UiT The Arctic University of Norway

Faculty of Health Science, Department of Medical Biology; Molecular Pharmacology and Toxicology Research Group

# Inhibition of bacterial and human zinc-metalloproteases

A putative strategy in the treatment of bacterial infections

Bibek Chaulagain

Master's thesis in Biomedicine [MBI-3911] November 2021

# Table of Contents

**Acknowledgements**

The present research work was performed as a part of the Master's degree in Biomedicine (MBI-3911) at the Department of Medical Biology, Faculty of Health Sciences, UiT The Arctic University of Norway, under the supervision of the Research group of Molecular Pharmacology and Toxicology. I am very grateful to my two supervisors during this thesis, Professor Ingebrigt Sylte and Professor Emeritus Jan-Olof Winberg, for the help in the laboratory work, and also in writing the thesis. They introduced me in the world of molecular modelling and enzyme kinetics as a young master student and helped me to explore my knowledge within this field. Similarly, I would also like to thank Fatema Rahman and Imin Wushur as they helped me a lot in the laboratory and molecular modelling. Likewise, I would also like to thank all the members of the Research group of Molecular Pharmacology and Toxicology for their support for providing me a good atmosphere to work and support in my work by sharing your ideas in this field.

Finally, I would like to thank all my family members and parents for their love and support throughout the period.

Thank you.

# Abstract

Antibiotic resistance is one of the major challenges in the present era and it is drastically increasing with the increase in time because of the overuse and misuse of antibiotics. Therefore, there is a demand of new antibiotics with new modes of action or other innovative strategies to overcome bacterial infections as soon as possible. The zinc metalloproteases Themolysin (TLN), Pseudolysin (PLN) and Aureolysin (ALN) are important bacterial virulence factors and the inhibition of these bacterial virulence factors is believed to be a new treatment option of bacterial infections. However, in order to have a therapeutic value, inhibitors of these enzymes should not interfere strongly with the activity of human zinc metalloproteases.

In the present thesis, 26 compounds were tested for the inhibition of TLN, PLN, ALN and the human matrix metalloproteases-14(MMP-14). The compounds were selected from a previous virtual screening project at the research group. The inhibition of the compounds was tested by measuring the enzyme activity of PLN, TLN ALN and MMP-14 after exposure of the test compounds. The time resolved fluorescence by the use of fluorogenic substrates was used to measure the enzyme activity. The results showed that some of the compounds inhibited the enzyme activity by 30%-40% and they were not considered as slow binders as there was no significant change in activity with respect to the time. Compounds with highest rate of inhibition in enzyme assays were selected and proceed for molecular modeling studies by docking and MMGBSA calculations. The best compounds were compared with a known strong inhibitor of the zinc- metalloproteases in the molecular modeling part.

## ABBREVIATIONS

| | |
|---|---|
| [E] | Concentration of enzyme |
| [I] | Concentration of inhibitor |
| [S] | Concentration of substrate |
| µl | Microliter |
| µM | Micromolar |
| 2D | Two- dimensional |
| 3D | Three-dimensional |
| ALN | Aureolysin |
| ChEMBL | A manually curated database of bioactive molecules having drug like features |
| DMSO | Dimethyl sulfoxide |
| $IC_{50}$ | The concentration of a compound that results in 50% inhibition of an enzymes reaction velocity against a substrate |
| $K_m$ | Michaelis-Menten constant: the concentration of substrate which gives an enzymaticreaction rate equal to the half of the maximum rate of enzymatic reaction ($V_m/2$) |
| kDa | Kilo-Dalton |
| MEROPS | Database on proteolytic enzymes |
| MMP | Matrix Metalloproteases |
| PLN | Pseudolysin |

| | |
|---|---|
| TLN | Thermolysin |
| $v_0$ | Velocity of an enzymatic reaction without the inhibitor |
| $v_i$ | Velocity of an enzymatic reaction containing the inhibitor |
| $V_m$ | Maximum rate of enzymatic reaction |
| VLS | Virtual ligand screening |
| WHO | World health Organization |

# 1 Introduction

## 1.1 History of antibiotics

From the discovery of penicillin to the present modern era, antibiotics can be taken as one of the boons in the field of medicine. As antibiotics have played a vital role in control and eradication of the infectious diseases, which in turn is very important for the existence of the humankind (1). Before the invention and production of antibiotics, the mortality rate was very high due to lack of effective treatment (medications) and control of diseases. Then soon after the invention of the microscope in the 17$^{th}$ century and other technologies, scientists became interested in the causes, routes, infection steps and control of diseases caused by the microbes, which accelerated the invention of the new medications leading to the discovery of the antibiotics (2).

11Antibiotics are the biomolecules produced by microorganisms or synthesized in laboratory which inhibits the growth of or has capacity to kill the micro-organisms (3). Besides the use in human and veterinary medicine, antibiotics are used in different nonmedical fields like in fisheries, aquaculture, food preservatives, paint industry, alcohol industry and lots of other domestic uses like in field of agriculture (3). Due to the extensive use of antibiotics in different fields along with the medical field, different bacteria have developed a resistance against the treatment which is termed as antibiotic resistance. When any bacteria is exposed to antibiotic the resistance is developed by bacteria due to new spontaneous mutation(s) during the antibiotic treatment and the mutation(s) are transferred to other bacteria in the form of resistance gene with the help of genetic elements like plasmids (3, 4).

Although antibiotics are considered to be one of the most significant invention in the field of modern medicine, research have shown that some antibiotics like tetracycline were present in skeletal samples of Egyptian people from the late roman period (5) and in samples from human skeleton from Sudanese Nubia from 650-550 CE (6). Artemisinin was discovered as a drug to treat malaria infection in the 1970s, and has been used in treatment of many illness by Chinese herbalists for thousands of years (7). This showed that antibiotics and anti-parasitic drugs have been used since ancient times, only the form was different (8).

## 1.2 Antibiotic resistance

The main reason of antibiotic resistance can be considered a result of irrational use and the increasing use in medicine. The antibiotic resistance is leading to global threats as many of the bacteria are becoming resistant to the antibiotics so that it is difficult to treat the infection with the same antibiotics as before or with the same dose as before (9). Antibiotic resistance is found to be more common in the countries where there is high use of antibiotics (8). At the European continent, the use of antibiotics was found to be higher in southern parts than in the northern parts. In the years from 2012-2016 the use of antibiotics was found to be in a declining phase in Finland, Norway and Sweden, while an increasing trend was observed in Greece and Spain (9). The same trend of decreased use of antibiotics continued in Norway, Sweden and Denmark during 2016-2019 and the increasing trend continued in the Greece and Spain (10).

The World Health Organization (WHO) with a collaboration with the division of infectious diseases at the university of Tubingen, Germany divided the pathogens according to the necessity of new antibiotics as with critical, high or medium priority. The pathogens were divided into these three categories based on their deadliness, resistance to known antibiotics, transmission frequency, medium of transmission and their treatment options remaining till date. The critical group included bacteria with multiple drug resistance (resistance produced by any pathogen for more than one drug) that are in high threat in the hospitals leading to the use of critical care devices like ventilators. These bacteria include *Acinebacter, pseudomonas aeruginosa (P.aeruginosa),* and *Enterobacteriaceae* respectively. *P. aeruginosa* was classified as the second most critical bacteria for which new antibiotics are urgently needed. It is a demand of quickly obtaining new antibiotics with new modes of action or other innovative strategies to overcome these bacterial infections. The second and third category consisted of bacteria like *salmonella, streptococcus* like bacteria which are less dangerous and are single drug resistant (10).

## 1.3 Pseudomonas aeruginosa

*P. aeruginosa* is a gram-negative opportunistic bacterium first reported in humans in 1862 and is responsible for many kinds of diseases, including 10-15% of hospital acquired infections

(11). In addition to this, *P. aeruginosa* is also responsible for chronic lung infection in cystic fibrosis patients, bacteremia in severe burn cases and acute ulcerative keratitis (which is a major cause of mortality). And almost all kinds of *P. aeruginosa* infections are found to be associated with an immune-comprised host defense (12), like urinary tract infection and other infections (eyes, skin, soft tissues) (13). Present antibiotics are occasionally successful against *P. aeruginosa* infections because *P. aeruginosa* possesses both intrinsic and acquirable resistance mechanisms (14). Another reason for putative complications is that the bacteria forms biofilm which blocks the access of antibiotics to the bacteria and antibiotics gets trapped inside the biofilm (15). *P. aeruginosa* can survive in highly diversified conditions as it consists of highly variable metabolic versatility from soil to a living host. When a mixture of unfavored carbon is provided to *P. aeruginosa,* the carbon metabolism is regulated by catabolite repression control mechanism. This mechanism accelerates the catabolism of substrates like short chain fatty acids, amino acids and polyamines (16, 17). This helps *P. aeruginosa* to survive in anaerobic respiration with minimum utilization of energy (12). Other striking features of *P. aeruginosa* is its ability to grow up to 42°C temperature (12, 13) and survival in poor nutrition and hostile condition (16).

The genome of *P. aeruginosa* consists of a two compartment system (core genome and accessory genome) which encodes the outer membrane proteins which are involved in adhesion, motility, export of virulence factor and sensing to the environment. The genome has 5570 open reading frames (ORFs) and a genome size of 6.3Mbp (14). The core genome is highly conserved and covers about 90% of the total genome (18). The accessory genome are found to make a cluster in a certain loci and are named as “regions of genomic plasticity” which help in niche based adaptation (19).

### 1.4 Virulence factors as targets for development of new antibacterial drugs

In bacteria, proteases play a vital role in nutrition, growth and invasion into host that leads to the death of millions of people in the world (20, 21). In addition, several proteases are known to be bacterial virulence factors. Bacterial virulence factors are molecules synthesized by the bacteria which increases the capacity of bacteria to infect or damage a host tissue. They are known to facilitate bacterial colonization by inducing damage to a host tissue and actively

weakening the host immune response. Inhibition of bacterial virulence factor instead of targeting the bacterial growth is considered as a new alternative strategy in the development of antibiotics (22-24). Such compounds are found to have little effect on evolutional pressure for the development of antibiotic resistance, and little impact in normal flora of the human body. They may be used alone, or as an adjuvant to existing treatment. Many proteases are found to be bacterial virulence factors and are interesting antibacterial drug targets. However, there are no compounds which so far have been approved as antibiotics that target bacterial virulence (24). Virulence factor is a character of any microorganism which help them to replicate and disseminate within the host by overcoming the host defense mechanism (25) like urease in *cryptococcus neoformans.*

However, in order to have therapeutic potential the inhibitors should only inhibit the bacterial proteases and not interfere strongly with the function of human proteases. It has been estimated that there are more than 60000 different proteases (26).

### 1.5 Proteases

Proteases or peptidases are enzymes that have the capacity to catalyze the reaction and break down proteins into amino acids or small polypeptides and form new protein products. Proteases also help in the regulation of protein function (27). The process of protein break down is done by cleaving peptide bonds of proteins by hydrolysis (28). Proteases are broadly classified into two classes; exo-peptidases and endo-peptidases on basis of their hydrolysis of the proteins. Exopeptidases are proteases which cleave peptides at N-terminus or C-terminus releasing single amino acids, while endopeptidases cleave within the peptide chain. Figure 1 is a schematic illustration of a protein/peptide binding to the active site of an endo-peptidase. The MEROPS database classify the proteases (on the basis of the active site residues taking part in a catalytic reaction ) into eight classes including; aspartic (A), cysteine (C), glutamic (G), metallo (M), serine (S), threonine (T), aspargine (N) and mixed (M) (29). In humans, the proteases are involved in different physiological process of our body like in glaucoma (metalloproteases are expressed) (30), cell signaling, hemostasis, blood coagulation, wound repair (31), reproduction and angiogenesis (32). Any kind of dysregulation of the proteases may lead to different

diseases (33). There are approximately 570 proteases in humans, out of which 190 are metalloproteases (34).

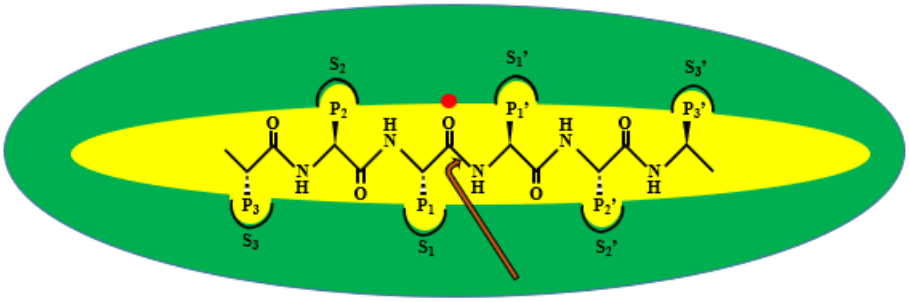



**Figure 1**: **Schematic illustration of the binding of a peptide substrate to the active site cleft in a protease.** The active site cleft with its sub-sites (yellow) is designated as S ($S_1$, $S_2$ etc) and the side-chains in the peptide substrate that interact with these sub-sites are designated P ($P_1$, $P_2$ etc). The unprimed sites are to the N-terminal and the primed sites are to the C-terminal side of the scissile bond (red arrow). Red dot represents the catalytic residue (zinc in most metalloproteases).

### 1.5.1 Matrix metalloproteases (MMPs)

Matrix Metalloproteases (MMPs) is a sub-family of calcium dependent metalloproteases containing a catalytic zinc ion and a structural zinc ion. The subfamily consists of both secreted and membrane associated proteases and belong to the M10 family of proteases (21). MMPs are built up of different domains, modules and motifs (Figure 2). The main characteristics of this family is that it consists of the HEXXHXXGXXH zinc binding motif and has a met-turn formed by a conserved methionine C-terminal to the zinc binding motif (35). The fourth ligand in the inactive pro-form is the cysteine of the PRCGXPD motif of the pre-domain, while a water molecule is the fourth ligand in the activated form. In humans, 23 different MMPs are found (22, 36). MMPs are endopeptidases which play vital roles in the degradation of the extracellular matrix and basement membrane. The activity of MMPs is found to be increased in different human cancer forms.

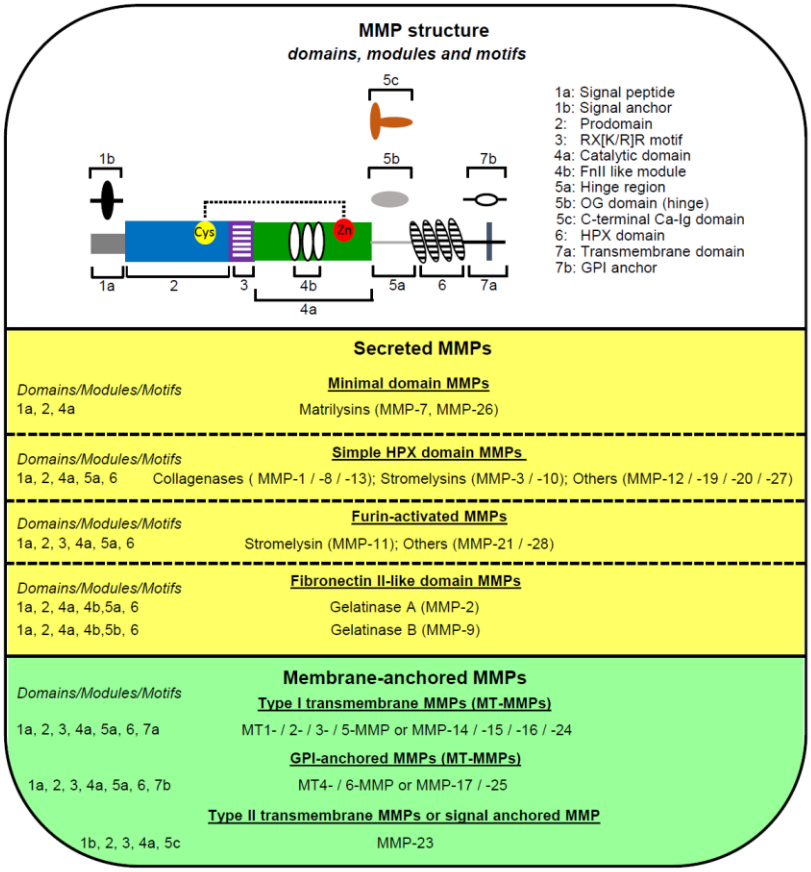



**Fig. 2**: Structure of secreted and membrane-anchored MMPs (The figure is adopted from Extracellular human and bacterial metalloproteases – challenges in design of specific metalloprotease inhibitors. Fatema Raman, Imin Wushur, Ingebrigt Sylte and Jan-Olof Winberg, Tidsskrift for Norsk-biokjemisk selskap, in press.)

### 1.5.1.1 Matrix metalloprotease-14 (MMP-14)

MMP-14 is a membrane bound collagenase which is an important protein in cancer invasion and metastasis, and collagenolysis is vitally dependent on MMP-14 (37). MMP-14 targets a wide range of extracellular matrix (ECM) proteins. Re-modeling of ECM proteins plays a significant role in skeletal muscle injury and cellular regeneration. The regulation of cellular regeneration and skeletal muscle injury is activated by MMP-14. MMP-14 helps in the activation of cytokines, chemokines and growth factors (38). MMP-9 is a secretory type metalloprotease (gelatinase B), whereas MMP-14 is a membrane type-1 metalloprotease (MT1-MMP) consisting of a transmembrane domain having the catalytic site located outside the cell, i.e. into the extracellular environment (21). All MMPs are comprised of different structural

domains including an N-terminal pro-domain followed by a catalytic domain, hinge region and C-terminal hemopexin domain (Figure 2). In MMP-14, the C-terminal hemopexin domain is followed by a transmembrane domain and the catalytic domain in MMP-9 consists of a unique module of three fibronectin-II like repeats (33). The structure of MMP-14 in complex with the tissue inhibitor 2 of matrix metalloproteases (TIMP-2) is shown in Figure 3. MMP-9 is activated by different naturally occurring proteases such as trypsin, MMP-2, MMP-3 or by organomercurial compounds like $HgCl_2$. MMP-9 can also be activated by different bacterial metalloproteases like PLN and TLN. Likewise, MMP-14, the other membrane type MMPs (MT-MMPs) as well as some of the secreted MMPs are activated in the cell by the serine protease furin, which cleaves a basic RX[K/R]R motif located in the C-terminal part of the pro-domain in these MMPs (see figure 2) (33).

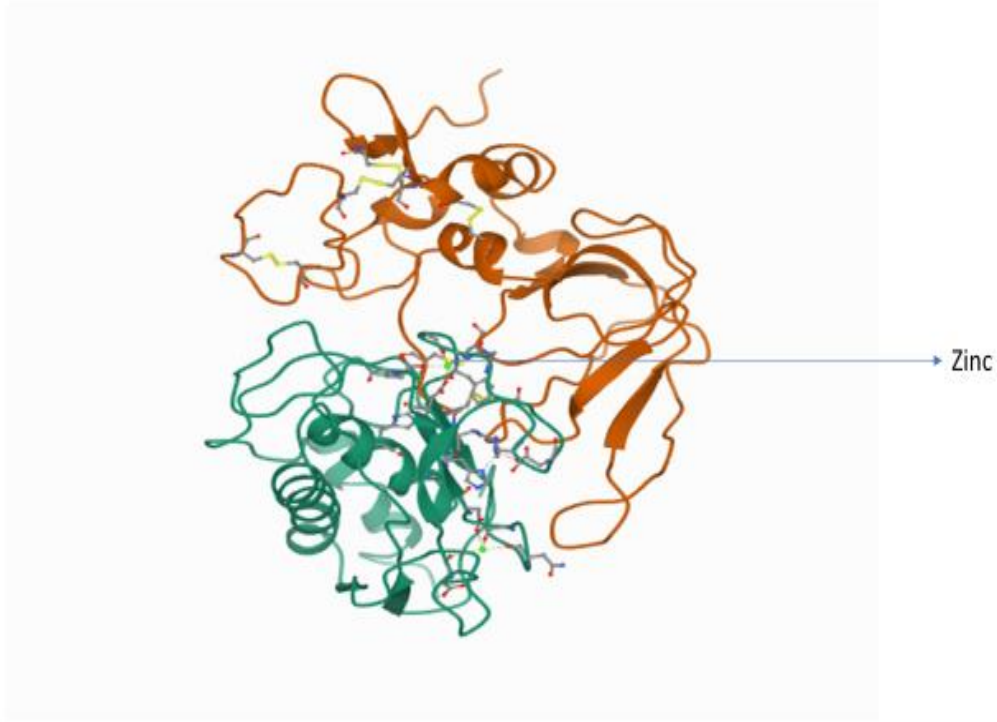



**Figure 3**: 3D structure of the catalytic domain of MMP-14, in which MMP-14 is bound to the Tissue Inhibitor of Metalloprotease-2 (TIMP-2) (PDB ID: 1BQQ). TIMP-2 (orange) is bound to the catalytic zinc (indicated by blue arrow) in MMP-14 (represented in green color).

