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Virtual drug screening study to discover novel ERAP1 allosteric site inhibitors for the treatment of ankylosing spondylitis (AS)

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ABSTRACT: Endoplasmic reticulum aminopeptidase 1 (ERAP1) is one of the key molecules in the antigen presentation process. To date, associations of ERAP1 with Ankylosing Spondylitis (AS) have been revealed with strong data. As such, to target the allosteric site of ERAP1 exhibits a therapeutic potential in the treatment of AS. In this paper, 9,800 ligands from "FDA-Approved Drugs", "World-not-FDA Approved Drugs", and "Drugs in Clinical Trials" datasets of ZINC15 database were screened to the allosteric site of ERAP1. The best scored drugs are filtered with ADME analysis, the toxicity and bioactivity profiles of the discovered drugs and the known inhibitors were investigated. Results revealed that ZINC000100052688 (Ventavis), ZINC000004217466, and ZINC000024760115 (Dactolicib) follow the Lipinski's rule of five and have -10.0 kcal/mole, -9.8 kcal/mole, and -9.7 kcal/mole binding affinities to allosteric site of ERAP1, respectively. Furthermore, ZINC000004217466 is the most promising since it has high protease and enzyme inhibitory activity with no toxicity. Due to that to date, only few chemical ligands recognizing ERAP1 regulatory site have been synthesized, to reveal possible repurposable drugs is quite promising, and ZINC000004217466 is the best candidate among 9,800 drugs since it has rather binding affinity, proper chemical properties, no toxicity, and high bioactivity in the inhibition of ERAP1 regulatory site.

KEYWORDS: Ankylosing spondylitis; Arthritis; Autoimmunity; ERAP1; Virtual drug screening.

1. INTRODUCTION

Ankylosing spondylitis (AS) is one of the most significant arthritis subtype sourcing from strong autoimmunity reaction [1]. While the main symptom of AS is characterized as long-term inflammation in the joints of the spine, this circumstance gives rise to spinal fusion (ankylosis), bone formation (syndesmophytes), mobility loss in spine, shoulders, and hips as well as severe back pain [2]. Despite the AS prognosis is based on undesired activity of antigen presentation process, the association of collaboratively acting genes which are human leukocytes antigen B27 (HLA-B27) and Endoplasmic reticulum aminopeptidase 1 (ERAP1) with AS have been revealed for many years [3].

ERAP1 is one of the key aminopeptidases acting a significant peptide trimming role in order to activate adaptive immunity. Once to trim the peptides, antigenic fragments are loaded into major histocompatibility complex 1 (MHC1) subclasses such as HLA-B27 [4]. The loaded antigens are recognized by T-cells and immune response including inflammatory cytokine secretion is initiated. It's previously demonstrated that AS positive patients have higher enzymatic activity of ERAP1 leading to immunodominance of HLA-B27 [5]. In addition, various SNPs affecting ERAP1 activity such as M349V, K528R, D575N, R725Q, and Q730E are frequently observed in AS positive patients [6]. Considering, ERAP1 is evaluated as a potent drug target in the prognosis of AS [7].

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Despite that various drug molecules targeting ERAP1's active site through its GLU183, GLY317, MET319, GLU354, PHE433, and TYR438 residues have been designed, only few studies aiming to target its allosteric regulatory site through ILE681, LYS685, and ARG 841 residues have been published [8,9]. As such discovery of commercially available drugs that may bind to regulatory sites of ERAP1 with higher affinity then the inhibitors targeting its active site is rather promising to AS treatment.

In this study, a total of 9,800 drugs composed of FDA-Approved Drugs, World-not-FDA Approved Drugs, and Drugs in Clinical Trials datasets of ZINC15 database have been screened to the regulatory site of ERAP1 via molecular docking approach. ADME properties of the best scored drugs have been analyzed and the ones following Lipinski's rule of five have been investigated in following characterization studies. Furthermore, their toxicity, drug likeness scores, and bioactivity profiles have been revealed with various computational techniques. Results have demonstrated that ZINC000100052688 (Ventavis) used in the treatment of pulmonary arterial hypertension (PAH) from FDA-Approved Drugs [10], ZINC000004217466 with unknown activity from World-not-FDA Approved Drugs, and ZINC000024760115 (Dactolicib) which is PI3K inhibitor [11] have the highest binding affinities which are -10.0 kcal/mole, -9.8 kcal/mole, and -9.7 kcal/mole, respectively, amongst the best scored drugs following Lipinski's rule of five. In particular, toxicity and bioactivity analysis have put forward that ZINC000004217466 has highest no toxicity, high protease, and enzyme inhibitory activity. In the light of the findings ZINC000004217466 is assessed as promising to be used in AS treatment as ERAP1 regulatory site inhibitor.

