18	ZINC000009187177	11.10	0.03
19	ZINC000004733241	16.59	0.00

20	ZINC000009812433	4.51	0.05
21	ZINC000008763069	7.39	0.07

Ligand-protein Interaction analysis

Ligand-protein interaction analysis showed compound 10 fitting into the hydrophobic cavity in the b' domain selected pocket as shown by Figure 3A and establishing a triple polar bonds with Lys 287 and a single polar bond with Pro 234 and Leu 235 as shown by Figure 3 B.

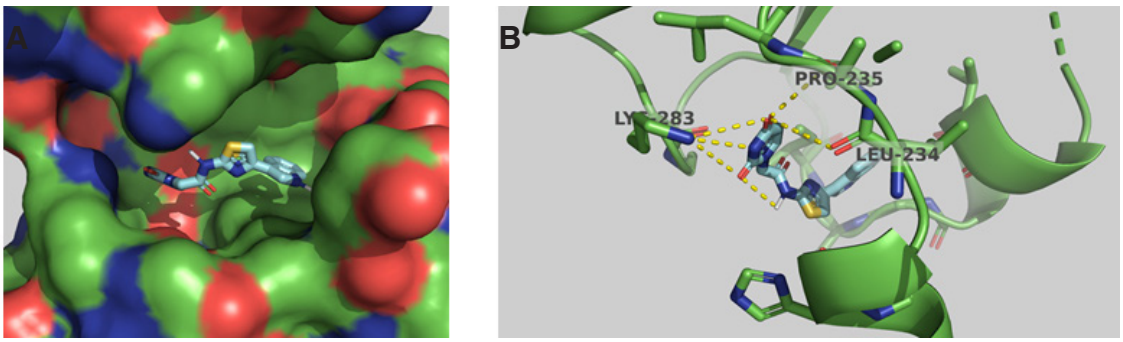


Figure 3. (A) Compound 10 predicted docking pose. (B) Ligand interaction of compound 10 with surrounding residues of the interacting pocket.

Discussion

PDI is an emerging drug target in various diseases including cancer, inflammation and infectious diseases. The role of PDI in inflammation has been investigated and characterized by several studies. PDI was shown to be required for thrombosis, hemostasis and vascular inflammation (Cho, 2013). In fact, PDIA1, ERp5, ERp72 and ERp57 were reported to be involved in thrombus initiation *in vivo* (Furie & Flaumenhaft, 2014). In the laser-induced vascular injury model, Jasuja *et. al.* (Jasuja et al., 2010) showed that PDIA1 is secreted from activated endothelial cells and bound platelets to contribute to thrombus growth. Indeed, Hahm et al. showed in a recent study that neutrophil PDIA1 is required for neutrophil adhesion and crawling in the in vivo model of tumor necrosis factor-α–induced vascular inflammation, and that αMb2 integrin-mediated adhesion and crawling of neutrophils is regulated by extracellular PDIA1 r during vascular inflammation (Hahm et al., 2013). Inhibition of PDIs using blocking antibodies inhibited leukocytes adhesion, platelet thrombus formation and fibrin generation (Cho et al., 2008; Jasuja et al., 2010; Khalaf et al., 2021).

Quercetin-3-Rutinoside; known as Rutin, constitutes a potent anti-thrombotic agent both *in vitro* and *in vivo*, was also reported as a specific inhibitor of PDI reductase activity (Jasuja et al., 2012). Wang *et al.* (Wang et al., 2013) reported that Rutin binds directly to the b' domain of PDIA1 with an equal stoichiochemistry ratio, and restricts flexibility of the protein to allow a more compact conformation. The major binding site of Rutin was shown to be within PDIA1 b' fragment and b'x fragment infusion in mouse thrombus model reversed inhibition of platelet thrombus formation by Rutin (Wang et al., 2013).