### 1.5.2 Bacterial metalloproteases

Bacterial proteases are found in all eight classes of proteases. Most of the metalloproteases are zinc metalloproteases (39). According to the presence of sequences around the HEXXH motif bacterial zinc metalloproteases are classified into five distinct families; thermolysin, astacin, serratia, matrixin and reprolysin metalloproteases (40). The zinc metalloprotease family contains a large family called the thermolysin family or the M4 family, which falls under the MA clan of metalloproteases. The M4 family contains bacterial enzymes including PLN (LasB) from *P. aeruginosa*, TLN from *Bacillus thermoproteolyticus* and ALN from *staphylococcus aureus* and all belong the subclan MA(E) (41, 42). TLN is used by the industry in the synthesis of aspartame (artificial sweetener) (43, 44).

#### 1.5.2.1 Pseudolysin

PLN (LasB) (Figure 4) is the most important secreted bacterial virulence factor of *P. aeruginosa* (45) and has a capacity to digest denaturated proteins like fibrin, casein and hemoglobin. In addition to this, in *P. aeruginosa* infection it can decrease proteins of significant biological roles like elastin, laminin, cytokines, fibrinogen, coagulation factor XII. PLN is secreted by type II secretion pathway which is signal sequence dependent and involves a two-step secretion from the inner to the outer membrane. Secretion of PLN can be minimized by increasing the concentration of glucose while iron, ammonium sulfate and some antibiotics also have ability to decrease its production (46).

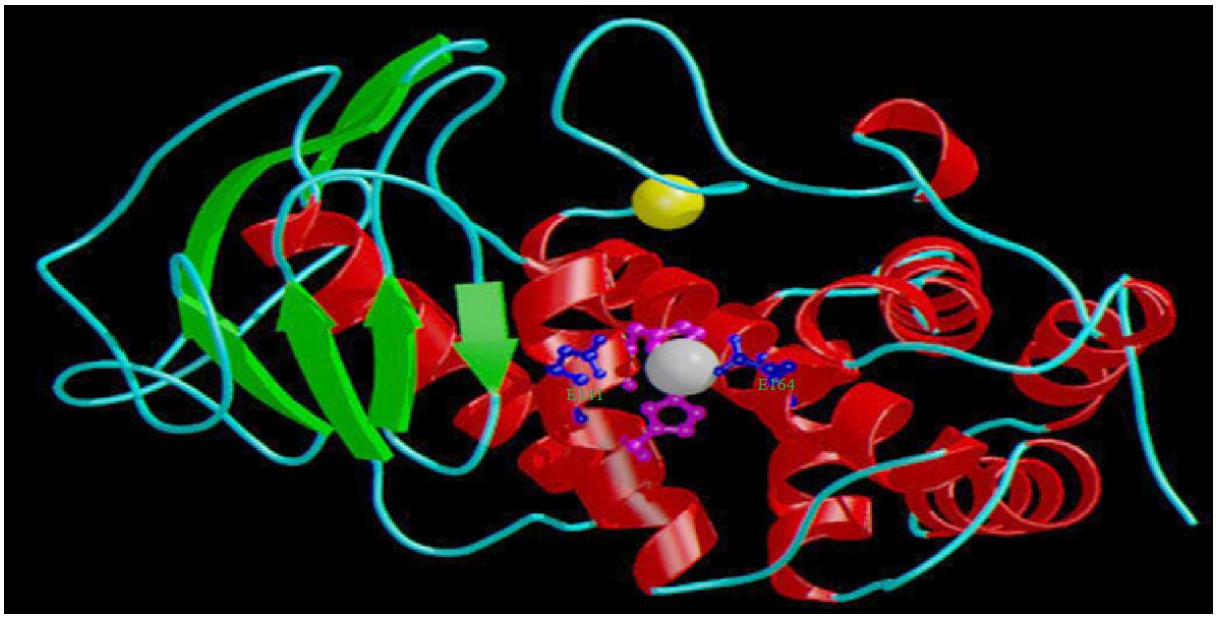



**Figure 4**: 3D Structure of PLN (resolution 1.5Å) of *P. aeruginosa* obtained by X-ray diffraction studies (figure adopted from the MEROPS database). The catalytic zinc is light grey in color and calcium in yellow color in CPK sphere. The ligands of zinc are shown in ball and stick representation. His-140 and His-144 are shown as purple color and Glu-164 is shown in blue. The catalytic Glu-141 is shown in blue.

### 1.5.2.2 Thermolysin

The structure of TLN is shown in Figure 5. TLN is also a member of M4 family of metalloproteases and can be seen in bacteria and fungus which is being biologically active at a pH of 7. TLN binds the catalytic zinc by a tetrahedrally co-ordination of by three amino acids (two histidines and a glutamic acid) and a water molecule(47). TLN cleaves at the N-terminal side of bulky amino acids like Leu, Phe and Val and other amino acids like Met, His, Tyr, Ser as these amino acids interacts at sub-site ($S_1$') in the enzyme (see figure 1) (48). PLN and griselysin also favor binding of hydrophobic residues to their $S_1$' sub-sites, although they have a larger preference for aromatic residues than TLN (49, 50).

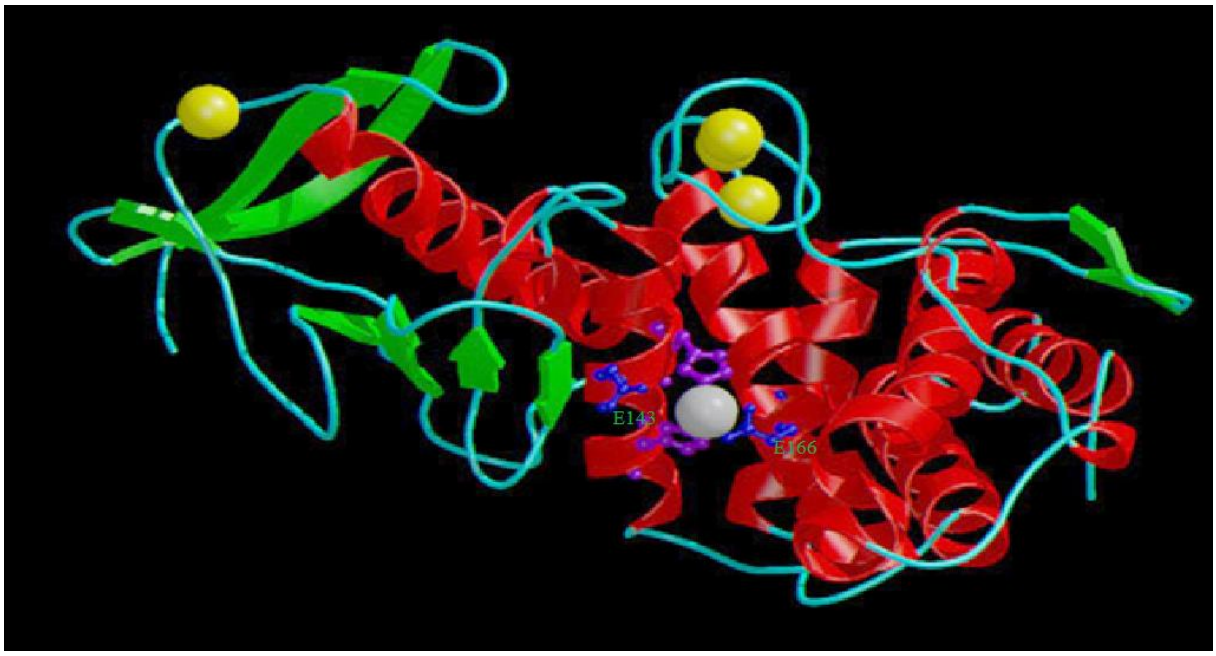



**Figure 5**: 3D Structure of Thermolysin from MEROPS database obtained by X-ray diffraction method. The catalytic zinc is represented as light grey in color and structural calcium in yellow in color. The ligands of zinc (His-142, His-146 and Glu-166) and catalytic Glu-143 are shown in ball and stick representation. The histidines in purple and the glutamic acids in blue color, respectively.

### 1.5.2.3 Aureolysin

Aureolysin (ALN) is a single chain protein having a molecular weight of 28 kDa. The structure of ALN is shown in Figure 5. For the catalytic activity, zinc is required, but it can also be replaced with cobalt (giving a protease which is more active than native enzyme). The richest source of aureolysin is strain V8 which is found in staphylococcus aureus (involved in cleavage of peptide bonds from COOH side) (51).

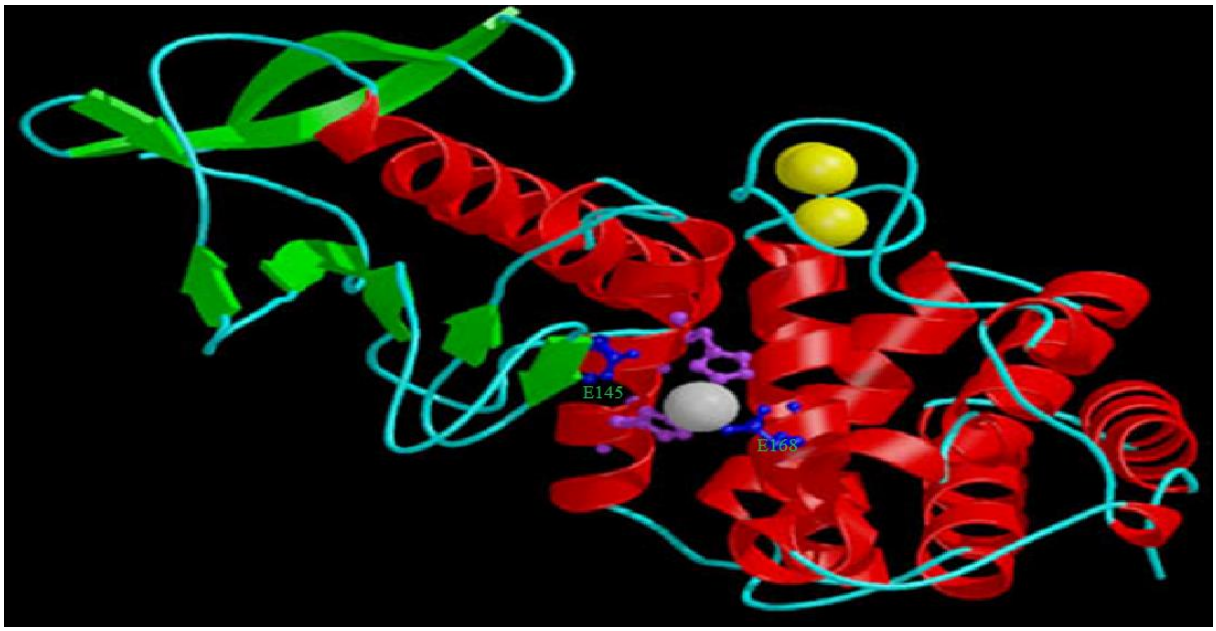



**Figure 6**: 3D structure of Aureolysin from MEROPS database obtained by X-ray diffraction method. The catalytic zinc is represented as light grey in color and structural calcium as yellow in color. The ligands of zinc (His-144, His-148 and Glu-168) and the catalytic glutamic acid (Glu-145) are shown in ball and stick representation. The histidines in purple and the glutamic acids in blue color, respectively.

### 1.5.3 Similarities between the bacterial proteases

There are a lot of similarities and differences between these three bacterial proteases if we look at the 3D-structures between them. One of the major structural difference is that PLN consists of a structural calcium and TLN have three of them. Likewise, another difference is that PLN has more space for binding to a substrate as compared to TLN. Even though, PLN is not more recognized as TLN, it has some difference in the size of $S_1$' pocket leading to the difference in substrate specificity and has larger substrate binding cleft then in TLN. (41, 52, 53). PLN has a bigger sized binding pocket then TLN (54). ALN also have similar structure as compared to the TLN but it shows a closed active site cleft confirmed when viewed without any inhibitor (55).

## 1.6 Molecular modeling

Molecular modeling is a collection of computational techniques used to study the molecular structure and behavior of biological molecules. The methods are used to stimulate, predict and analyze the properties and behavior of molecules in an atomic level. This has become one of the major applied strategy in the field of computational chemistry, drug design, computational biology and materials science (56).

### 1.6.1 Molecular Docking

Molecular docking is an application of molecular modeling which deals with the prediction of binding modes and affinities of proteins or enzymes with small molecules (ligands) by proving a search algorithm and numerical score (scoring function) (57). Molecular docking is one of the most commonly used technique in drug design as it has the ability to anticipate the binding conformation of ligands to its binding site with the strength expressed in a numerical value on the basis of different favorable intramolecular interactions like hydrophobic interactions, hydrogen bonds and ligand interactions (58). Molecular docking can be viewed as a "lock-and-key" model in which protein is referred as a "lock" and ligand as "key" as which they fit only in their orientation. But this concept has been replaced by another analogy "hand-in- globe" as in the docking process both the ligand and protein can be made flexible. In an induced fit docking process, the ligand and protein change their confirmation and maintain their optimum fit but in the XP docking the ligand change its conformation and the target is rigid (59). The main purpose of molecular docking is to predict ligand-receptor complexes using computational methods. This can be performed by two connecting steps which involves the attachment of the conformations of ligands into the active site of the protein and ranking those conformations using a scoring function. In ideal condition, sampling algorithms meets the experimental binding mode and scoring function ranks the highest among all the conformations (60). In addition to the use in drug discovery by identifying new target compounds to a particular protein, molecular docking can be used in in-silico as well as other experimental techniques like a combination of docking and other computational techniques with some experimental data can be used in study of drug metabolism by grab some important information

from cytochromeP450 (61). Similarly, this technique was also used to find inhibitors for the enzyme (DNA gyrase) by de-novo design of the same enzyme (62).

Major obstacles in docking studies are the receptor flexibility and movement of secondary elements of receptors, ligands and catalysts. Although, some methods can overcome this by introducing structural flexibility, but with the global flexibility there is a requirement of an effective way to obtain and selection of reliable protein structures for which can fit in protein embedment. Another limitation of this method is high computational cost in CPU hours (60).

### 1.6.2 MMGBSA calculation

In a molecular docking, scoring function is used for the evaluation of the docking results but these functions usually simplify the calculations with many approximations because of the speed of the work (screening of one million compounds in 90 minutes using 15 thousand CPUs) (63). So, the docking program leaves behind the accuracy by focusing on the speed of the work. Although in most of the cases docking programs are used for the ligand binding interactions there is no availability of an ideal scoring function for all types of proteins and biomolecules. So, by using an imprecise scoring function, most of the times it fails to give the correct estimation of binding energies as compared to the experimental value. In such case, for the confirmation of the accurate binding free energies rescoring steps are performed (64, 65). Therefore, in such case MMGBSA method is used for the rescoring as it has fastest force field method for free energy binding method in comparison to the other force field methods like thermodynamic integration (66, 67). So, MMGBSA is the most widely used method in the free energy calculation and gives more accurate and reliable results then traditional molecular docking (68-70).

## 1.7 Enzyme kinetics

Enzymes are the biological molecules capable of altering the chemical reactions occurring in living organisms. If any of the enzymes are dysregulated or failed to work, it may lead to the dysfunction of any system or even to the death of an organism. Likewise, most toxins and

venoms from different bacteria and animals consists of enzymes (71). So, lots of the pharmaceutical companies are developing and testing the compounds for the inhibition of the target enzymes of the pathogenic organisms. For this process we need to know every possible information of the enzyme like its substrate specificity, whether it is single or multi-substrate enzyme, $K_m$ and $k_{cat}$ values of substrates, catalytic residues in the active side of the enzyme. What assay should be used, is it a cell based or an in-vitro assay (72-74).

### 1.7.1 Enzyme inhibition

The rate of an enzymatic reaction can be altered by the presence of any kind of compounds (moderators). There can be three scenarios in such case: the rate of reaction can be either increased or decreased or no change. If the rate reaction is increased, it is called enzyme activation and if it is decreased it is known as enzyme inhibition and those compounds are termed as inhibitors. The inhibitors are widely used in the field of human and veterinary medicine as well as in the production of pesticides and pharmaceutical agents (75).

The inhibitor can bind both reversible and irreversible to the active site of an enzyme. In ideal conditions, the irreversible reaction is usually slow and reversible reaction is fast when we compare with the turn- over rate of enzymes (Figure 7). But there are also reversible inhibitors that have a slow binding to the enzyme. The relationship between the dissociation constant of the enzyme inhibitor complex ($K_i$) and total concentration of the enzyme ($E_t$) determines whether the reversible inhibitor binds tightly to the enzyme or not. If the ratio of ($E_t / K_i$) is 0.01 or less then the analysis of the inhibition activity is done on the basis of Michaelis-Menten equation or if the ratio is more than 0.01 the Michaelis-Menten is not valid as the concentration of the free inhibitor in the solution is not the same as total concentration of the inhibitor (76, 77).In the case of reversible inhibition, reversible inhibitors are used for the detection of enzyme reaction mechanism, i.e. if there is a binding order of substrates to an enzyme with two or more substrates and if so, which is the binding order. Figure **7** shows a two substrate reaction (S1 and S2) binding to the active side of the enzyme and the forming a ternary enzyme-substrate (ES1S2) complex and after the catalysis they form a ternary enzyme product complex(EP1P2) and finally from which the product P1and P2 leave the enzyme (78).

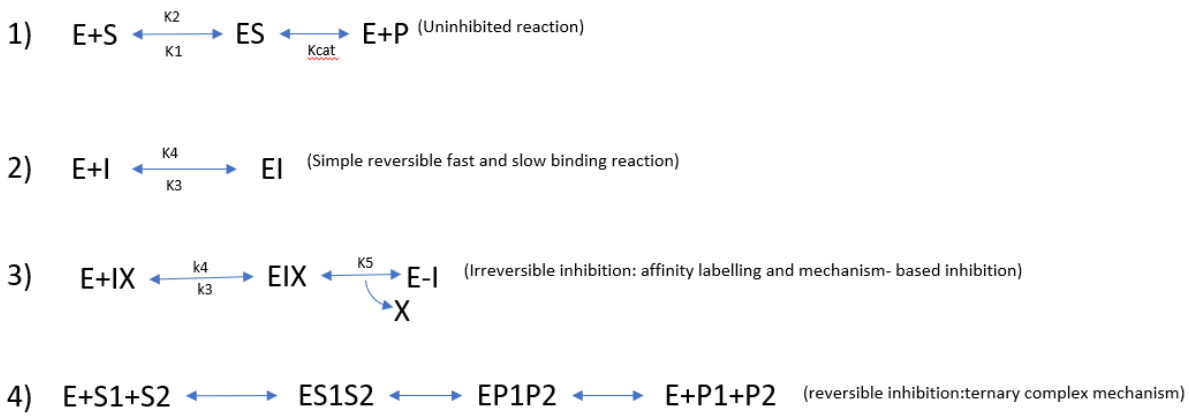



**Figure 7**: Representation of time dependent inhibition reaction:(1) Turn-over of the enzyme without the inhibitor. (2) Maintenance of equilibrium in simple reversible inhibition. K3 and K4 are low in a fast reaction and high in the case of slow reaction. (3) Irreversible inhibition of enzyme due to formation of covalent bond between enzyme and a reactive group of the inhibitor, which inactivates the enzyme and form a covalent adduct E-I. (4) tertiary complex mechanism in which two substrates S1 and S2 bind on active site and form enzyme substrate complex (ES1S2) and enzyme product complex (EP1P2) by catalysis and finally alt products P1 and P2 leaving the enzyme.