2. RESULTS AND DISCUSSION

As an aminopeptidase enzyme, ERAP1 acts a peptide trimming role in the antigen presentation process and its over activity is one of the primary reasons of AS prognosis [18]. To date, a few ERAP1 active site inhibitors have been synthesized, yet they have not reached clinical trial phases [19]. In addition, the target of the allosteric regulatory site of ERAP1 is quite promising for inhibition of its activity has been highlighted in literature with several publications [9]. However, only the binding profiles of two chemical compounds which are MNZ and ERAP1-IN-1 to the ERAP1 regulatory site have been demonstrated [9,20]. In order to discover possible drug molecules that might be used in AS treatment, a drug library including totally 9,800 ligands composed of FDA-Approved Drugs, World-not-FDA Approved Drugs, and Drugs in Clinical Trials datasets of ZINC15 database was created and screened to regulatory site of ERAP1 with molecular docking approach. The data including binding affinities of best scored drugs, their datasets, Lipinski's rule of five violations and the interacting amino acids of ERAP1 are demonstrated in Table 1.

Once to reveal best scored drugs from a designed drug library study has been validated by repeating the developed strategy with known ERAP1 inhibitors. For instance, MNZ inhibitors which are found in allosteric regulatory site of retrieved ERAP1's crystal structure of the protein have been re-docked to the same region considering the essential amino acid residues which are GLU183, GLY317, MET319, GLU354, PHE433, and TYR438 [21,22]. The interactions of MNZ with ERAP1 in both crystal structure and re-docked form are demonstrated in Figure 1. In addition, another allosteric site inhibitor which is ERAP1-IN-1 [20] has also been docked to allosteric site regulatory domain of ERAP1 to reveal common amino acids that might be recognized by known inhibitors. Furthermore, since discovering novel drugs with higher binding affinities than the active site inhibitors is such a promising approach, Bestatin [21] and DG013A [22] compounds whose ERAP1 inhibitory activity is known were docked to the active site of crystal structure of the protein (PDB ID: 6T6R). The data belonging to all inhibitors are listed in Table 2. Accordingly, while GLU 183, GLY 317, MET 319, GLU 320, HIS 353, GLU 354, PHE 433, and TYR 438 amino acid residues of ERAP1 are recognized by both DG013A and Bestatin as well as ILE 681, LYS 685, and ARG 841 are recognized by both MNZ and ERAP1-IN-1. To reveal common amino acids keeps a light to further drug design research aiming to block active residues which are found within allosteric regulatory and active sites of the enzyme. The interactions observed between known inhibitors except MNZ and the protein are demonstrated in Figure 2.

Table 1. Molecular docking results of 10 best scored drugs.

10 Best Scored Drugs				
Ligand Name	**Score (kcal/mol)**	**Dataset**	**Lipinski's Rule of Five Violations**	**Receptor Residues Interacting with Ligands**
ZINC000012358610	-11.1	Drugs in Clinical Trials	3 Violations	PHE 674, ILE 681, PRO 682, LEU 733, LEU 734, VAL 737, LEU 769
ZINC000011616925	-11.0	FDA Approved Drugs	1 Violation	PHE 674, ILE 681, LYS 685, LEU 733, LEU 734, ARG 807, ARG 841
ZINC000100052688	-10.0	FDA Approved Drugs	No	PHE 674, ASN 678, ILE 681, GLN 730, LEU 733, LEU 838, ARG 841, GLN 881
ZINC000164760756	-9.9	FDA Approved Drugs	2 Violations	PHE 674, ASN 678, LYS 685, LEU 769, PHE 803, ARG 807
ZINC000004217466	-9.8	World-not-FDA Approved Drugs	No	LEU 677, ASN 678, ILE 681, GLN 730
ZINC000051951669	-9.8	Drugs in Clinical Trials	2 Violations	LEU 733, LEU 734, VAL 737, ARG 841, GLN 881
ZINC000072190152	-9.8	Drugs in Clinical Trials	2 Violations	LEU 734, GLN 881
ZINC000003780340	-9.7	Drugs in Clinical Trials	2 Violations	PHE 674, LEU 677, ILE 681, LYS 685, LEU 734, ARG 841
ZINC000024760115	-9.7	Drugs in Clinical Trials	No	PHE 674, ILE 681, LYS 685, LEU 733, VAL 737, ARG 841, GLN 881
ZINC000043133316	-9.7	Drugs in Clinical Trials	2 Violations	PHE 674, LEU 677, ASN 678, LEU 734, ARG 841, GLN 881

Moreover, as an aminopeptidase enzyme, ERAP1 may recognize the peptides to trim in the antigen presentation process. As such, previously published an oligopeptide which might be recognized has been docked to the active site of ERAP1 with protein-protein docking study in HDock server. The peptide structure with amino acid sequence KHHAFSFK was exported from another ERAP1 crystal structure (PDB ID: 6RQX) [23] and prepared with the protein preparation protocol in USCF Chimera software. Once to protein preparation, peptide structure was docked to the active site of ERAP1 in order to verify peptide's location in crystal structure used in the study (PDB ID: 6T6R) and the docking score of the peptide was computed as -177.81 and confidence score was computed as 0.6356. According to HDock computations, since that docking score is quite negative and confidence score is found within 0.5-0.7 interval, it's assessed that the peptide is recognized by ERAP1 and the protein-peptide complex has been created with quite efficiency [15]. In addition, it's revealed that peptides might interact with SER 316, ALA 318, PHE 433, TYR 438, GLN 800, PHE 803, GLN 834, and SER 869 residues of ERAP1. The interactions of the peptide with ERAP1 active site are demonstrated in Figure 3.