To identify novel PDIA1 specific inhibitors from the screening of chemical compounds

dataset, we used an analytical approach inspired by the structural insights observed through the study of PDIA1 inhibition by Rutin. A pocket that can be used for inhibitor binding prediction was identified in the b' domain of the protein through an analysis of pocket distribution in the full-length protein structure of reduced human PDI (PDB ID: 4EKZ). The pocket, surrounded by charged and polar residues, contains a cavity with a hydrophobic environment. We used docking simulation to screen a library of 10 millions Drug-like compounds and we identified a series of 21 Drug-like compounds that bind to the predicted cavity with a high score. We then performed an enzyme-based assay to screen the selected compounds for PDI reductase activity inhibition. One of the compounds, the 2-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl)-N-[4-(1H-indol-3-yl)-1,3-thiazol-2-yl]acetamide (Compound 10), was confirmed to be active and showed an IC_{50} of 90 µM against PDI reductase activity. According to ChEMBL 20 database, no previous activity was reported so far for this compound. Interaction analysis showed compound 10 interaction with the hydrophobic cavity of the binding site mainly through the Indole group. H-bonds were predicted between oxygen atoms of pyrimidine moiety and PDI's Leu 287 and Glu 238 residues.

Investigating yeast PDI dynamics showed PDI's Interdomain flexibility (Tian et al., 2008; Tian et al., 2006) to be essential for protein catalytic activity. This flexibility is thought to be the main reason behind PDI's resistance to crystallization. Mobility between the b' and the a' domain was more pronounced than between a and b domains among all PDIs with x-linker, (Kozlov et al., 2010). Despite the fact that a and a' domains are essential for the enzyme's catalytic activity, substrate binding domain b' plays a central role in unfolded substrate recognition. In addition, a significant divergence of b' domain was found among PDI superfamily, which might help to explain substrate specificity (Kozlov et al., 2010). Structural investigation revealed several residues that are involved in substrate recognition and binding located between helices α1 and α3 of b' hydrophobic cavity (Byrne et al., 2009). In this study, we identified a compound that is predicted to establish hydrophobic bonds with several of these residues, as well as polar contacts with charged residues around the cavity. We hypothesize our compound binds to the b' domain pocket and locks the the protein complex and hence limits protein flexibility between b' and a' domains which results in PDIA1's catalytic activity inhibition.

Conclusion

In this study, we reported a virtual screening workflow coupled to an experimental functional screening assay. We identified a novel pharmacologically active antagonist of PDIA1 reductase activity which may lead to a new class of selective PDI inhibitors for further investigation in target disease models.

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تحديد الخصم الجديد لنشاط مختزل PDIA1 باستخدام الفحص الافتراضي

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المُستَخلَص

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تشتهر عائلة بروتين (PDI) بمساعدة البروتينات حديثة الصنع على التشكل في الشبكة الإندويلازمية (ER) لمعظم أنواع الخلايا، كما لوحظ لها كذلك عدة وظائف خارج الخلية بما في ذلك تسهيل التصاق الخلايا وعملية تخثر صفائح الدم. بالإضافة إلى أهميتها البيولوجية، فإن ملاءمة PDIs للفحص عالي الإنتاجية وسهولة تصنيعها، تجعل منها أهدافا جيدة في تطوير دواء ضد العديد من الأمراض. في الواقع، يتوفر عدد قليل من مثبطات PDI والتي تظهر خصوصية وفعالية منخفضة.

في محاولة لتحديد مثبطات أكثر فعالية ومحددة لـ PDIA1، أجرينا فحصاً افتراضياً لقاعدة مركبات كيميائية كبيرة (10^7 مركب). وبعد هذا الفحص، استخدمنا فحصاً تجريبياً لتثبيط نشاط الأختزال الخاص لـ PDIA1 للتحقق من صحة المركب الرئيسي ولمزيد التحسين منه بإستخدام تقنيات المعلوماتية الحياتية وتصميم الأدوية الحسابية. حيث تبين انه يمكن استخدام الجزيء المحدد كمرشح رئيسي لتطوير الأدوية المضادة للالتهابات ومنع تجلط الدم الشرياني.

مفاتيح الكلمات: PDI، تجلط الدم، الفحص الافتراضي، الالتحام الجزيئي، الالتهاب.