### 1.7.2 $IC_{50}$ value and $K_m$ value

The half minimal inhibitory concentration ($IC_{50}$) is the minimum amount of inhibitor required to decrease the reaction velocity of the enzyme by its half (50%). It is usually expressed in the form of dose response curve (79). A dose response curve is a curve which represents the change in function of any exogenous substance with the change of concentration of the substance(80). The relation between the concentration of inhibitor on reaction velocity (v) in relation with $IC_{50}$ value is shown in equation (1); where $v_i$ and $v_0$ are the reaction velocity with and without inhibitor, respectively.

$$v_i/v_0 = 1 / (1+([I])/IC_{50})) \quad \text{——————(1)}$$

The $IC_{50}$ value is not an actual dissociation constant because it strongly depends on the concentration of the substrate for both single and multi-substrate reactions. The higher the $IC_{50}$ value, the weaker the inhibitor binds to the enzyme (78, 81).

To determine a dose response curve, it is crucial to use a concentration of substrate which is equal or below the $K_m$ value if the inhibitor competes with the substrate to bind at the active site of the enzyme. For competitive inhibition, the relation between $IC_{50}$ value and $K_i$ value can be expressed as in the equation 2; where $K_m$ is the Michael-Menten constant and $K_i$ is the dissociation constant of the enzyme-inhibitor complex and [S] is the substrate concentration (74, 82).

$$IC_{50} = K_i (1+[S]/ K_m)) \quad (2)$$

The $K_m$ value (Michael-Menten constant) is the substrate concentration in which the reaction rate (v) is half of the maximum of the rate of the reaction ($V_{max}$). $K_m$ value is the measurement of the apparent affinity of the enzyme for the substrate; low $K_m$ value indicates that the enzyme is functioning more efficiently at lower substrate concentration and vice-versa. The Michael-Menten equation is expressed in the equation (3); where [S] is the substrate concentration, $K_m$ is the Michael-Menten constant, $V_{max}$ is the maximum rate of reaction and v is the reaction velocity(83, 84).

$$v = V_{max}[S]/ (K_m+[S]) \quad (3)$$

## 1.8 Aims of the study

The main aim of the thesis is to test different compounds which could possibly be inhibitors of the zinc metalloproteases (PLN, TLN, ALN) which are the virulence factors of *P. aeruginosa*. In addition, the compounds were tested for their inhibition of MMP-14 to check whether they would inhibit human proteases or not. If the compounds were found to inhibit the metalloproteases by more than 50%, they should be further studied to determine $IC_{50}$ and $K_i$ values. In this thesis 26 compounds (table 1) were selected from VLS and tested against PLN, TLN, ALN and MMP-14. The main objective is to discover compounds which can strongly bind to the bacterial metalloproteases without any or limited effect on the human metalloproteases. Another objective was to learn methods that are applicable in preclinical drug discovery.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 List of the chemicals and equipment used in the experiment:

1. DMSO
2. ES001
3. ES005
4. PLN
5. TLN
6. ALN
7. MMP-14
8. ES001 and ES005
9. Hepes
10. $CaCl_2 \bullet 2H_2o$
11. Brij-35
12. Milli-Q water
13. Laboratory weight balance
14. Cuvettes
15. pH-Meter
16. 96-well plates
17. Aluminum foils
18. Micropipettes(0-1000µl), multichannel pipettes(0-200µl)
19. Volumetric flasks
20. Eppendorf tubes
21. Perkin Elmer LS50 Spectrofluorimeter
22. Clariostar microplate reader
23. Computer with software (Schrodinger, Graph pad prism-6, Microsoft office)

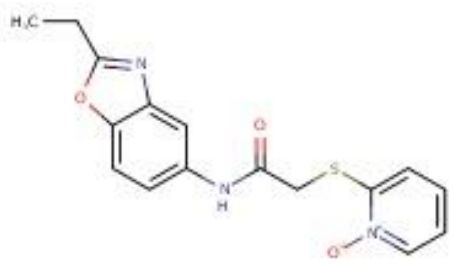
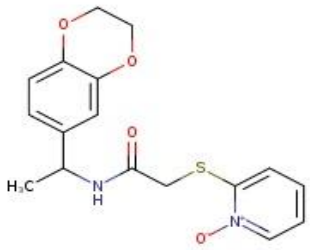
#### 2.1.2 General information about the chemicals and compounds

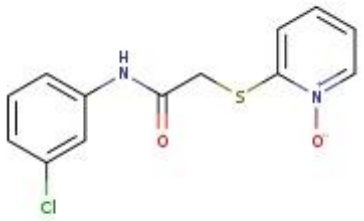
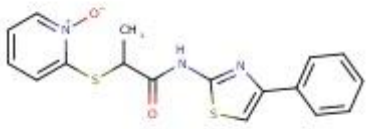
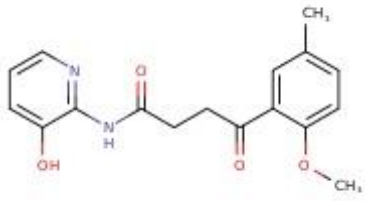
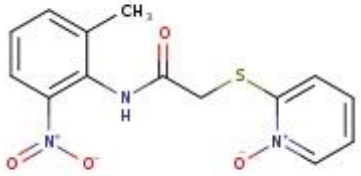
DMSO (Dimethylsulfoxide) was ordered from Merck(Darmstsdt, Germany). Hepes was from Sigma (St Louis,USA). MMP-14, Thermolysin and Pseudolysin was purchased from Calbiochem (San Diego, USA). Aureolysin was supplied by Bio-Centrum limited (Krakow, Poland). Similarly, McaPLGL(Dpa)AR-NH2 (ES001) and McaRPPGFSAFK(Dnp)-OH (ES005) were imported R&D Systems (Minneapolis, MN, USA). All the assays were performed by using a CLARIOstar microplate reader exported from CLARIOstar® BMG LABTECH, Germany.

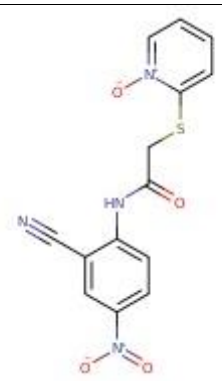
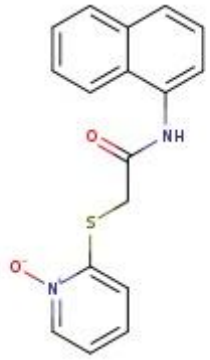
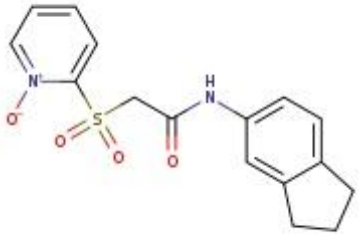
All 26 compounds (table 1) used for testing as inhibitors of PLN, TLN, ALN and MMP-14 were first selected from molecular modelling (VLS) study and ordered from Molport, Riga, Latvia.{sales@molport.com}

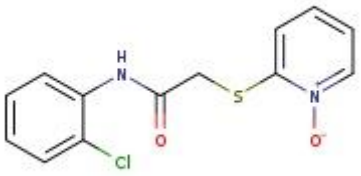
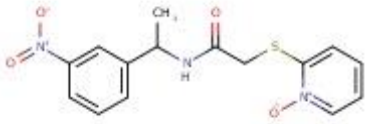
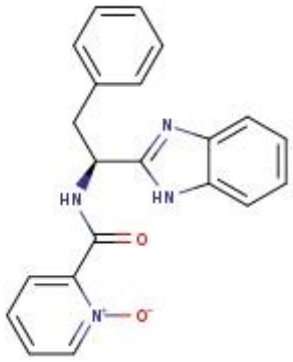
In addition, the compound "Galardin" (figure 8) was used as a control in the molecular modelling studies. Galardin is a known inhibitor of zinc metalloproteases (35).

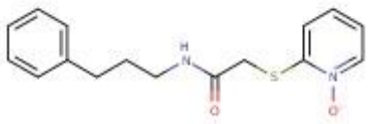
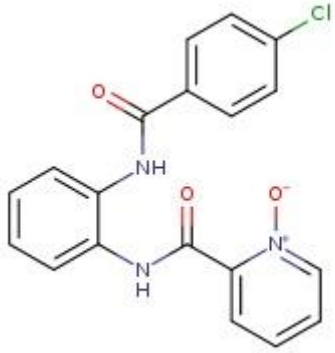
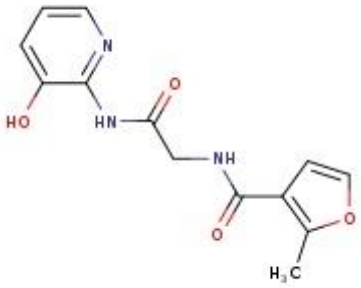
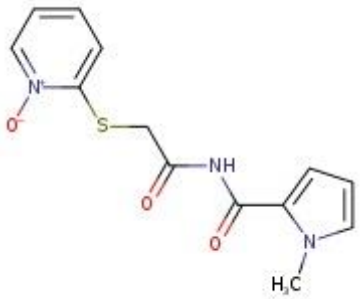
**Table 1.** The 26 compounds tested in the present study. Their Molport ID, compound ID in the present work and two-dimensional structure are indicated in the table.

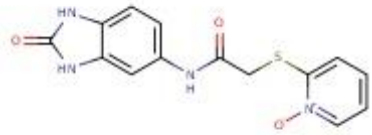
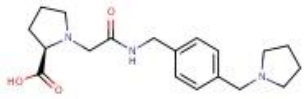
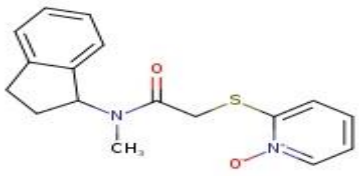
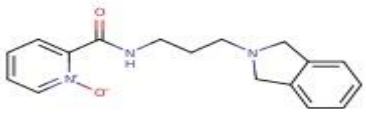
| **Molport ID** | **Compound ID** | **Structure** |
|---|---|---|
| | | |
| 027-694-799 | Compound 1 |  |
| 009-222-606 | Compound 2 |  |

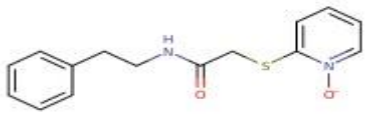
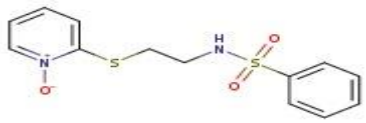
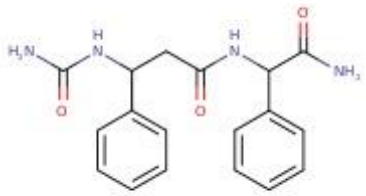
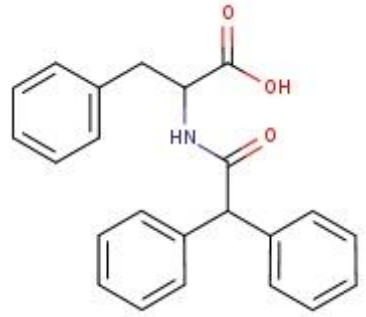
| | | |
|---|---|---|
| 004-254-412 | Compound 3 |  |
| 009-242-175 | Compound 4 |  |
| 009-226-420 | Compound 5 |  |
| 009-242-151 | Compound 6 |  |

| | | |
|---|---|---|
| 004-254-706 | Compound 7 |  |
| 030-017-687 | Compound 8 |  |
| 004-259-596 | Compound 9 |  |
| 009-073-866 | Compound 10 |  |

| | | |
|---|---|---|
| 027-942-684 | Compound 11 |  |
| 028-759-255 | Compound 12 |  |
| 004-254-409 | Compound 13 |  |
| 019-665-318 | Compound 14 |  |

| | | |
|---|---|---|
| 004-254-754 | Compound 15 |  |
| 009-346-000 | Compound 16 |  |
| 010-176-168 | Compound 17 |  |
| 004-276-484 | Compound 18 |  |

| | | |
|---|---|---|
| 004-270-223 | Compound 19 |  |
| 042-117-341 | Compound 20 |  |
| 027-675-266 | Compound 21 |  |
| 029-939-652 | Compound 22 |  |

| | | |
|---|---|---|
| 004-254-606 | Compound 23 |  |
| 009-337-761 | Compound 24 |  |
| 020-103-247 | Compound 25 |  |
| 002-462-292 | Compound 26 |  |

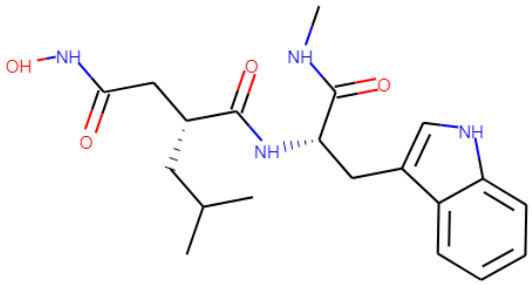



**Figure 8**: 2D Structure of galardin exported from ChEMBL.

## 2.2 Methods

### 2.2.1 Preparation of assay buffer (0.1 M Hepes, 10 mM $CaCl_2$, 0.005% Brij-35, pH 7.5)

One hundred ml of assay buffer was prepared as follows: 50 ml of Milli-Q water was added to 2.38 g Hepes (MW=238.3 g / mole) and 0.147 g $CaCl_2x2H_2O$ (MW=147.02 g / mole). To this solution, 16.7 µl of 30% Brij-35 was added and the pH was adjusted to 7.5 by titration with 10 M NaOH in Milli-Q water. Thereafter, Milli-Q water was added to give a final volume of 100 ml, the pH checked and if necessary further adjusted with 10 M NaOH to give a pH of 7.5. The buffer is stored at 4 °C.

### 2.2.2 Preparation of compounds

All the compounds from Molport were in powder form. These compounds were dissolved in 100% DMSO to give a stock solutions with a concentration of either 10 mM or 20 mM as described in table 2. The volume of DMSO added were based on the following formula:

Volume of DMSO to be added = (quantity of substance available*purity)/ molecular weight of compound

In most of the cases a stock solution of 10 mM was prepared but in some of the cases a stock solution of 20 mM was prepared because the volume of the glass bottle with a compound received from Molport was too small for the DMSO volume to give a concentration of 10mM.

**Table 2**. Volume of 100% DMSO added to the powder compounds to give the desired concentration of the compounds. All the information about the purity, molecular weight and quantity was taken from the molport.

| **Compound name** | **Quantity(mg)** | **Molecular Weight(g/mole)** | **Purity** | **Volume of DMSO (ml) to give 10mM** | **Volume of DMSO (ml) to give 20mM** |
|---|---|---|---|---|---|
| 027-694-799 | 20 | 329.37 | ≥90 | 5.465 | 2.732 |
| 009-222-606 | 20 | 346.4 | ≥90 | 5.196 | 2.599 |
| 004-254-412 | 20 | 294.75 | ≥90 | 6.106 | 3.053 |
| 009-242-175 | 20 | 357.45 | ≥90 | 5.035 | 2.517 |
| 009-226-420 | 20 | 314.341 | ≥90 | 5.726 | 2.863 |
| 009-242-151 | 20 | 319.34 | ≥90 | 5.636 | 2.818 |
| 004-254-706 | 20 | 330.32 | ≥90 | 5.449 | 2.724 |
| 030-017-687 | 20 | 244.254 | ≥90 | 7.369 | 3.684 |
| 004-259-596 | 20 | 310.37 | ≥90 | 5.780 | 2.900 |

| | | | | | |
|---|---|---|---|---|---|
| 009-073-866 | 20 | 332.37 | ≥90 | 5.415 | 2.707 |
| 027-942-684 | 20 | 339.351 | ≥90 | 5.3042 | 2.652 |
| 028-759-255 | 4.7 | 333.36 | ≥90 | 1.268 | 0.634 |
| 004-254-409 | 20 | 294.75 | ≥90 | 6.106 | 3.053 |
| 019-665-318 | 18.1 | 358.401 | ≥90 | 4.545 | 2.272 |
| 004-254-754 | 20 | 302.39 | ≥90 | 5.952 | 2.97 |
| 009-346-000 | 20 | 367.79 | ≥90 | 4.894 | 2.447 |
| *010-176-168* | 20 | 275.264 | ≥90 | 6.539 | 3.269 |
| 004-276-484 | 20 | 291.33 | ≥90 | 6.178 | 3.089 |
| 004-270-223 | 20 | 316.34 | ≥90 | 5.690 | 2.845 |
| 042-117-341 | 10 | 345.443 | ≥90 | 2.605 | 1.302 |
| 027-675-266 | 20 | 314.4 | ≥90 | 5.725 | 2.862 |
| 029-939-652 | 20 | 297.358 | ≥90 | 6.053 | 3.026 |
| 004-254-606 | 20 | 288.37 | ≥90 | 6.241 | 3.120 |
| 009-337-761 | 20 | 310.39 | ≥90 | 5.800 | 2.900 |
| 020-103-247 | 20 | 340.383 | ≥90 | 5.288 | 2.644 |
| 002-462-292 | 100 | 359.425 | ≥95 | 26.431 | 13.215 |

### 2.2.3 Assay method

In the reaction velocity assays, we tested the inhibitory activity of compounds using a concentration of 100 µM and 1.0% DMSO. The compounds were first diluted to 1 mM in assay buffer, which results in a DMSO concentration of 10%. A brief description of how this new stock solution was prepared is that compounds was dissolved at a concentration of 20 mM in 100% DMSO (first stock). So, to make our desired concentration of the compound, 50 µl of the solution was taken from the stock and it was mixed with 50 µl of 100% DMSO (second stock). And in the second step, 900 µl of HEPES buffer was added to the second stock. This resulted in a 10 times dilution of both compound and DMSO (third stock). This third stock was stored safe at 4°C and used in the assay in 96 well plates.

Each day it was necessary to determine how much the stock solution of the proteases should be diluted in order to obtain an optimal concentration for the inhibitory assays and its controls. These assays were performed with a Perkin Elmer LS50 Spectrofluorimeter ($\lambda_{ex}$=320 nm, $\lambda_{em}$=405 nm, slit width=10 nm) and the reactions were followed for 1 min (10 data points per second) at 37 °C. The assay (total volume of 100 µl) consisted of assay buffer, 4 µM substrate (ES001 for MMP-14 and ES005 for TLN, PLN and ALN) and various concentrations of enzyme.

After many variable concentrations of enzymes, one fixed concentration was selected which was used in the Clariostar plate reader where the reaction velocity was followed for 30 minutes at 37 °C.

We don’t know if some of these compounds are inhibitors of the proteases and if so, are they fast or slow binders, weak or strong binders. Therefore, the proteases were preincubated with and without the compounds for various time points (0, 15 and 30 min) at room temperature before the reaction velocity was determined. In the experiments with no preincubation (0 min), the enzymatic reaction was started by adding the enzyme to the mixture of either buffer, substrate and inhibitor or to buffer and substrate (control). In the experiments with 15 and 30 min preincubation, the enzymatic reaction was started by adding the substrate.

For each assay, four parallels were performed. Table 3 shows the amount of buffer, substrate, compound and enzyme used in each assay.

**Table 3**: Reaction mixtures for control and inhibitory assays. Shown is the amount of assay buffer, DMSO, compound/inhibitor (I), substrate (ES001/ES005) and enzyme in each well of a 96 well plate. Following approximate concentrations of the proteases was used per assay: TLN (0.1 nM), PLN (0.5 nM), ALN (1.4 nM) and MMP-14 (1.0 nM) Each experiment were performed with four parallels.

| | Control | | | | With Inhibitor (100 µM) | | | |
|---|---|---|---|---|---|---|---|---|
| Assay Buffer (µl) | 70 | 70 | 70 | 70 | 70 | 70 | 70 | 70 |
| 10% DMSO (µl)<br>In assay buffer | 10 | 10 | 10 | 10 | 0 | 0 | 0 | 0 |
| 1 mM [I] containing 10% DMSO in assay buffer (µl) | 0 | 0 | 0 | 0 | 10 | 10 | 10 | 10 |
| Enzyme (µl) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 40 µM of either ES005 or ES001 (µl) | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Total assay volume (µl) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

### 2.2.3.1 Settings of the Perkin Elmer LS50 spectrophotometer:

The assay was performed using a time driven application.

The excitation wavelength was fixed to 320 nm and emission wavelength was fixed to 405 nm. The excitation and emission slit was adjusted to 10 nm. The temperature was set to 37°C using a water-bath and a thermostatic cuvette holder.

### 2.2.3.2 Settings of the clariostar:

The fluorescence intensity was measured with a plate mode of slow kinetics. The excitation and emission wavelength was fixed to 320 and 405 nm, with slit widths of 10 nm. The focal height was 5 mm and one monochromator was used for the measurement. In the general settings menu, flying mode was selected with only one kinetic window, the number of cycles was 225 for 30 minutes of the reading and cycle time was 8 sec. The temperature was fixed to 37°C. The gain was adjusted to 2000. The reading direction was bidirectional, horizontal left-right from top to bottom. The concentration and substrate for each enzyme is described in table 4.