Once to validate the study, ADME properties of the best scored drugs were analyzed and the data was used as a filter to reveal ideal drugs with higher affinity than the inhibitors. Lipinski's rule of five points that an ideal drug should not have more than 5 H-bond donors, more than 10 H-bond acceptors, more than 500 kDA molecular weight (MW), and more than 5 octanol-water partition coefficients (CLogP) [17]. The results have shown that pulmonary arterial hypertension (PAH) drug ZINC000100052688 (Ventavis) [10] from FDA-Approved Drugs dataset, ZINC000004217466 with unknown activity from World-not-FDA Approved Drugs dataset, and PI3K inhibitor ZINC000024760115 (Dactolicib) [11] have the highest binding affinity to regulatory site of ERAP1 amongst the compounds following Lipinski's rule of five. The chemical structures of the revealed drugs and the known inhibitors are demonstrated in Figure 4. In addition, the physicochemical properties, solubility classes, and pharmacokinetics properties of them as well as the

inhibitors used in validation studies are listed in Table 3. It's concluded that while the discovered drugs exhibit better ADME profiles comparing the inhibitors, ZINC000004217466 from World-not-FDA Approved Drugs has the best ADME properties due to it is moderately soluble, has high gastrointestinal tract (GI) and blood brain barrier (BBB) absorptions as well as no CYP isoform inhibitory effects.