**Table 4**: Assay concentration of substrate and total assay volume in the Clariostar microplate reader using 96 well plates.

| Enzyme | Assay volume | Inhibitor concentration | Substrate | Substrate concentration |
|---|---|---|---|---|
| TLN | 100 µl | 100 µM | ES005 | 4 µM |
| PLN | 100 µl | 100 µM | ES005 | 4 µM |
| ALN | 100 µl | 100 µM | ES005 | 4 µM |
| MMP-14 | 100 µl | 100 µM | ES001 | 4 µM |

## 2.3 Molecular modeling

For all molecular modeling, the Schrödinger software was used. The Glide program was used for docking of the compounds (*Glide Schrödinger Release 2020-2*; Schrödinger, LLC: New York, NY, USA, 2020)

### 2.3.1 Docking and MMGBSA calculation

For the docking and MMGBSA calculation compound 5, 10, 17 and galardin were selected. After generation of different enantiomers, altogether 14 molecules were docked. Galardin was taken as a control as a known high affinity inhibitor of zinc metalloproteases. The computational experiment was performed by using maestro 2020 software.

### 2.3.2 Ligand preparation

The structure of the selected compounds was drawn by using a 2D sketcher as implemented in Maestro Schrodinger software. The lig-prep program was used to convert the drawn 2D structure into 3D structure which can be further used for computational studies. For this process the pH was set to 7 with a putative deviation of ±1. The carboxylic acids were deproteinated, the tertiary amino acids were made positively charged, and phosphonates were partially deproteinated in the process and all other default setting of maestro was used.

### 2.3.3 Protein preparation and grid map generation

The protein was prepared using the protein preparation wizard from Maestro. Altogether four proteins (TLN, PLN, ALN, MMP-14) were prepared. At first, the enzymes were exported in the working space from PDB with their respective PDB codes as shown in table 5. Then missing hydrogen bonds were added, polar hydrogen bonds were optimized and crystallographic water molecules beyond 5Å from hetero group were deleted. Then, minimization process was performed to avoid unwanted contacts. To generate the grid map for docking, the zinc atom of

the catalytic site was selected as centroid, and a grid map was generated including residues within 20 $Å^3$ of the zinc atom.

**Table 5**: The PDB codes of the structures used for docking.

| Enzymes | PDB code |
|---|---|
| TLN | 5dpe |
| PLN | 1u4g |
| ALN | 1bqb |
| MMP-14 | 1bqq |

### 2.3.4 Docking

For all ligands, ligand docking with a SP (standard precision) was performed. The ligand sampling was made flexible. The number of poses per ligand was set to 5 for performing post docking minimization. Docking was performed one ligand at a time.

### 2.3.5 MMGBSA calculation

Prime MMGBSA was performed. All amino acids within 5Å of the ligand was included in a conformational sampling by using an energy minimization sampling approach. All other default settings were followed to perform the MMGBSA calculation.

# 3 Results

## 3.1 Calculating inhibition activity of the compounds for zinc metalloproteases

In this study molecular modeling and enzyme kinetics were used to calculate the inhibition activity of the enzymes (PLA, TLN, ALN and MMP-14) with respect to the 26 compounds shown in table 1. Figures 8-15 show that none of the compounds reduced the activity by more than 50%, and the inhibition did not increase with the increase in preincubation time (0-30 min) of enzyme and compound. This was the case for all enzymes (PLN, TLN, ALN, MMP-14). Therefore, none of the compounds could be considered as slow binders. The calculations are shown in appendix 1-4 for TLN, PLN, ALN and MMP-14, respectively.

For TLN compound 5, 10 and 17 were found to inhibit the activity with approximately 40%, and compounds 4, 9, 15, 20, 21 and 22 showed only a negligible inhibition, so they are considered as weak binders of TLN (figures 8 and 9). In the case of PLN some compounds (3, 15, 20 and 23) did not show any inhibition at all or a negligible inhibition (<5%). Compound 17 gave the strongest inhibition (approximately 20%). So, compound 17 was selected for MMGBSA calculation with PLN (figures 10 and 11). Compounds 5, 10 and 17 inhibited the activity of ALN with approximately 30% which was the highest inhibition of ALN, so they were selected for MMGBSA calculation (figures 12 and 13). The highest inhibition activity of MMP-14 was with the compounds 5, 10 and 17, that all reduced the activity by approximately 30%, and they were selected for the MMGBSA calculations. The compounds 4, 13, 14, 25 and 26 showed very low inhibit the MMP-14 activity (figures14 and 15).

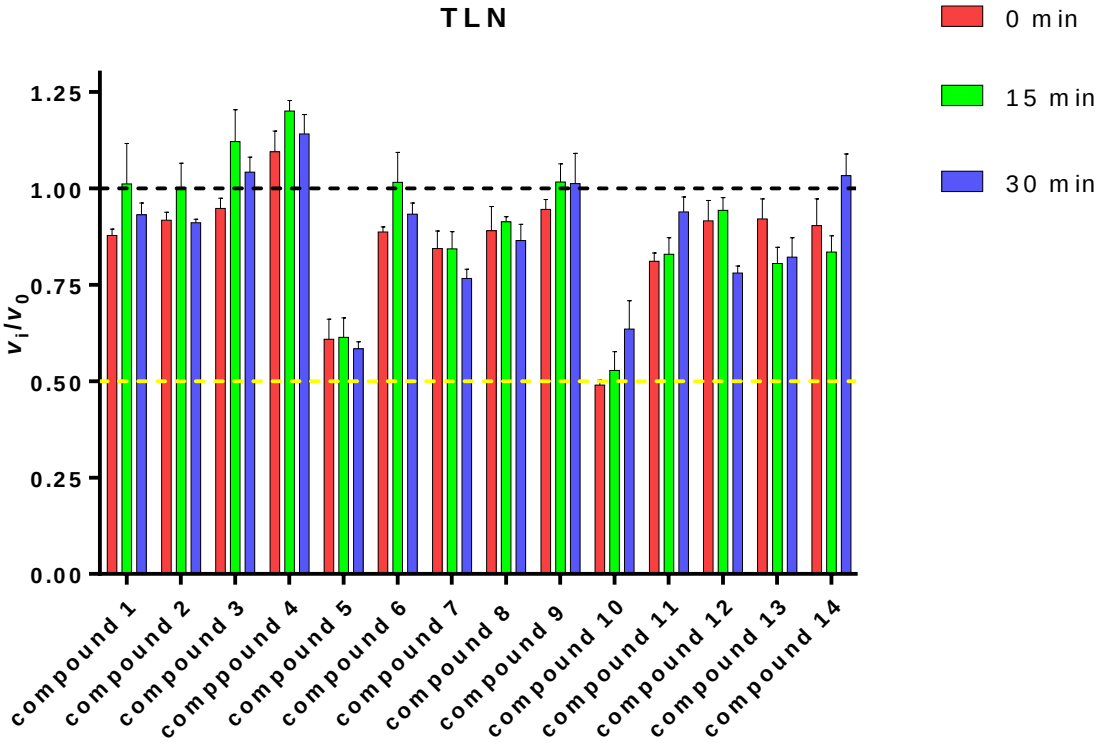



**Figure 8**: Inhibitory effects of TLN activity of compounds 1-14 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the TLN activity by more than 50%. Compounds 5 and 10 were selected for docking and MMGBSA calculation as these compounds were found to inhibit the TLN activity approximately 40%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

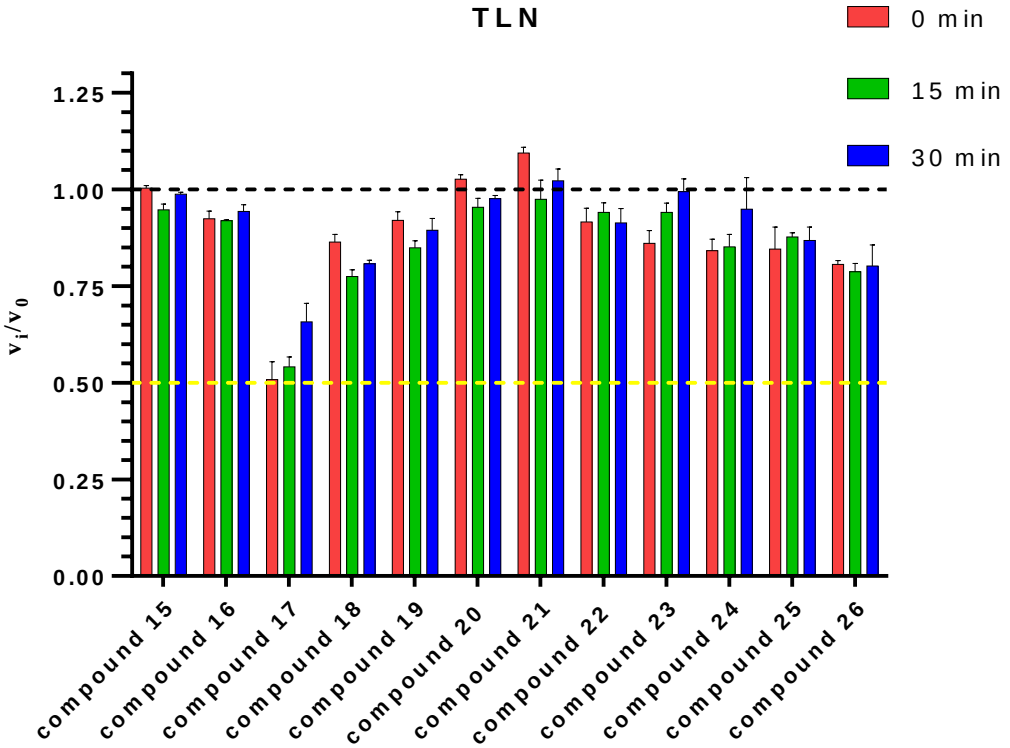



**Figure 9**: Inhibitory effects of TLN activity of compounds 15-26 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the TLN activity by more than 50%. Compound 17 was selected for docking and MMGBSA calculation as this compound was found to inhibit the TLN activity approximately 40%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

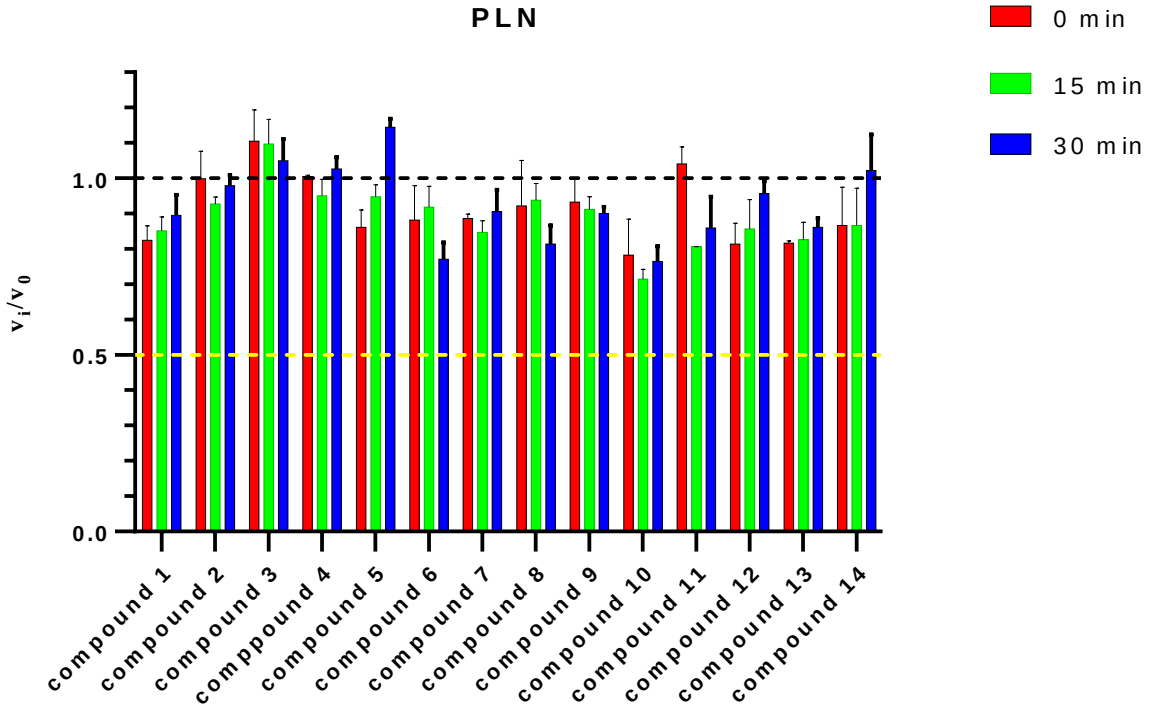



**Figure 10**: Inhibitory effects of PLN activity of compounds 1-14 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the PLN activity by more than 50%. Non compounds were selected for docking and MMGBSA calculation as none of the compounds were found t significantly inhibit the PLN activity. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

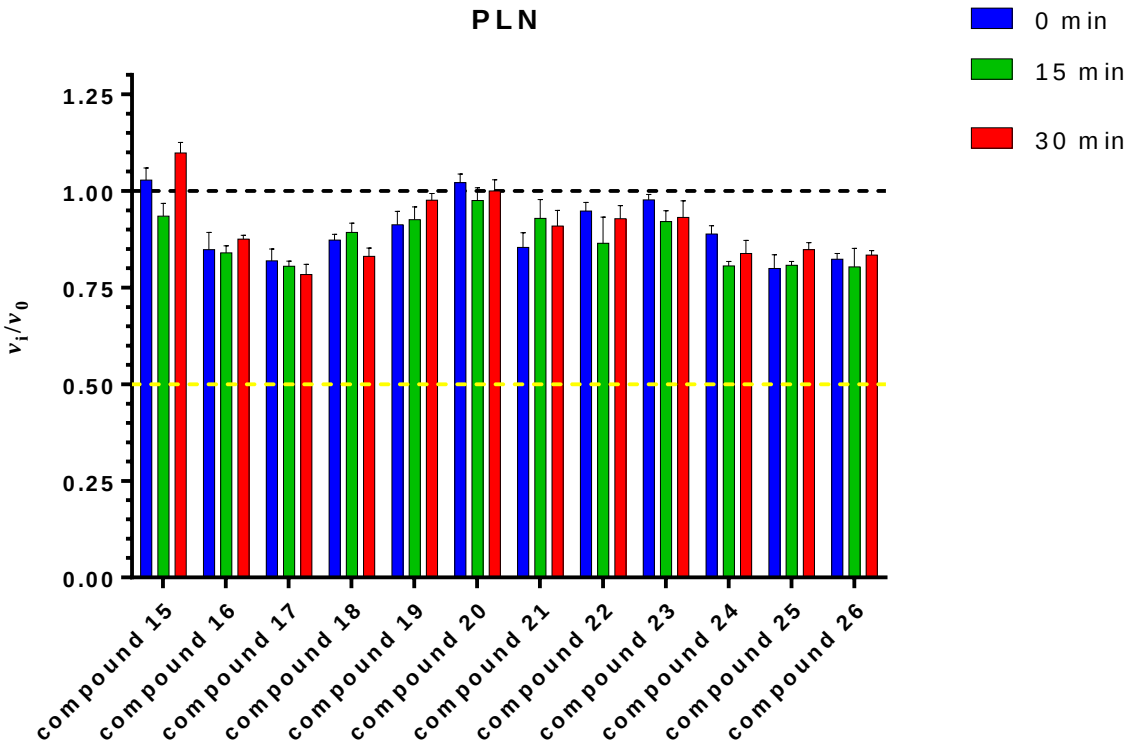



**Figure 11**: Inhibitory effects of PLN activity of compounds 15-26 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the PLN activity by more than 50%. Compound 17 was selected for docking and MMGBSA calculation as this compound was found to inhibit the PLN activity approximately 25%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

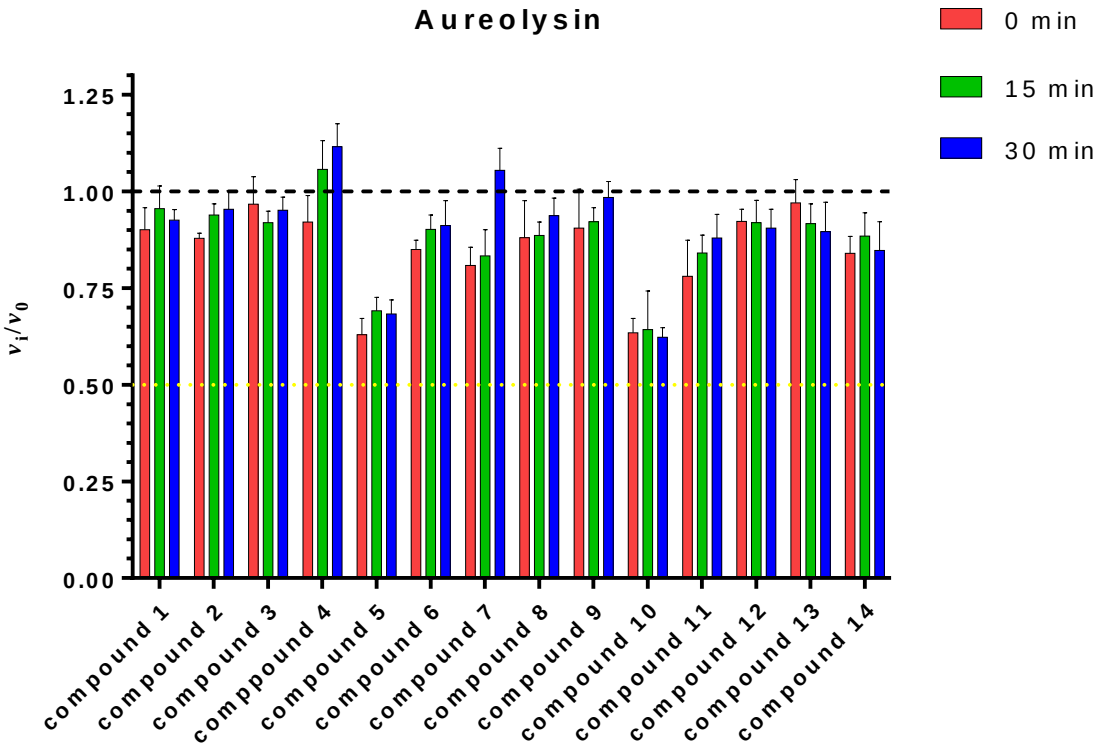



**Figure 12**: Inhibitory effects of ALN activity of compounds 1-14 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the ALN activity by more than 50%. Compounds 5 and 10 were selected for docking and MMGBSA calculation as this compound was found to inhibit the ALN activity approximately 40%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

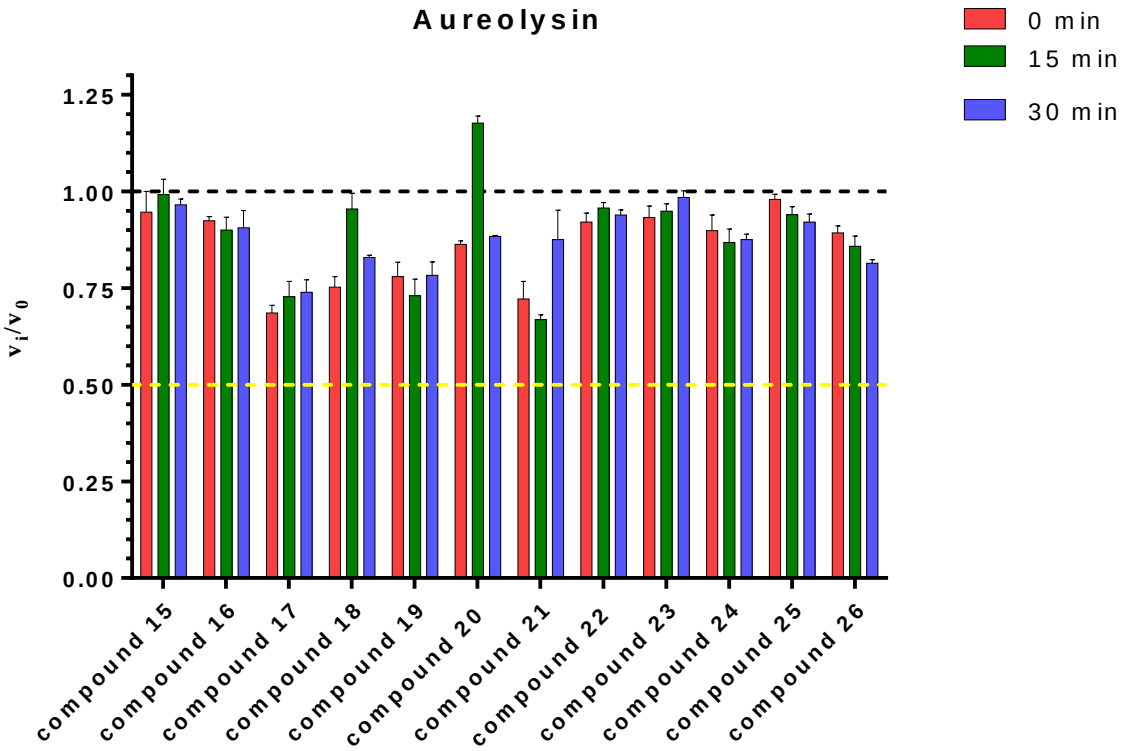



**Figure 13**: Inhibitory effects of ALN activity of compounds 15-26 from Table1 using a fixed concentration of 100 µM at different time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the ALN activity by more than 50%. Compound 17 was selected for docking and MMGBSA calculation as this compound was found to inhibit the ALN activity approximately 40%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

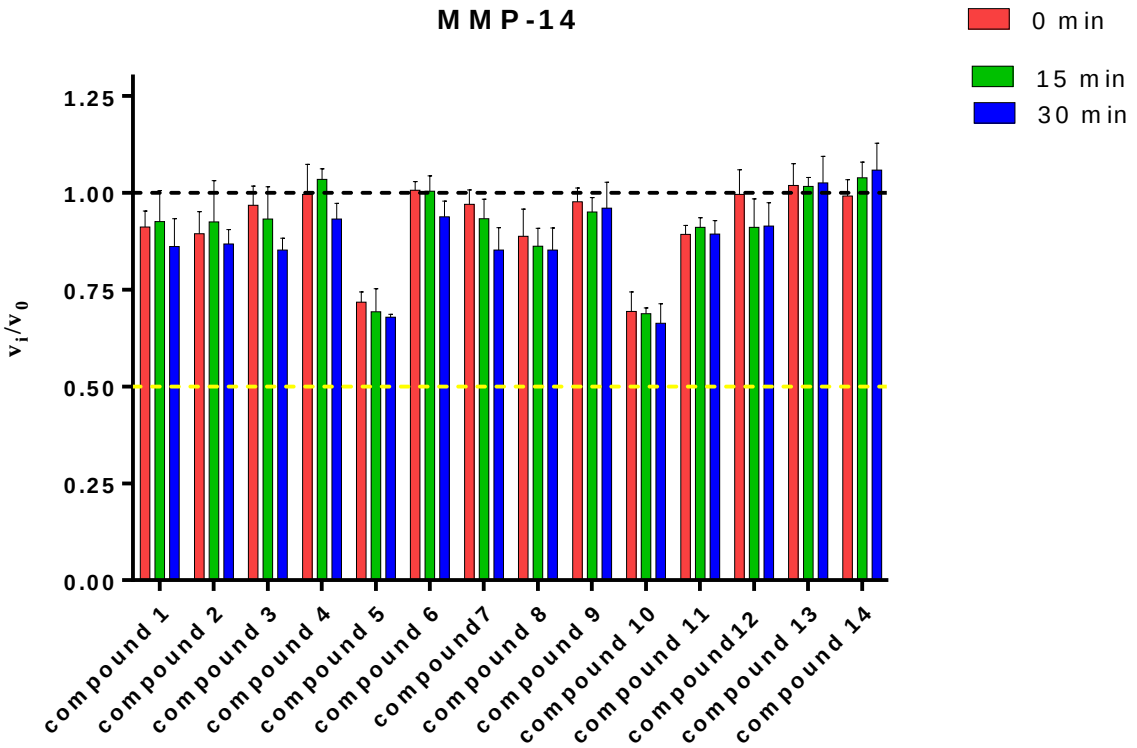



**Figure 14**: Inhibitory effects of MMP-14 activity of compounds 1-14 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES001 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the MMP-14 activity by more than 50%. Compound 5 and 10 was selected for docking and MMGBSA calculation as this compound was found to inhibit the MMP-14 activity approximately 30%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

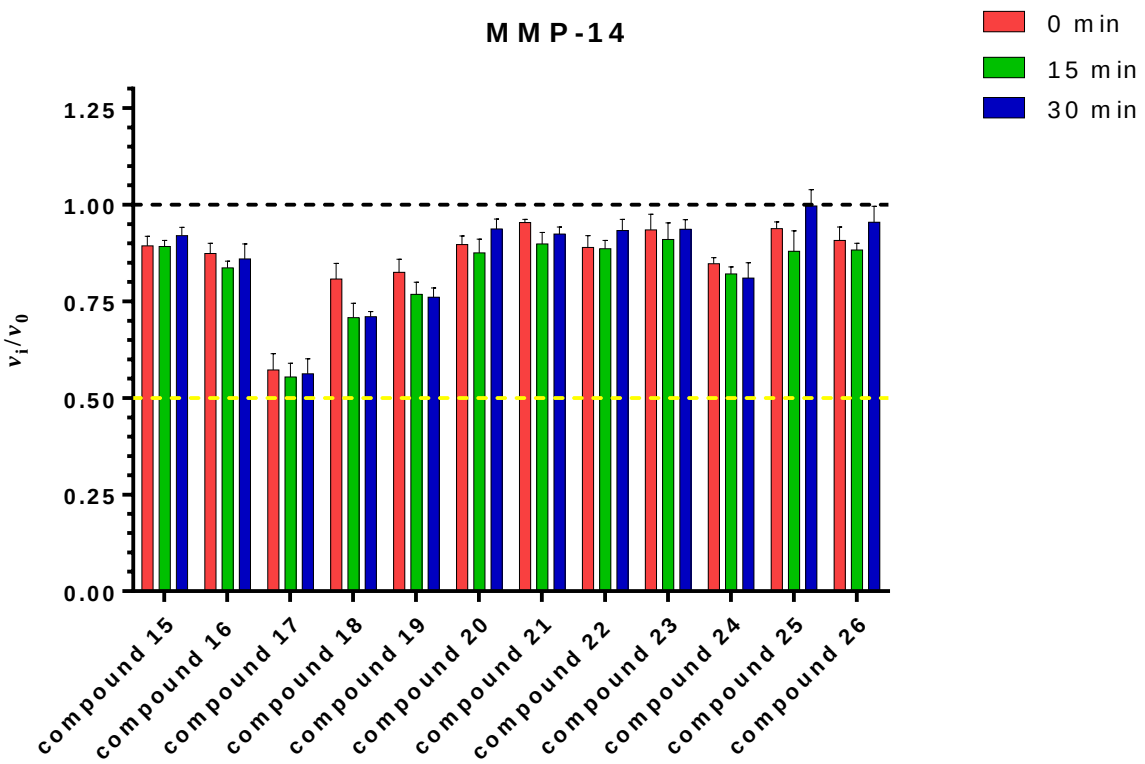



**Figure 15**: Inhibitory effects of MMP-14 activity of compounds 15-26 from Table1 using a fixed concentration of 100 µM at different preincubation time intervals (0, 15 and 30 minutes). The inhibition experiments were performed by using a fixed concentration of substrate ES005 (4µM) and Hepes buffer pH 7.5 as described in Materials and Methods. None of the compounds were found to inhibit the MMP-14 activity by more than 50%. Compound 17 was selected for docking and MMGBSA calculation as this compound was found to inhibit the MMP-14 activity approximately 30%. The yellow horizontal line represents the 50% inhibition and black horizontal line represents no inhibition at all.

### 3.2 Molecular Modeling

After the selection of compounds based on the wet lab experiments, compounds were taken for the MMGBSA calculation. The selected compounds were compound 5, 10 and 17 for the enzymes TLN, ALN and MMP-14, while only compound 17 was selected for the PLN. In addition to this, one compound (galardin) was taken as a control. Galardin is a known high affinity inhibitor of the zinc metalloproteases (85). Then these compounds were docked with their respective enzymes and MMGBSA calculation was performed which is shown from figure 17-25 and table 10.

After the MMGBSA calculation the 3D interactions of the enzymes with ligands were observed in the workspace. This was done to compare the molecular interactions of different enzymes with the ligands. Tables 6-9 show that the ligands interacted differently with the enzymes. Different amino acids were involved in ligand binding in the enzymes.

#### 3.2.1 Molecular interactions between TLN and ligands

For docking and MMGBSA calculation of TLN, compounds 5, 10, and 17 were selected and their binding mode compared with that of the known inhibitor galardin. Amino acids of TLN directly involved in ligand binding to the docked compounds are shown in table 6. When TLN was docked with galardin, Glu-166 was forming a hydrogen bond with the oxygen atom of galardin, and His-231 was forming a hydrogen bond with same oxygen atom of galardin. The catalytic zinc atom was forming a metal coordination with an oxygen atom of galardin separated by a distance of 2.10Å (table 6) making strong interactions. In addition, Asn-112 formed hydrogen bonds with two NH groups of galardin, Ala-113 formed a hydrogen bond with one NH group. Asp-226 was forming Pi-cation interactions with the 5-membered ring structure of galardin (figure 16). The MMGBSA score for TLN with galardin was -15.87 kcal/mol (table 10).

When TLN was docked with compound 17, the zinc atom was forming a coordination with two different oxygen atoms (a distance of 2.38Å to the closest oxygen atom, table 6) of the

compound, while His-231 was forming a hydrogen bond with one of the oxygen atoms and Pi cationic interactions with the six membered ring structure of the compound. Arg-203, Asn-112, Ala-113 were also forming hydrogen bonds with oxygens and NH groups of the compound (figure 17). The obtained MMGBSA score was -6.4 kcal/mol (table 10).

Similarly, a MMGBSA score of -8.4 kcal/mol was obtained when compound 10 was docked with TLN (table 10). The zinc atom was forming a coordination with an oxygen atom of the compound at a distance of 2.13Å (table 6). His-231 and Glu-166 were both forming hydrogen bonds with the zinc coordinating oxygen atom. Arg-203 interacted directly with two oxygen atoms of compound 10. Glu-166 and His-146 were both forming stacking interactions with a six membered ring structure of the compound. Ala-113 was also forming a hydrogen bond with the NH group of the compound (table 10 and figure 18).

Likewise, docking of compound 5 with TLN gave a MMGBSA score of -6.11 kcal/mol (table 10). The zinc atom (separated by nearest oxygen atom by 2.16Å) and Glu-166 both interacted with the same oxygen atom of the compound (figure 19). Asn-112 and Ala-113 were forming a hydrogen bond with the same NH group of the compound. Glu-143 was also forming a salt bridge with a negatively charged oxygen atom of the compound (figure 19).

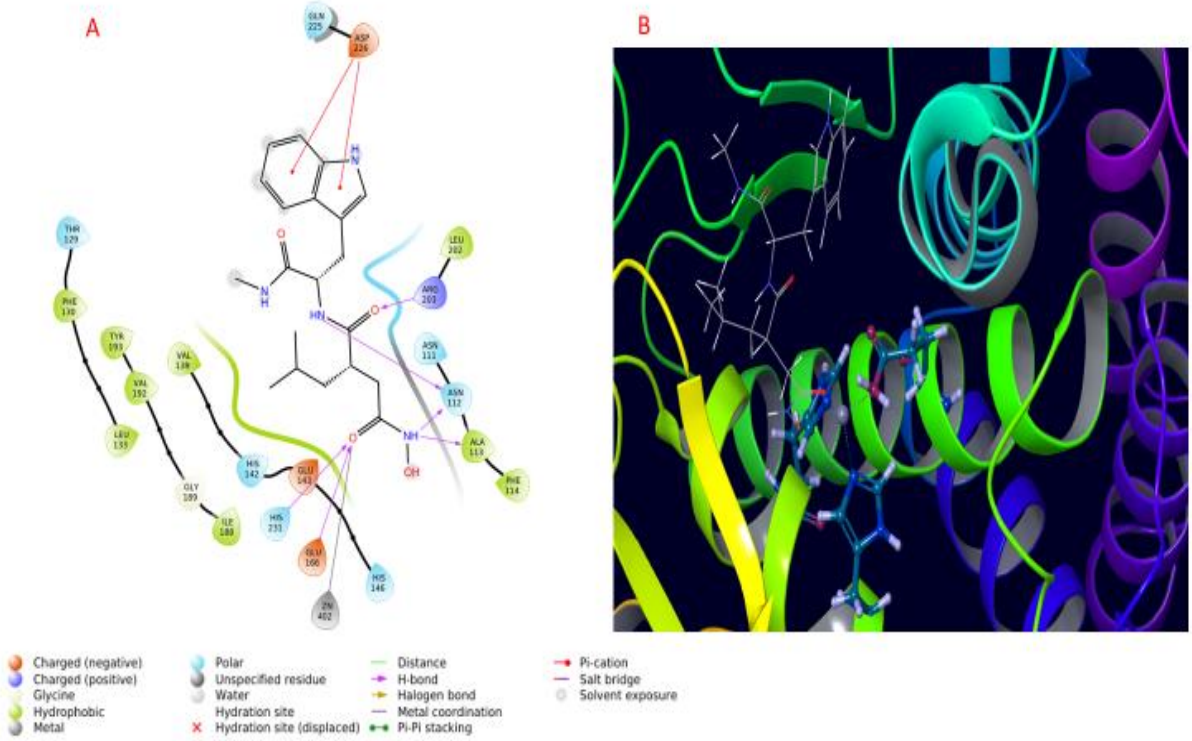



**Figure 16**: The TLN-galardin complex after MMGBSA calculations. Figure (A): ligand interaction diagram of TLN with galardin. All the interactions of galardin with the TLN are indicated by different colors of the arrows at the bottom of the diagram. Figure (B): 3D representation of the interaction of TLN with galardin. Zinc is displayed in dark grey color. The side chains of the most important amino acids for ligand binding are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

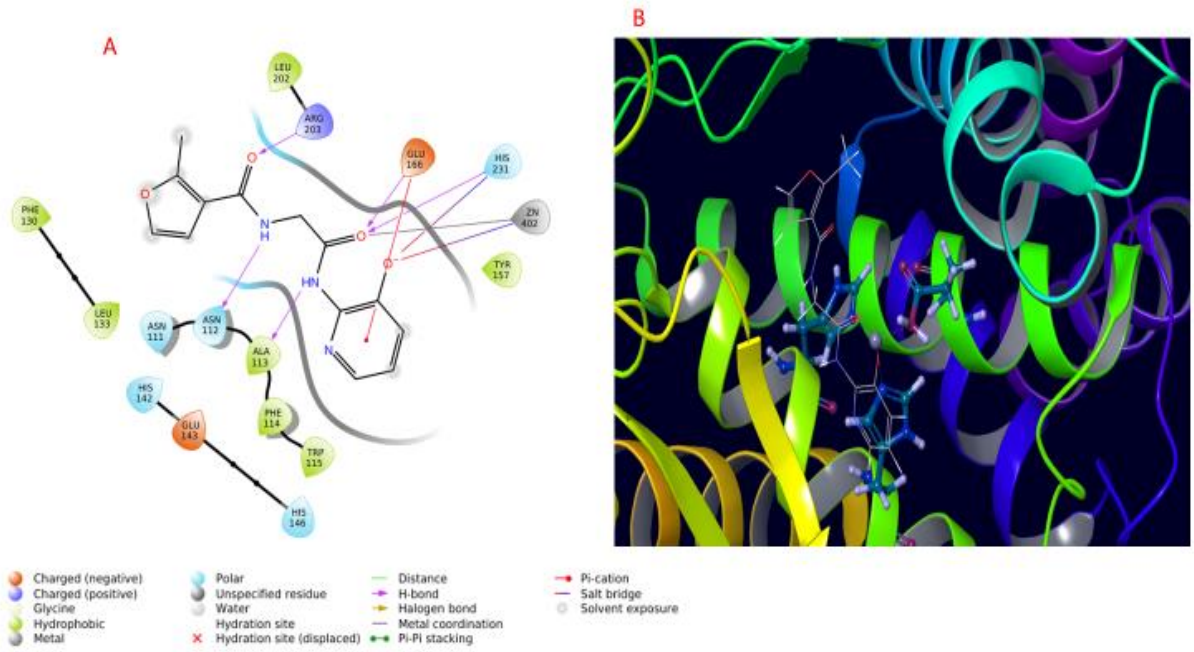



**Figure 17**: The TLN-compound 17 complex after MMGBSA calculation. Figure (A): ligand interaction diagram of TLN with compound 17. All interactions of the compound with the PLN are indicated by different colors of the arrows at the bottom of the diagram. Figure (B): 3D representation of the interaction of TLN with compound 17. Zinc is displayed in dark grey color. The side chains of the most important amino acids for the binding of the ligand are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

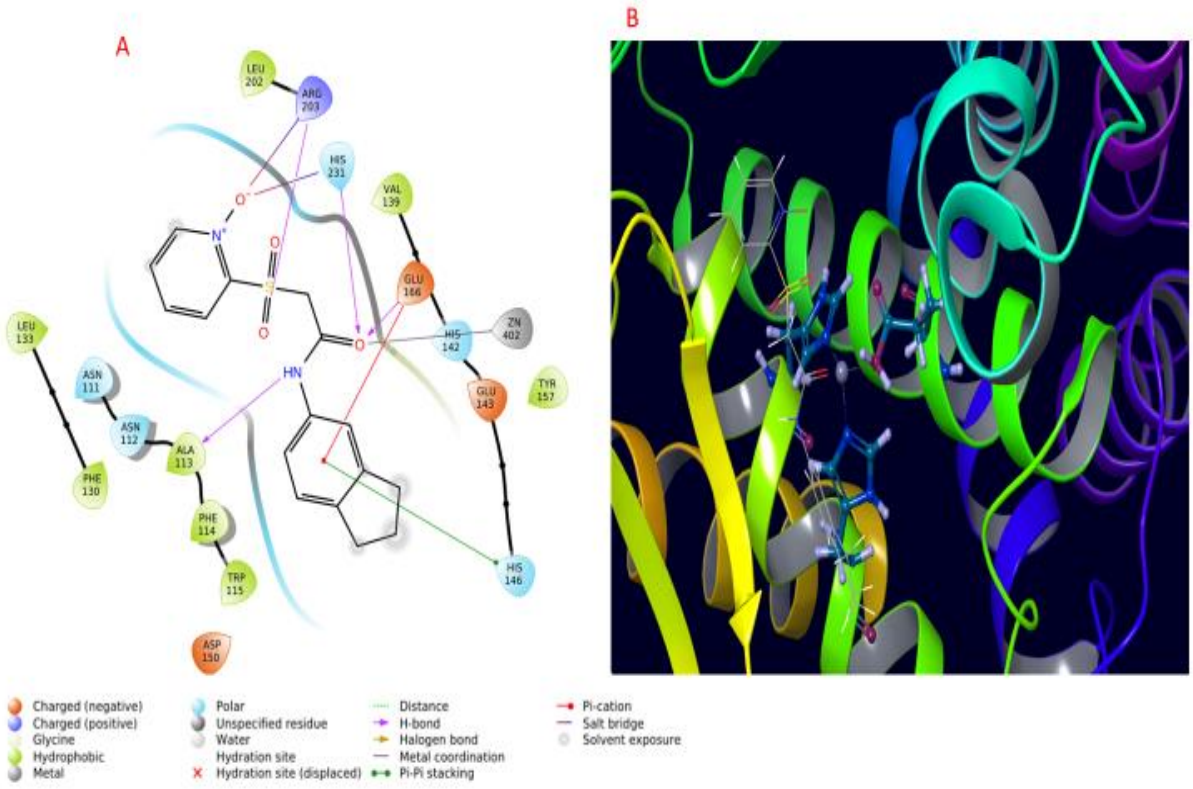



**Figure 18**: The TLN-compound 10 complex after MMGBSA calculations. Figure (A): ligand interaction diagram of TLN with compound 10. All interactions of the compound with the PLN are indicated by different colors of the arrows at the bottom of the diagram. Figure (B): 3D representation of the interaction of TLN with compound 10. Zinc is displayed in dark grey color. The side chains of the most important amino acids for the binding of the ligand are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