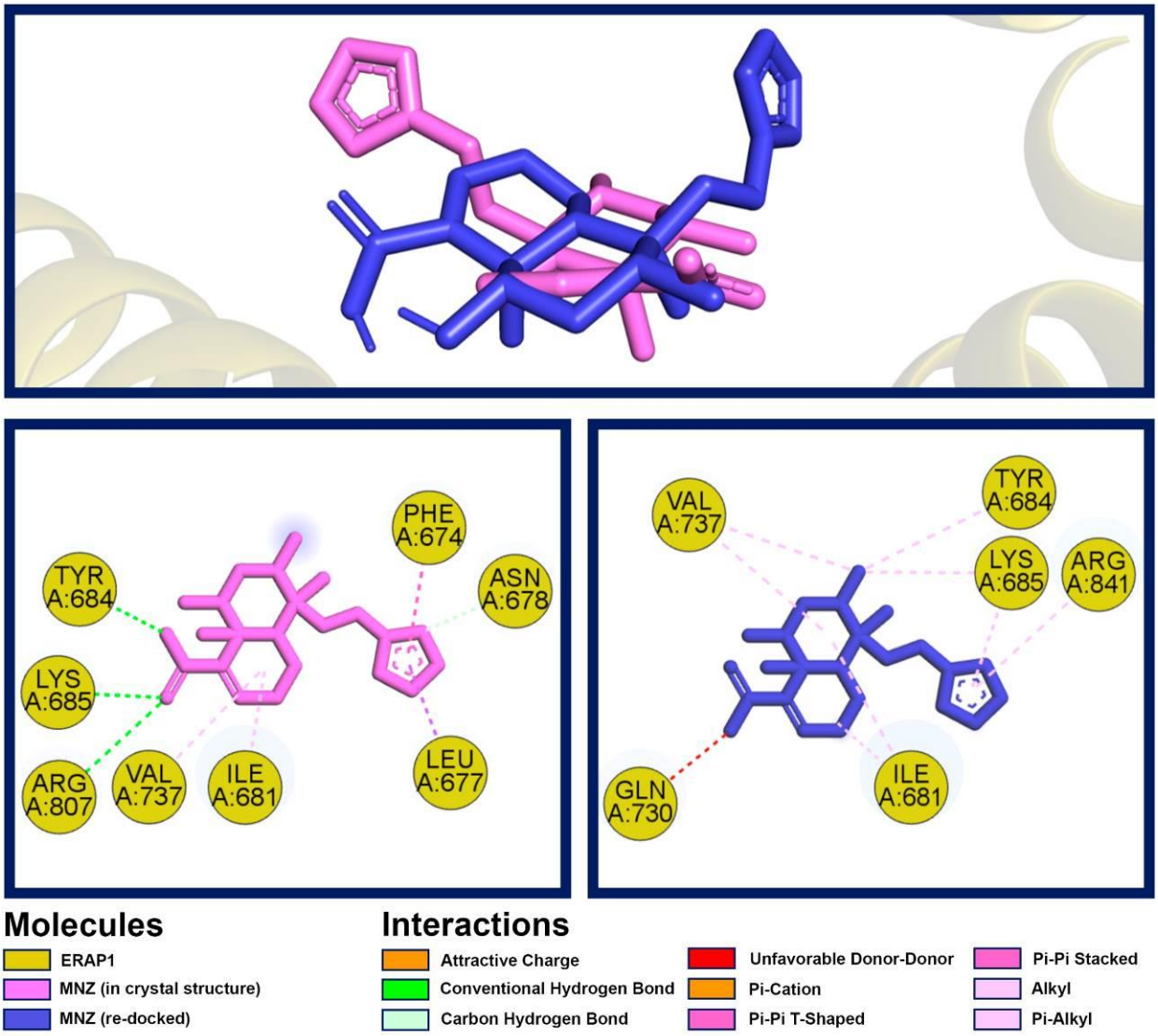

Figure 1. 2D and 3D illustration of the interactions of ERAP1 with MNZ inhibitor in both crystal structure (PDB ID: 6T6R) and re-docked form

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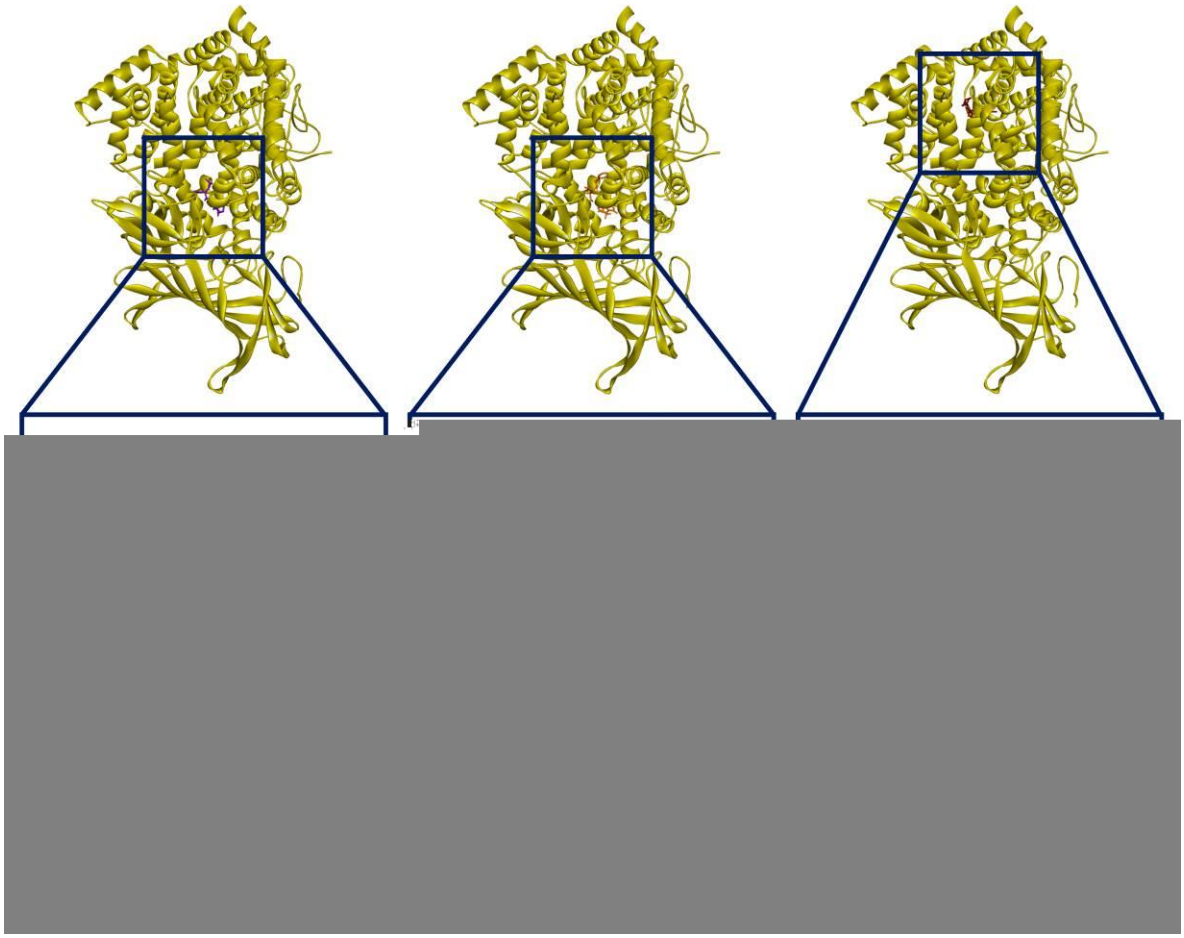

Figure 2. The interactions of ERAP1 (PDB ID: 6T6R) with Bestatin and DG013A active site inhibitors as well as ERAP1-IN-1 allosteric site inhibitors exhibiting -8.8, -8.2, -7.9 kcal/mol binding affinities, respectively.