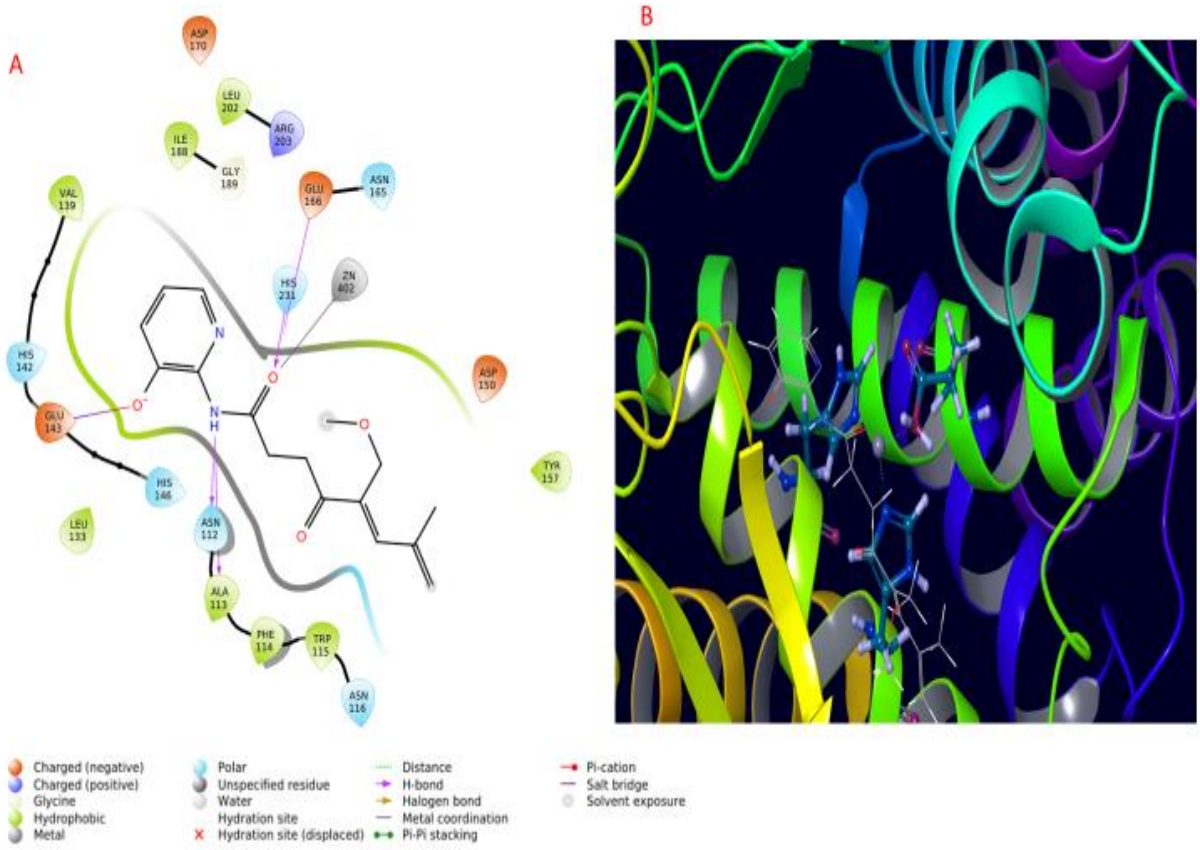



**Figure 19**: The TLN-compound 5 complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of TLN with compound 5. All interactions of the compound with the PLN are indicated by different colors of the arrows at the bottom of the diagram. Figure (B): 3D representation of the interaction of TLN with compound 5. Zinc is displayed in dark grey color. The side chains of the most important amino acids for the binding of the ligand are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

**Table 6**: Amino acids of TLN directly involved in ligand interactions.

| Ligands | Amino acids in TLN | Atomic distance between catalytic zinc and closest oxygen atom (Å) |
|---|---|---|
| Galardin | Glu-166, His-231, Ala-113, Asn-112, Arg-203, Asp-226 | 2.10 |
| Compound 17 | Glu-166, Asn-112, His-231, Arg-203, Ala-113 | 2.14 |
| Compound 10 | Glu-116, Ala-113, Arg-203, His-231, His-146 | 2.15 |
| Compound 5 | Glu-166, Asn-112, Ala-113, Glu-143 | 2.14 |

### 3.2.2 Molecular interactions between PLN and ligands

For docking and MMGBSA calculation of PLN, only compound 17 was selected, and the binding modes was compared with the binding mode of the known inhibitor galardin. When PLN was docked with galardin, Glu-141 was forming a hydrogen bond with the OH group of galardin, and Arg-198 was forming a hydrogen bond with an oxygen atom of galardin. The catalytic zinc atom was forming a metal coordination with an oxygen of galardin. In addition, Asn-112 formed hydrogen bonds with two oxygen atoms in galardin, while Ala-111 formed a hydrogen bond with an NH group of galardin (figure 20). When PLN is docked with compound 17, Arg-198 was forming a hydrogen bond with an oxygen in compound 17, Glu-141 and Ala-113 were forming hydrogen bonds with NH groups, and the catalytic zinc was coordinated with two oxygen atoms of compound 17. Hid-140 was forming pi-pi stacking with the five membered ring system of the compound (figure 21).

The detail description is shown in the table 7 and figure 20-21.

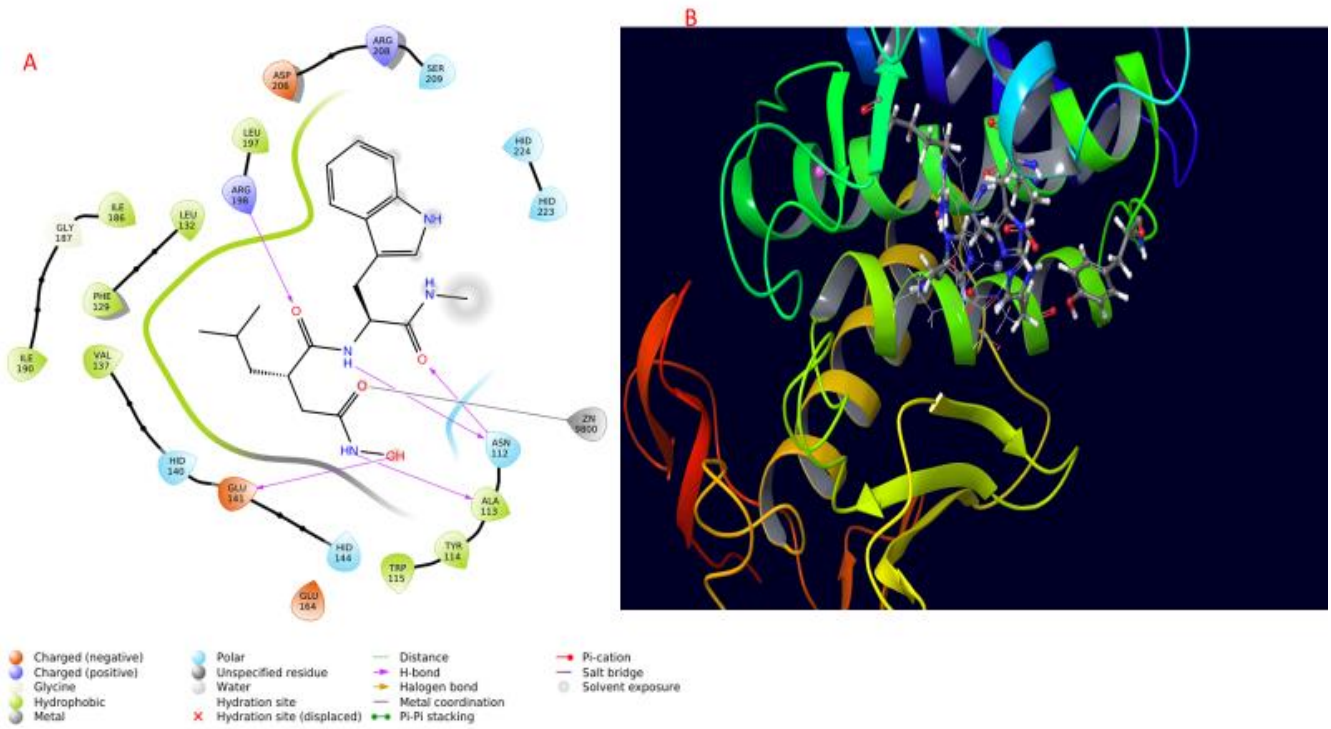



**Figure 20**: The PLN-galardin complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of the interaction of PLN with galardin. All interactions of galardin with the PLN are indicated by different colors of the arrows at the bottom of the diagram. Figure (B): 3D representation of the interaction of PLN with galardin. Zinc is displayed in dark grey color. The side chains of the most important amino acids for the binding of the ligand are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

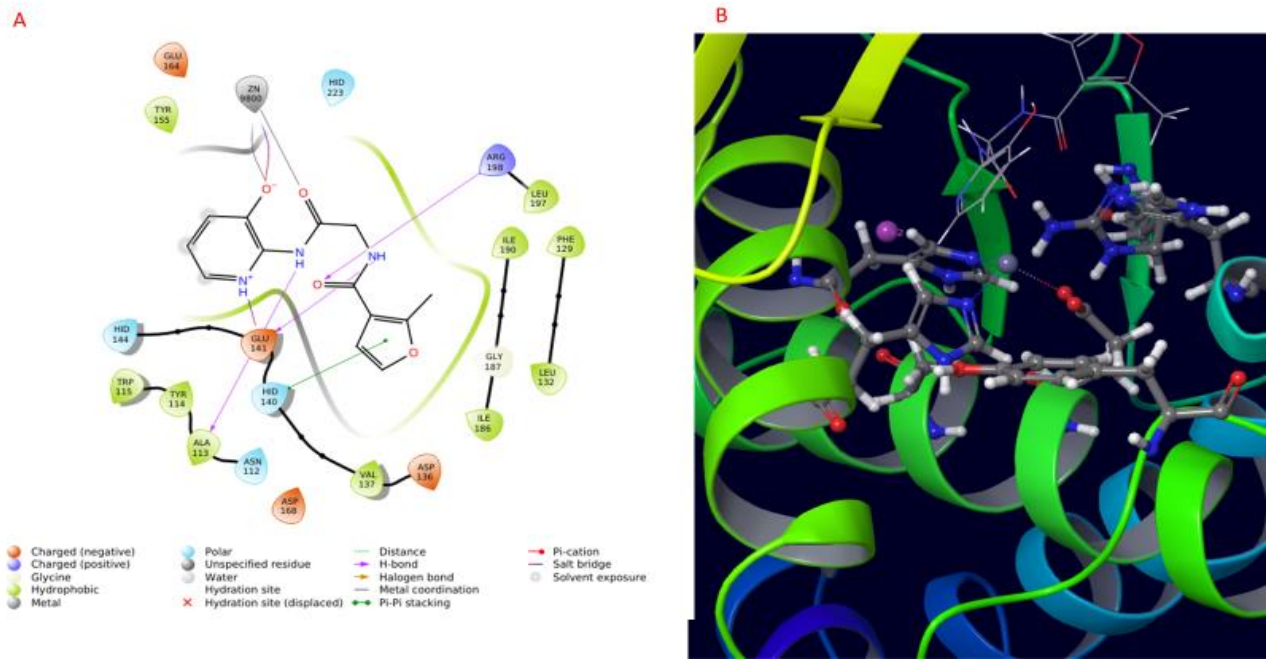



**Figure 21**: The PLN-compound 17 complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of PLN with compound 17. All interactions of compound 17 with the PLN are indicated by different colors of the arrows at the bottom of the diagram. Figure (B): 3D representation of the interaction of PLN with compound 17. Zinc is displayed in dark grey color. The side chain of the important amino acids for the binding of the ligand are displayed with the following color coding of the atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

**Table 7**: Amino acids of PLN directly involved in ligand binding.

| Ligands | Amino acids in PLN | Atomic distance between catalytic zinc and closest oxygen atom (Å) |
|---|---|---|
| Galardin | Glu-141, Arg-198, Asn-112, Ala-113 | 2.09 |
| Compound 17 | Ala-113, Glu-141, Hid-140, Arg-198 | 2.16 |

### 3.2.3 Molecular interactions between ALN and ligands

For docking and MMGBSA calculations of ALN, compounds 5, 10, 17 were selected, and compared with the binding mode of the known inhibitor galardin. When ALN was docked with galardin, the amino acids Ala-115 and Trp-117 were forming hydrogen bonds with NH-groups of galardin, while Asn-114 was forming a hydrogen bond with an oxygen of the compound (figure 22). The zinc atom was forming a metal coordination with an oxygen of the galardin at an atomic distance of 2.15Å. The MMGBSA score was -24 kcal/mol (table 10).

When ALN was docked with compound 17, the amino acid Ala-115, was forming a hydrogen bond with a NH group of the compound (figure 23). Hid-144, Hid-148 and Tyr-159 were forming a Pi-Pi stacking interactions with the two ring systems of the compound. The catalytic zinc atom was forming a strong metal coordination with an oxygen atom of the compound separated by a distance of 2.18Å (Table 8, figure 23). The MMGBSA score of the ALN-compound 17 complex was -9.19 kcal/mol (table 10).

The docking mode of compound 10 with ALN is shown in figure 24. The amino acids Glu-145 and Glu-168 were forming salt bridges with the $N^+$ of the compound, and Hid-148 and Tyr-159 were forming Pi-Pi stacking within the benzene ring of the compound. The catalytic zinc atom was forming a strong metal coordination with two oxygen atoms of the compound separated by a distance of 2.24Å (table 8). The MMGBSA score was -2.83 kcal/mol (table 10).

The binding mode of compound 5 after MMGBSA calculations is shown in figure 25. The amino acids Arg-200 was forming a hydrogen bond with an oxygen atom of the compound. Tyr-159 and Hid-148 were forming the Pi-Pi stacking with the benzene ring of the compound. The catalytic zinc atom was forming coordination with two oxygen atoms at a distance of 2.18 Å to the closest oxygen atom (table 8). The MMGBSA score was 0.98 kcal/mol (table 10). The detail description is shown in the table 8 and figure 22-25.

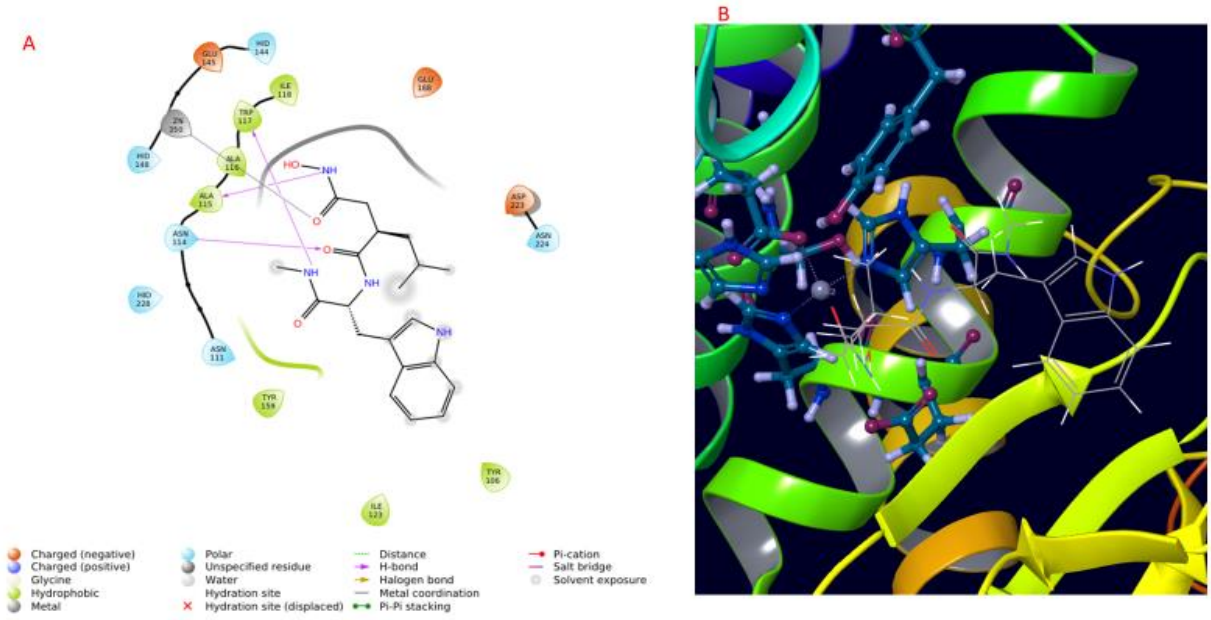



**Figure 22**: The ALN-galardin complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of the interaction of ALN with galardin. All types of interactions of galardin with ALN are indicated by different colors of the arrows shown at the bottom of the diagram. Figure (B): 3D representation of the interaction of ALN with galardin. Zinc is displayed in dark grey color. The side chains of the most important amino acids for ligand binding are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

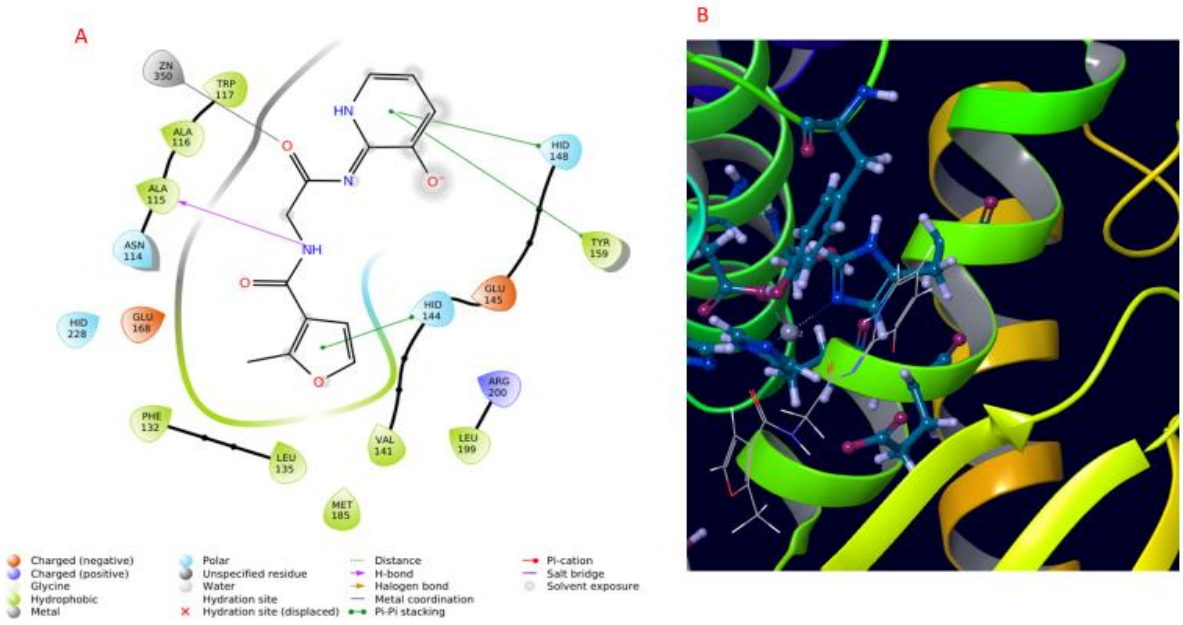



**Figure 23**: The ALN-compound 17 complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of ALN with compound 17. All interactions of compound 17 with the PLN are indicated by different colors of the arrows shown at the bottom of the diagram. Figure (B): 3D representation of the interaction of ALN with compound 17. Zinc is displayed in dark grey color. Side chains of the important amino acids for ligand binding are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

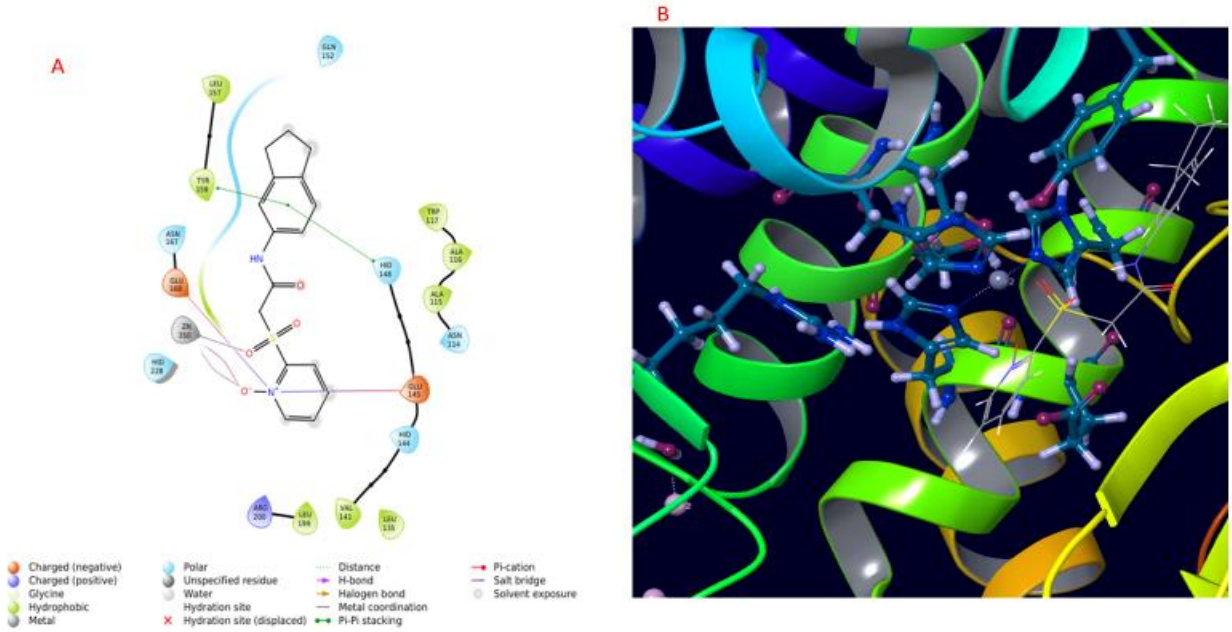



**Figure 24**: The ALN-compound 10 complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of the interaction of ALN with compound 10. All interactions of compound 10 with ALN are indicated by the arrows as shown at the bottom of the diagram. Figure (B): 3D representation of the interaction of ALN with compound 10. Zinc is displayed in dark grey color. The side chains of the most important amino acids for the binding compound 10 are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

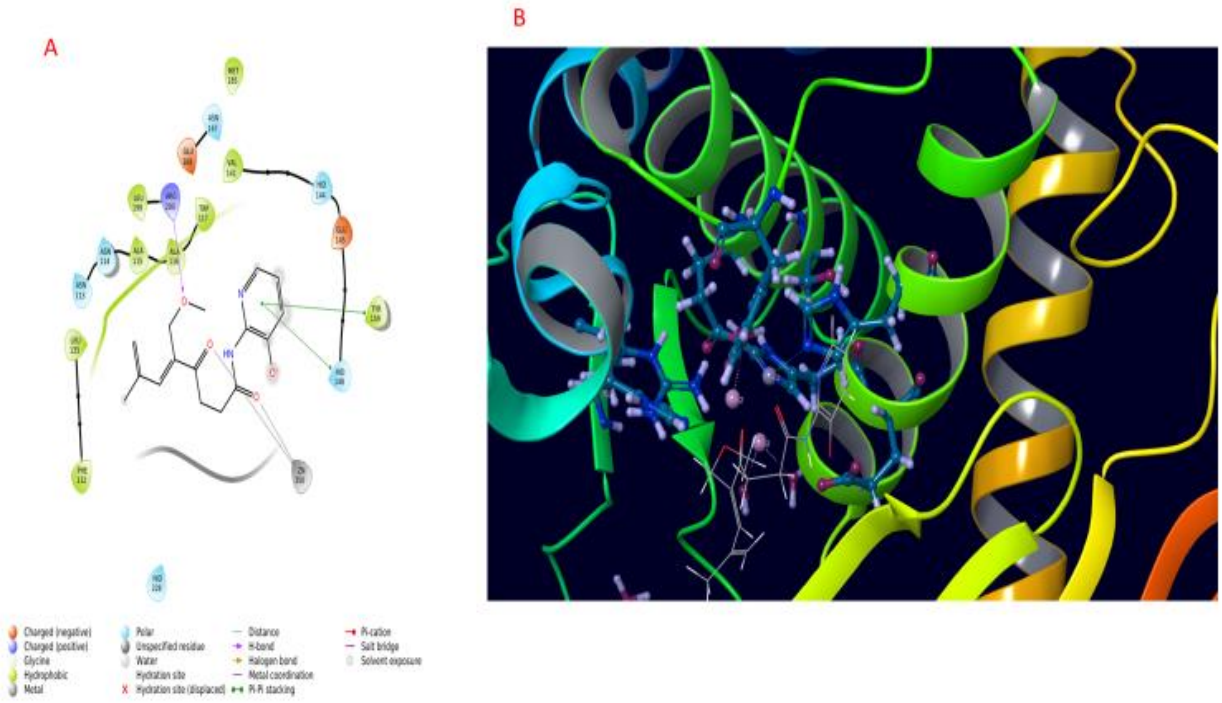



**Figure 25**: The ALN-compound 5 complex after MMGBSA calculations. Figure (A): 2D ligand interaction diagram of ALN with compound 5. All the interactions of compound 5 with ALN are indicated by different colors of the arrows as shown at the bottom of the diagram. Figure (B): 3D representation of the interaction of ALN with compound 5. Zinc is displayed in dark grey color. The side chain of the most important amino acids for the binding of compound 5 are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

**Table 8**: Amino acids in ALN directly involved in ligand binding.

| Ligands | Amino acids in ALN | Atomic distance between catalytic zinc and closest oxygen atom (Å) |
|---|---|---|
| Galardin | Asn-114, Ala-115, Trp-117 | 2.15 |
| Compound 17 | Hid-144, Hid-148, Ala-115, Tyr-159 | 2.18 |
| Compound 10 | Hid-148, Glu-145, Glu-145, Tyr-159 | 2.24 |
| Compound 5 | Hid-148, Tyr-159, Arg-200 | 2.18 |

### 3.2.4 Molecular interactions between MMP-14 and ligands

For the docking and MMGBSA calculations of MMP-14, compounds 5, 10, 17 were selected and compared with the binding mode of galardin. The most important amino acids for ligand binding to MMP-14 are indicated in table 9. When MMP-14 was docked with galardin (figure 26) the amino acids, Tyr-261 and Leu-199 were forming hydrogen bonds with the two different oxygen atoms of the compound. Gly-197, Ala-200 and Pro-259 were forming a hydrogen bond with different NH groups the compound. Similarly, Glu-240 was also forming a hydrogen bond with OH group the compound. The zinc atom was forming coordination with an oxygen atom of galardin. The MMGBSA score was -31.3 kcal/mol indicating a very strong binding (table 10).

The binding mode of compound 17 after MGBSA calculation is shown in figure 27. The amino acids Ala-200 was forming hydrogen bond with a NH group of the compound. Hid-239 was forming a Pi-cationic interaction with NH group of the compound. Hid-249, Phe-204 and Hid-243 were forming a Pi-Pi stacking within the five membered ring structure of the compound. The zinc atom was forming a coordination with an oxygen atom at a distance of 2.14 Å. The MMGBSA score was -1.4 kcal/mol (table 10). The binding mode of compound 10 after MMGBSA calculations is shown in figure 28. The amino acids Glu-240 formed a salt bridge with nitrogen atom of the six membered ring structure of the compound. Tyr-267 was forming a Pi-Pi stacking with the six membered ring structure of the compound. The zinc atom was

coordinating with an oxygen atom, at a distance of 2.14Å (table 9). The MMGBSA score was -9.12 kcal/mol (table 10). The binding mode of compound 5 after MMGBSA calculations is shown in figure 29. The amino acids Leu-199 was forming a hydrogen bond with an oxygen atom within the compound. Hid-243 was forming a Pi-Pi stacking with the six membered ring structure of the compound. Zinc atom was forming metal coordination with two different oxygen atoms of the compound separated by a distanced of 2.16 Å. The MMGBSA score was -2.5 kcal/mol. The detail description is shown in the table 9 and figure 26-29.

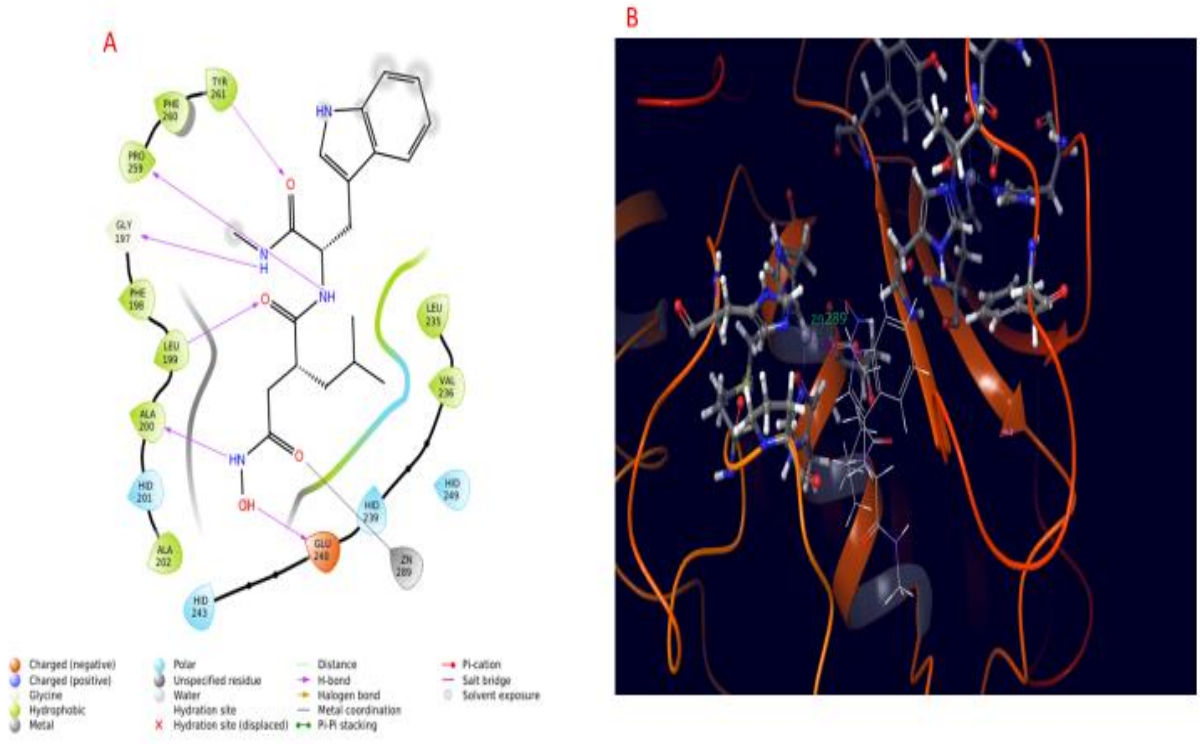



**Figure 26**: The MMP-14-galardin complex after MMGBSA calculation. Figure (A): 2D ligand interaction diagram of MMP-14 with galardin. All interactions of galardin with MMP-14 are indicated by the arrows shown at the bottom of the diagram. Figure (B): 3D representation of the interaction of MMP-14 with galardin. Zinc is displayed in dark grey color. The side chain of the most important amino acids for the binding of galardin are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

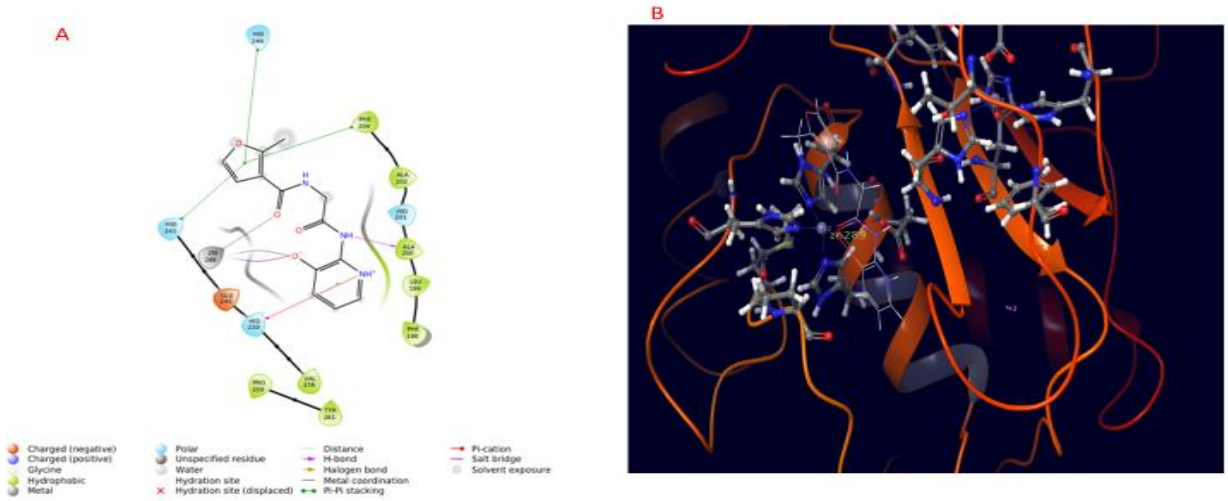



**Figure 27**: The MMP-14 compound 17 complex after MMGBSA calculation. Figure (A): 2D ligand interaction diagram of MMP-14 with compound 17. All interactions of the compound with MMP-14 are indicated by arrows shown at the bottom of the diagram. Figure (B): 3D representation of the interactions of MMP-14 with compound 17. Zinc is displayed in dark grey color. The side chains of the most important amino acids for ligand binding are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

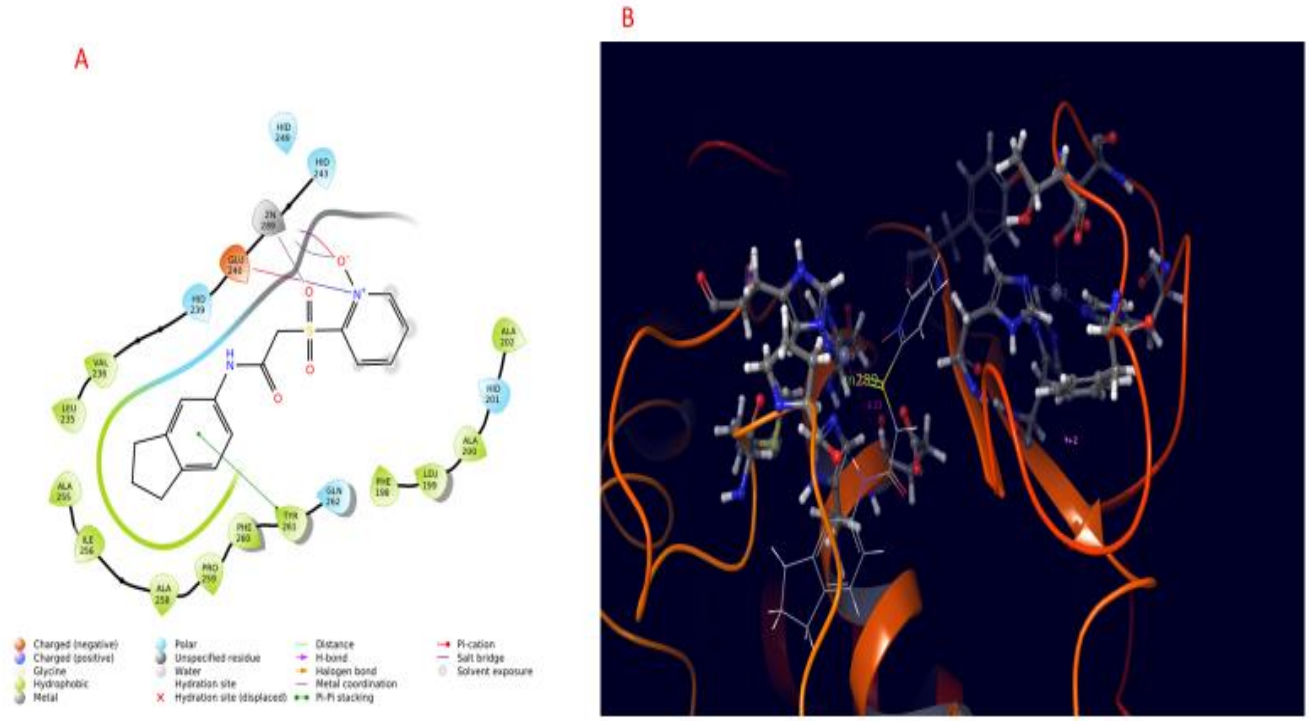



**Figure 28**: The complex of MMP-14 with compound 14 after MMGBSA calculation. Figure (A): 2D ligand interaction diagram of MMP-14 with compound 10. All interactions of the compound with the MMP-14 are indicated the arrows shown at the bottom of the diagram. Figure (B): 3D representation of the interactions of MMP-14 with compound 10. Zinc is displayed in dark grey color. The most important side chains for binding of the ligand are displayed with the following color coding of the atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

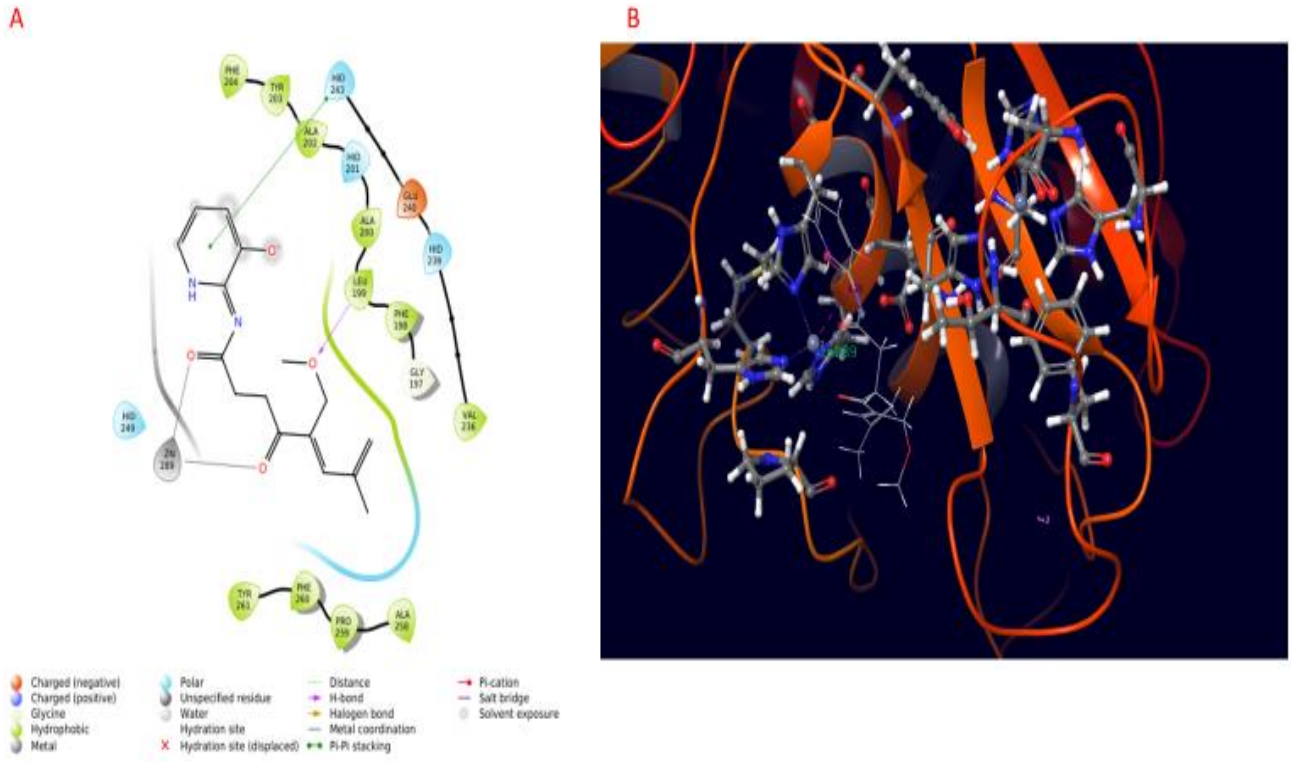



**Figure 29**: The MMP-14 - compound 5 complex after MMGBSA calculation. Figure (A): 2D ligand interaction diagram of MMP-14 with compound 5. All interactions of the compound with MMP-14 are indicated by the arrows shown at the bottom of the diagram. Figure (B): 3D representation of the interactions of MMP-14 with compound 5. Zinc is displayed in dark grey color. The most important side chains for ligand binding are displayed with the following color coding of atoms: oxygen: red, nitrogen: blue, hydrogen: grey, carbon: white and color coding of the ligand atoms: oxygen: red, nitrogen: blue, carbon: white.