Table 2. Molecular docking score of the commercially available inhibitors.

Inhibitors				
Ligand Name	**Binding Affinity (kcal/mol)**	**Receptor Residues Interacting with Ligand**	**Binding Region**	**Ref.**
DG013A	-8.8	GLN 181, GLU 183, PRO 184, GLY 317, MET 319, HIS 353, GLU 354, ARG 430, PHE 433, TYR 438	Active Site	[22]
Bestatin	-8.2	GLU 183, GLY 317, MET 319, GLU 320, GLU 354, GLU 376, PHE 433, TYR 438	Active Site	[21]
ERAP1-IN-1	-7.9	PHE 674, LEU 677, ILE 681, PRO 682, LYS 685, VAL 737, ARG 807, ARG 841	Regulatory Region	[20]
MNZ	-7.3	ILE 681, LYS 685, GLN 730, ARG 841	Regulatory Region	[9]

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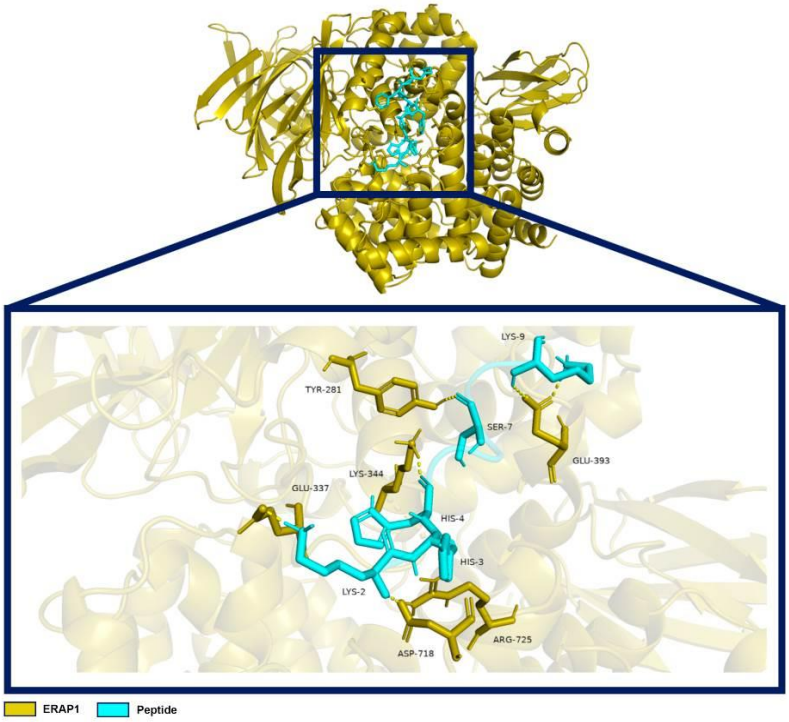

Figure 3. The interactions between ERAP1 aminopeptidase enzyme and the oligopeptide with KHHAFSFK amino acid sequence.

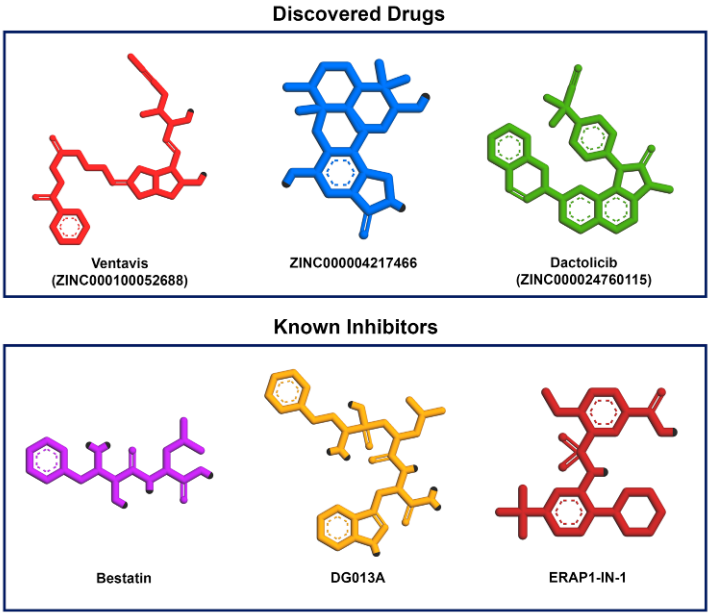

Figure 4. Chemical structures of recently discovered drugs from virtual drug screening study and commercially available inhibitors.

ZINC000100052688 (Ventavis) from FDA-Approved Drugs dataset is the drug which is used in pulmonary arterial hypertension (PAH) at clinical trial phase 3 [24]. As a synthetic prostacyclin (PGI2) analogue, the working principles of Ventavis is based on inhibition of platelet aggregate formation and to

reduce blood pressure in various conditions [25]. The results have demonstrated that Ventavis has the highest binding affinity with -10.0 kcal/mole value to regulatory site of ERAP1 by the interacting with its PHE 674, ASN 678, ILE 681, GLN 730, LEU 733, LEU 838, ARG 841, and GLN 881 amino acids via conventional hydrogen bonds, carbon hydrogen bonds, pi-sigma, alkyl, and pi-alkyl interactions. ZINC000004217466 from the World-not-FDA Approved Drugs dataset has no known effect over any diseases. Molecular docking-based study has shown that it has the second-best binding affinity with -9.8 kcal/mole value among the compounds having proper ADME properties. Since its activity had not been enlightened to date, possible ERAP1 inhibitory role of the drug is indicated with this data. In addition, it's revealed that ZINC000004217466 might interact with LEU 677, ASN 678, ILE 681, and GLN 730 residues of ERAP1 via conventional hydrogen bonds and pi-alkyl interactions. At last, it's revealed that ZINC000024760115 (Dactolicib) has -9.7 kcal/mole binding affinity to ERAP1's regulatory site and today, its activity over negative breast cancer and pancreatic neuroendocrine tumors is investigated as a PI3K/mTOR inhibitor [26,27]. Analysis have shown that this imidazoquinoline derivative is able to interact with PHE 674, ILE 681, LYS 685, LEU 733, VAL 737, ARG 841, and GLN 881 residues of ERAP1 via conventional hydrogen bonds, pi-sigma, alkyl, and pi-alkyl interactions. Accordingly, higher binding affinities of recently discovered drugs and the similarities between the interactions of them and the known inhibitors indicate the high potential of the drugs in the inhibition of ERAP1 activity through recognition of its allosteric regulatory site. 2D and 3D interaction profiles of the discovered drugs with ERAP1 are demonstrated in Figure 5.

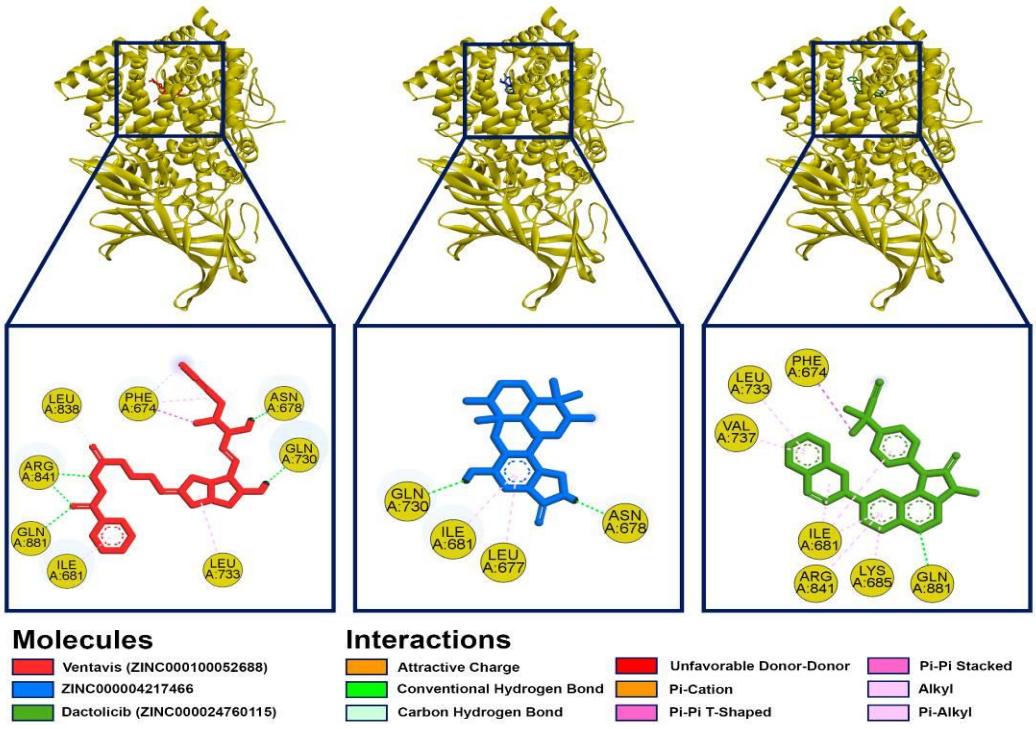

Figure 5. 3D and 2D illustrations of ERAP1 interactions with recently discovered drugs; ZINC000100052688 (Ventavis), B) ZINC000004217466, and C) ZINC000024760115 (Dactolicib), respectively.

The drug likeness parameters and possible toxicity profiles of recently discovered drugs and the known inhibitors were predicted with OSIRIS Property Explorer tool ("Molecular Properties Prediction - Osiris Property Explorer," n.d.). The results have demonstrated that ZINC000004217466 has the highest drug likeness and highest drug score after ERAP1-IN-1. Furthermore, a few toxicity profiles such as mutagenicity, tumorigenicity, and reproductive effect were observed in only Dactolicib. In addition, Molinspiration Cheminformatics v2020 online server might predict bioactivities of chemical compounds were referred to in literature ("Calculation of Molecular Properties and Bioactivity Score – Molinspiration Cheminformatics

v2020" n.d.). The computations of the server point to inactivity of the compounds having less than -0.5 value, moderate bioactivity of the compounds having the score within -0.5 – 0.0 interval, and high bioactivity of the compounds having the score within 0.0 – 0.5 interval [31]. Since ERAP1 is an aminopeptidase, only protease and enzyme inhibitory activities of the compounds were investigated. The findings have shown that ZINC000004217466 has the highest enzyme activity among all substances and the highest protease inhibitory activity among the selected drugs and the inhibitors recognizing the regulatory site of ERAP1 (Table 4).

Table 3. ADME analysis of three best scored compounds following Lipinski's rule of five and the inhibitors.

ADME Properties								
Properties		**Best Scored Drugs**			**Inhibitors**			
Physico-chemical properties	**Ligand Name**	ZINC000100052688	ZINC000004217466	ZINC000024760115	DG130A	Bestatin	ERAP1-IN-1	MNZ
	Formula	C30H38O5	C23H31NO4	C30H23N5O	C27H37N4O4P	C16H24N2O4	C20H28O4	C20H21F324O5S
	Molecular Weight (g/mol)	478.62	385.50	469.54	512.58	308.37	332.43	458.45
	Molar Refractivity	138.99	111.71	143.85	143.88	83.31	93.49	111.46
	TPSA (topological polar surface area)	83.83 Å²	78.79 Å²	76.50 Å²	161.11 Å²	112.65 Å²	70.67 Å²	104.32 Å²
	Log S (SILICOS-IT)	-5.00	-4.74	-6.44	-2.40	-0.75	-4.17	-5.87
Solubility Class	**SILICOS-IT Solubility (mg/ml)**	4.83e-03	6.94e-03	1.69e-04	2.03e-00	5.42e-01	2.27e-02	6.18e-04
	SILICOS-IT Solubility (mol/l)	1.01e-05	1.80e-05	3.60e-07	3.96e-03	1.76e-01	6.82e-05	1.35e-06
	Solubility Class	Poorly Soluble	Moderately Soluble	Poorly Soluble	Poorly soluble	Very soluble	Moderately soluble	Moderately soluble
Pharmacokinetics	**GI absorption**	High	High	High	Low	High	High	Low
	BBB permeant	No	Yes	No	No	No	Yes	No

P-gp substrate	Yes	Yes	No	Yes	No	Yes	No
CYP1A2 inhibitor	No	No	No	No	No	No	No
CYP2C19 inhibitor	No	No	Yes	No	No	No	Yes
CYP2C9 inhibitor	Yes	No	Yes	No	No	No	Yes
CYP2D6 inhibitor	Yes	No	No	Yes	No	No	Yes
CYP3A4 inhibitor	Yes	No	No	Yes	No	No	Yes

Table 4. Bioactivity and toxicity prediction of the selected drugs and commercially available inhibitors.

Bioactivity and Toxicity Analysis								
Ligand	**Protease Inhibitor**	**Enzyme Inhibitor**	**Druglikeness**	**Drug-Score**	**Mutagenicity**	**Tumorigenicity**	**Irritant Effect**	**Reproductive Effect**
ZINC000100052688	0.25	0.31	-7.78	0.14	No	No	No	No
ZINC000004217466	0.31	0.74	0.87	0.58	No	No	No	No
ZINC000024760115	-0.05	0.34	-6.38	0.05	Yes	Yes	No	Yes
DG013A	1.25	0.51	-10.96	0.34	No	No	No	No
Bestatin	1.02	0.37	-9.18	0.46	No	No	No	No
ERAP1-IN-1	0.29	0.70	0.31	0.61	No	No	No	No
MNZ	-0.08	-0.12	-5.28	0.3	No	No	No	No

3. CONCLUSION

AS is one of the most significant arthritis types characterized by long term inflammation, ankylosis, syndesmophytes, mobility loss and severe pain in the spine. One of the primary drug targets in the AS treatment is ERAP1 having peptide trimming role in antigen presentation to activate immune response. Besides that, there are a few compounds which are not at clinical trial phases having binding affinity to the active site, various studies have shown that targeting the regulatory site might inhibit over activity of ERAP1. As such, the drugs from FDA-Approved Drugs, World-not-FDA Approved Drugs, and Drugs in Clinical Trials datasets of ZINC15 database were screened to ERAP1 regulatory site through molecular

docking. The efficiency of the study was validated with re-docking of MNZ and docking of commercially available inhibitors. Once to validate the study, best scored drugs were filtered with ADME properties, and results have shown that ZINC000100052688 (Ventavis), ZINC000004217466, and ZINC000024760115 (Dactolicib) have the highest binding affinities among the compounds following Lipinski's rule of five. Furthermore, protease and enzyme inhibitory activities, and toxicity profiles of the selected drugs and the inhibitors have shown that ZINC000004217466 has the highest enzyme and protease inhibitory activity and no toxicity. In light of this information, ZINC000004217466 whose activity is unknown is assessed to be used in AS treatment as ERAP1 regulatory site inhibitor. However, the findings should be verified with further molecular dynamics (MD) simulations, *in vitro* and *in vivo* studies.

4. METHODS

4.1. Virtual Drug Screening

The crystal structure of ERAP1 with (4aR,5S,6R,8S,8aR)-5-(2-(Furan-3-yl)ethyl)-8-hydroxy-5,6,8a-trimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylic acid (MNZ) inhibitor was retrieved from Protein Data Bank (PDB: 6T6R), and prepared with UCSF Chimera 1.16 software's Dock Prep package [12]. Drug library including 9,800 drugs was created with retrieving FDA-Approved Drugs (1,615 drugs), World-not-FDA Approved Drugs (4,288 drugs), and Drugs in Clinical Trials (3,897 drugs) datasets from ZINC15 database. Both prepared protein and drug library were imported to PyRx Virtual Screening tool, and drugs were minimized with energy minimization module [13]. The minimized drugs were docked to allosteric regulatory site of ERAP1 with AutoDock Vina [14] package of the software by targeting MNZ binding region considering the PHE 674, LEU 677, ASN 678, ILE 681, TYR 684, LYS 685, VAL 737, and ARG 807 residues with the grid box coordinates x= 25.075, y= 50.072, and z= 15.065 and 15x15x15 dimensions. The results were exported as .csv files of each dataset and .pdb files of 10 best scored drugs' modes with 0 rmsd/ub and 0 rmsd/lb were exported. Biovia Discovery Studio was used to analyze the interactions between the drugs and ERAP1 crystal structure.

4.2. Validation

The efficiency of the molecular docking approach was validated by MNZ (PubChem CID: 51693778) and ERAP1-IN-1 inhibitor (PubChem CID: 4798291) whose binding profile to regulatory site of ERAP1 had been demonstrated, previously, by following the same procedure. Furthermore, in order to reveal binding affinity of ERAP1 active site inhibitors, commercially available Bestatin (PubChem CID: 72172) and DG013A (PubChem CID: 72193911) drugs were docked to active site in order to recognize GLU183, GLY317, MET319, GLU354, PHE433, and TYR438 residues with the grid box coordinates x= 28.109, y= 28.351, and z= 12.358 and 20x20x20 dimensions. In addition, in order to discover possible PPI molecules and interacting amino acid residues of ERAP1's active site has been revealed with protein-protein docking study. As such, the peptide with KHHAFSFK amino acid sequence was exported from another ERAP1 crystal structure (PDB ID: 6RQX). The peptide was prepared with the previous protein preparation protocol and docked to the active site of 6T6R structure's including GLU183, GLY317, MET319, GLU354, PHE433, and TYR438 residues with HDock online server [15]. The interactions within the protein interface were analyzed with PyMol software.

4.3. ADME Properties

Absorption, distribution, metabolism, and excretion (ADME) properties of the best scored 10 drugs as well as the inhibitors used in validation study were investigated with swissADME server [16]. According to Lipinski's rule of five, an ideal drug should not have more than 5 H-bond donors, more than 10 H-bond acceptors, more than 500 kDA molecular weight (MW), and more than 5 octanol-water partition coefficients (CLogP) [17]. Once various physicochemical and pharmacokinetics properties had been analyzed, the three ligands following Lipinski's rule of five with highest binding scores and the commercially available inhibitors were characterized in further studies.

4.4. Toxicity and Bioactivity Analysis

Various possible toxicity properties such as tumorigenicity, mutagenicity, irritant and reproductive effects as well as drug likeness property and drug scores of three best drugs following Lipinski's rule of five and commercially available inhibitors were investigated with OSIRIS Property Explorer tool ("Molecular Properties Prediction - Osiris Property Explorer," n.d.). In addition, bioactivity profiles of the same chemical compounds were predicted with Molinspiration Cheminformatics v2020 server ("Calculation of Molecular Properties and Bioactivity Score – Molinspiration Cheminformatics v2020" n.d.). Due to the fact that ERAP1 is an aminopeptidase enzyme, only protease and enzyme inhibitory activities of the compounds were investigated.

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Conflict of interest statement: There is no conflict of interest with any individual and data.

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