**Table 9**: Amino acids directly involved in ligand binding to MMP-14.

| Ligands | Amino acids in MMP-14 | Atomic distance between catalytic zinc and closest oxygen atom (Å) |
|---|---|---|
| Galardin | Tyr-261, Pro-259, Gly-197, Leu-199, Ala-200, Glu-240 | 2.13 |
| Compound 17 | Hid-249, Phe-204, Hid-243 Hid-239, Ala-200 | 2..14 |
| Compound 10 | Glu-240, Tyr-261 | 2.14 |
| Compound 5 | Hid-243, Leu-199 | 2.15 |

### 3.3 MMGBSA calculation

After the 3D analysis of the docking and MMGBSA, MMGBSA score was also our point of interest. A large negative value indicates that the compound has strong affinity for the enzyme. The values were obtained from the Schrodinger software after the analysis on the basis of the settings and conditions for docking and MMGBSA, as described in the methods section. After the analysis, galardin which is a known high affinity inhibitor of zinc metalloproteases was found to have the most negative value of all selected compounds. Galardin had a MMGBSA score of -16.12 kcal/mol for PLN, -15.87 kcal/mol for TLN, -24.00 kcal/mol for ALN and -31.38 kcal/mol for MMP-14. For PLN, the MMGBSA value of compound 17 was -3.45 kcal/mol. For the enzyme ALN, an MMGBSA value of 0.98 kcal/mol was obtained for compound 5, -2.83 kcal/mol for compound 10 and -9.19 kcal/mol for compound 17. For MMP-14, an MMGBSA value of -1.4 kcal/mol was obtained for compound 5, -9.42 kcal/mol for compound 10 and -2.5 kcal/mol for compound 17. The obtained result is shown in table 10.

**Table 10**: Obtained MMGBSA values (kcal/mol) of selected compounds from figure 7-14 (compound 5, 10, 17 and galardin) with PLN, ALN, TLN and MMP-14.

| Compound | PLN | TLN | ALN | MMP-14 |
|---|---|---|---|---|
| Galardin | -16.12 | -15.87 | -24 | -31.38 |
| Compound 5 | NC* | -6.11 | 0.98 | -1.4 |
| Compound 10 | NC* | -6.4 | -2.83 | -9.42 |
| Compound 17 | -3.45 | -8.4 | -9.19 | -2.5 |

*NC- not calculated.

# 4 Discussion

## 4.1 Structural differences in active site of zinc metalloproteases

In the case of bacterial zinc metalloproteases, the active site cleft of different enzymes has structural similarities with human zinc metalloproteases. Therefore, a major challenge for developing drugs targeting bacterial zinc metalloproteases is to identify compounds that only inhibit bacterial zinc metalloproteases without inhibiting human metalloproteases. Binding to human zinc metalloproteases may give off-target effects and putative drug side effects. If we look at the S1' sub-pocket of the human and bacterial zinc metalloproteases studied in the present project, it can be seen they favor both small and large hydrophobic substrate side chains at P1' position of the substrate (figure 1). One of the differences identified between the M4 family and the MMPs is a conserved arginine residue (corresponding to Arg-198 in PLN, Arg-203 in PLN and Arg-200 in ALN) situated at the edge of S1' sub-pocket in the M4 enzymes, which is absent in MMPs. Many enzyme kinetic and molecular modeling studies indicate that arginine may be an essential amino acid for high affinity binding to of inhibitors for M4 enzymes (86). In the present study docking and MMGBSA calculations indicated that this arginine is important for binding of galardine to PLN and TLN, but not for ALN. This is in agreement with previous studies indicating that galardine is a stronger inhibitor of PLN and TLN than of ALN (85). However, some of the low affinity compounds in the present study interacted with the arginine as well.

## 4.2 Dual role of MMPs

The role of the MMPs in different pathological conditions as well as in the physiological functions of our body is also one of the important elements that may hinder in the development of drugs against the bacterial virulence factors studied in the present thesis. MMPs are involved in different physiological processes (reproduction, immune response) in our body, and are expressed in different pathological conditions (viral infections, cancer) (87). Since the role of the MMPs in the pathological conditions were discovered before their role in the physiological process, around half a century ago different pharmacological companies started to develop the specific inhibitors by inhibiting the activity of MMPs by focusing on specific diseases. But the

majority of the inhibitors were not approved as drugs because of side effects. There may be many reasons for their disapproval including lack of selectivity. Another reason may be the dual role of the MMPs in different pathological conditions, where one or many MMPs can increase a disease or protect against a disease. This dual role is mainly due to availability of many substrate attachment sites on the enzyme, in which one substrate can help in progression and other substrate leading to the protection of the disease in different stages of disease (33, 88, 89).

### 4.3 Interactions of ligands with zinc metalloproteases

In the present study we have tested 26 compounds obtained by a previous VLS campaign in the research group, using PLN as the target (unpublished). In the VLS campaign, the compounds were selected according to a relatively high docking score compared with known PLN inhibitors, and that they had a binding mode resembling known PLN inhibitors. In the present thesis, the compounds were tested for their inhibition of PLN, TLN, ALN and MMP-14 using enzyme inhibition studies and molecular modeling. The results of the inhibition kinetics experiments showed that none of the compounds inhibited the enzyme activity of the zine metalloproteases with more than 50%. Compounds 5, 10 and 17 (table 1) were found to inhibit the activity of TLN, ALN and MMP-14 by only 30% while only compound 5 was able to reduce activity of PLN by 40%. The VLS approach was based on a combined ligand based and structure-based approach. In the ligand-based approach pharmacophore models were generated based on 97 known PLN inhibitors from the literature. The dataset of known inhibitors contained structurally quite divergent molecules with a large range of affinities, ranging from 41 nM of phosphoramidone (PDB id: 3DBK) into the mM range. A better approach might have been to focus on the known compounds with highest affinity. However, such an approach would most probably result in hits with large structural similarities with well-known strong affinity inhbitors, but any new structural scaffolds for binding would not be obtained (90).

Compounds 5,10,17 in addition to high affinity binder galardin ( were docked into TLN, PLN, ALN and MMP-14 and MMGBSA calculations were performed in order to study the differences in binding modes between the high affinity compounds and the low affinity inhibitors (compound 5,10 and 17). To become a strong reversible inhibitor and PLN and TLN

amino acids corresponding to Arg198, Glu141 and Asn112 in PLN are presumed to interact with the ligand (90). The molecular docking studies and MMGBSA calculations suggested that galardin binds stronger to PLN and TLN than to ALN which is confirmed by previous experimental studies(85).

The docking of TLN with galardin showed that galardin fits nicely into the binding pocket (figure 16). The important amino acids involved in the interaction with the ligand were Glu-166, His-231, Ala-113, Asn-112, Arg-203 and Asp-226 in addition to zinc, and this interaction gave a MMGBSA score of -15.87 kcal/mol (figure 16 and table 10). When these interactions were compared with those of compound 17, the interaction of His-231, which forms a strong hydrogen bond with galardin and Asp-226 which stabilize a ring system of galardin (figure 16) are lacking for compound 17, which may explain the lower MMGBSA score (-8.4 kcal/mol) and affinity of compound 17 than of galardin (figure 17 and table 10). Similarly, if we look at the interaction of compound 10 with TLN the important amino acid Asn-112 for high affinity binding was lacking as compared to galardin which was forming a double hydrogen bond with two NH group of the ligand. So, the lack of this interaction of compound 10 may explain the lower MMGBSA score (figure 18 and table 10) and the lower affinity relative to galardin. Likewise, compound 5 lacks interactions with Arg-203 and His-231 in TLN, which were forming strong interactions with galardin.

Docking and MMGBSA calculation of PLN with galardin indicated that galardin fits into the binding pocket of PLN. The amino acids Arg-198, Glu-141, Ala-113 and Asn-112 were found to be the important amino acids involved in different molecular interactions contributing to a strong binding (figure 20). The molecular binding of PLN with compound 17 indicated several interactions, but only the interaction with Glu-141 was common with that of galardin, and all other interactions were different, which may explain the lower affinity and MMGBSA score (-3.45 kcal/mol) of compound 17 (figure 21 and tables 7, 10).

The binding mode of galardin against ALN was found to be similar to that of PLN but the interaction was found to be weaker than for PLN (table 10). Amino acids directly involved in the hydrogen bonding with galardin were Asn-114, Trp-117 and Ala-115 contributing to strong interactions. But due to the absence of the interactions of amino acids corresponding Glu-141 and Arg-198 in PLN (table 8) the interactions are somehow weaker than in PLN (figure 22). If

we look at the binding modes of compound 5, 10 and 17 also only one hydrogen bond with a single amino acid (Ala-115) while other main interactions were by Pi-Pi stacking with Tyr-159 and Hid-148, which might contribute to the much lower affinity than for galardin (figure 23). However, the docking also indicated that compound 10 formed salt bridges with Glu-145 and Glu-268 that should contribute to strong binding. It is therefore hard to explain the low affinity of compound 10 based on the docking mode (figure 24). In the case of compound 5 there was only one amino acid (Arg-200) involved in the formation of hydrogen bond with the compound, while other amino acids (Hid-148 and Tyr-159) were connected by Pi-Pi stacking that may altogether give quite low binding affinity for ALN (figure 25 and table 8).

When galardin was docked with MMP-14 it was found to fit properly into the binding pocket with a high MMGBSA score of -31.38 kcal/mol. The important amino acids involved in the interaction (hydrogen bonds) were Tyr-261, Pro-259, Gly-197, Leu-199, Ala-200 and Glu-240 (figure 26 and table 9). In the case of docking of compound 17 with MMP-14 only Ala-200 of those amino acids was found to be involved with galardin was interacting directly with compound 17, which may explain the weak inhibition and the low MMGBSA score of -2.5 kcal/mol (figure 27 and tables 9, 10). Similarly, in the case of docking of compound 10 with MMP-14 only amino acid Glu-240 was involved in Pi-cationic interaction with the ligand, and Tyr-261 was forming a Pi-Pi interactions with the ligand giving a MMGBSA score of -9.42 kcal/mol, which is quite weak compared with galardin (figure 28, tables 9, 10). For compound 5 only Leu-199 of those involved in galardin binding was interacting directly with compound 5, while other interactions seen for galardin were absent, which may explain the low MMGBSA score of -1.4 kcal/mol and the low inhibition compared to galardin (figure 29, tables 9, 10).

### 4.4 Challenges in developing inhibitors for zinc metalloproteases

The results from MMGBSA showed that there was a variation of results in the score for different enzymes. In-spite of having the highest inhibition in the wet lab, some compounds showed a low MMGBSA score. There might be many reasons for this. The most probable reasons could be that the inhibitors (compounds) could not fit on the active site of the enzyme. Some of the compounds which were chosen from VLS were not available from Molport exactly in the same structure/enantiomer as suggested by VLS as proper inhibitors. Some of the

compounds that were chosen for experimental testing therefore had small structural deviations from the molecule suggested by the VLS study as proper inhibitor. Another reason that may influence the result of the inhibition kinetics experiment was putative human errors. In almost every step like dilution, pipetting and mixing the manual process is done by using pipettes by hand which may vary the result. There is also a chance of cross contamination between the reagents which also may give wrong results (91). The use of multi- channel pipette, uncalibrated pipette and variable volume pipette may also vary the result (92, 93).

There might be several other reasons for finding the specific inhibitors for human and bacterial metalloproteases because of unique features in each of the proteases. So, to find the specific inhibitors we have to overcome the different challenges in the design of the inhibitors. The reasons for the variance of the result might be due to difference in the specificity of the substrate between the metalloproteases in which some are found to be overlapping and some to be totally different. If we look at the history of the nomenclature of the MMPs, they were first named according to their main substrate (collagenase, gelatinase) but later they were named matrix metalloproteases after the discovery of cleaving of extracellular matrix proteins by them. The number tag is given following the timeline of discovery (94). In the broad sense it can be viewed that MMPs have overlapping substrate specificity but if we view the detail structure of cleft of the active site, it can be seen similar but not exactly the same (95).

### 4.5 Possible solutions to overcome the challenges

To overcome these challenges and reduce the possibility for human errors, there should be novel strategies with the advancement in the working techniques by minimizing the use of primitive methods and increasing the use of automation techniques (96). Another approach may be to target the exosites (site outside the cleft of active site which helps in substrate binding and orientation), which would help to control the in-vivo selectivity of MMPs (97, 98). It has been found that each MMPs have their own inhibitory sites (hotspots) which can also be a site for selective inhibition. Designing the inhibitors targeting such hotspots help in rigidification of flexibility of the cavities (protein surface) lead to the development of selective inhibitors for MMPs (99, 100). Similarly, formation of antibody based inhibitors which target the catalytic zinc complex is also a technique that may help to increase the therapeutic potential and to

minimize the risk factors in the designing of new inhibitors for MMPs (101). Likewise, macromolecular inhibition of the MMPs by utilizing the pro-domain also help in development of selective and potent inhibitors of MMPs (102).

Although the above techniques for the designing of the inhibitors are selective but those selected inhibitors might have other disadvantages like newly designed inhibitors might not be tissue specific and they might affect in pathological pathways during homeostasis. So, for the further improvement the technique can be combined with protein engineering and directed evolution technique. This technique is proved to be widely used in study of enzyme substrate specificity, protein evolution and protein-protein interaction because of its reliability and specificity (103-105). For enhancing the specificity and ability of the inhibitors, engineering and targeting the Tissue Inhibitors of Metalloproteases (TIMPs) is also one of the techniques used in designing various inhibitors with unique specificity and reducing the side effects in different members of MMPs (106, 107). Since the most commonly used strategy for development of potent inhibitors for zinc metalloproteases depends on the core zinc atom and peptide and nonpeptidic scaffold for additional interactions for the increasement of selectivity and potency, this technique mostly fails in the development of potent inhibitors for zinc metalloproteases as zinc metalloproteases consists of highly conserved domains. Another strategy to enhance the selectivity and potency of the zinc metalloproteases can be variation of zinc binding groups which decrease the chance of missing the target due to interplay between enthalpy and entropy for same scaffold. So, variation of zinc binding sites is also one of the preferable techniques for development of effective inhibitors(108).

## 5. Conclusion

The present project is focused on the identification of compounds that can be developed into putative drugs by the use of molecular modeling and enzyme kinetics studies. None of the compounds were identified as putative drug candidates for the MMPs and the M4 family. We found at most only 40% of inhibition by compound 17 against PLN. The inhibition activities of the compounds 5, 10 and17 for TLN, ALN and MMP-14 were around 30%. Most of the tested compounds did not inhibit the enzymes at all. So, compounds 5, 10, and 17 were considered as weak inhibitors, not good enough for being taken for the further steps to calculate $IC_{50}$ value and $K_i$ values. There are many challenges in designing of compounds as possible drugs that do not hamper any other physiological function. The dual role of MMPs in pathological and physiological conditions is one of the major obstacles as they can act as drug and anti-drug targets. In the case of developing an inhibitor for a bacterial zinc metalloprotease a major challenge is to design a compound that should only strongly bind to the bacterial enzymes and not interfere in any physiological function of the human (should not bind on the active site of the human zinc metalloproteases).

## 6. Future prospective

In the coming future this project can be continued with different prospects. Since the compounds identified as weak inhibitors in this project are not structurally similar to known inhibitors in the literature, we can search for different structural analogs of the most promising low affinity inhibitors (for example compound 17, 10 or 5) in order to obtain compounds with stronger inhibition of the zinc metalloproteases. This can be done by using different databases (like Molport, ChEMBL and zinc databases of known drugs) which may provide putative structural analogs of the compounds, which then can be tested in lab, and by using molecular modeling. Likewise, another possibility can be use of structure- based drug design for structural modification of the most promising compounds in the binding site after docking, and then obtain the new molecule by synthesis before testing in assays. Another prospective can be testing of present compounds and compounds obtained from searching analogs and structural based drug design with additional human and bacterial metalloproteases.

**References**


1. Aminov RI. A brief history of the antibiotic era: lessons learned and challenges for the future. Front Microbiol. 2010;1:134.
2. Mohr KI. History of Antibiotics Research. In: Stadler M, Dersch P, editors. How to Overcome the Antibiotic Crisis : Facts, Challenges, Technologies and Future Perspectives. Cham: Springer International Publishing; 2016. p. 237-72.
3. Meek RW, Vyas H, Piddock LJV. Nonmedical Uses of Antibiotics: Time to Restrict Their Use? PLOS Biology. 2015;13(10):e1002266.
4. Uddin TM, Chakraborty AJ, Khusro A, Zidan BMRM, Mitra S, Emran TB, et al. Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. Journal of Infection and Public Health. 2021.
5. Cook M, Molto E, Anderson C. Fluorochrome labelling in Roman period skeletons from Dakhleh Oasis, Egypt. Am J Phys Anthropol. 1989;80(2):137-43.
6. Bassett EJ, Keith MS, Armelagos GJ, Martin DL, Villanueva AR. Tetracycline-labeled human bone from ancient Sudanese Nubia (A.D. 350). Science. 1980;209(4464):1532-4.
7. Cui L, Su XZ. Discovery, mechanisms of action and combination therapy of artemisinin. Expert Rev Anti Infect Ther. 2009;7(8):999-1013.
8. Aminov R. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. Frontiers in Microbiology. 2010;1(134).
9. Machowska A, Stålsby Lundborg C. Drivers of Irrational Use of Antibiotics in Europe. Int J Environ Res Public Health. 2018;16(1):27.
10. Organization WH. WHO publishes list of bacteria for which new antibiotics are urgently needed. 2017.
11. Bodey GP, Bolivar R, Fainstein V, Jadeja L. Infections Caused by Pseudomonas aeruginosa. Reviews of Infectious Diseases. 1983;5(2):279-313.
12. Lyczak JB, Cannon CL, Pier GB. Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist1*Address for correspondence: Channing Laboratory, 181 Longwood Avenue, Boston, MA 02115, USA. Microbes and Infection. 2000;2(9):1051-60.
13. Gellatly SL, Hancock RE. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathog Dis. 2013;67(3):159-73.
14. Codagnone M, Cianci E, Lamolinara A, Mari VC, Nespoli A, Isopi E, et al. Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. Mucosal Immunol. 2018;11(1):35-49.
15. Kung VL, Ozer EA, Hauser AR. The accessory genome of Pseudomonas aeruginosa. Microbiol Mol Biol Rev. 2010;74(4):621-41.
16. Frimmersdorf E, Horatzek S, Pelnikevich A, Wiehlmann L, Schomburg D. How Pseudomonas aeruginosa adapts to various environments: a metabolomic approach. Environmental Microbiology. 2010;12(6):1734-47.
17. Suh SJ, Runyen-Janecky LJ, Maleniak TC, Hager P, MacGregor CH, Zielinski-Mozny NA, et al. Effect of vfr mutation on global gene expression and catabolite repression control of Pseudomonas aeruginosa. Microbiology (Reading). 2002;148(Pt 5):1561-9.

18. Wolfgang MC, Kulasekara BR, Liang X, Boyd D, Wu K, Yang Q, et al. Conservation of genome content and virulence determinants among clinical and environmental isolates of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2003;100(14):8484-9.
19. Juhas M, van der Meer JR, Gaillard M, Harding RM, Hood DW, Crook DW. Genomic islands: tools of bacterial horizontal gene transfer and evolution. FEMS Microbiol Rev. 2009;33(2):376-93.
20. Ballok AE, O'Toole GA. Pouring salt on a wound: Pseudomonas aeruginosa virulence factors alter Na+ and Cl- flux in the lung. Journal of bacteriology. 2013;195(18):4013-9.
21. Maeda H. Role of microbial proteases in pathogenesis. Microbiol Immunol. 1996;40(10):685-99.
22. Dickey SW, Cheung GYC, Otto M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. Nat Rev Drug Discov. 2017;16(7):457-71.
23. Munguia J, Nizet V. Pharmacological Targeting of the Host-Pathogen Interaction: Alternatives to Classical Antibiotics to Combat Drug-Resistant Superbugs. Trends Pharmacol Sci. 2017;38(5):473-88.
24. Hauser AR, Mecsas J, Moir DT. Beyond Antibiotics: New Therapeutic Approaches for Bacterial Infections. Clin Infect Dis. 2016;63(1):89-95.
25. Cross AS. What is a virulence factor? Critical Care. 2008;12(6):196.
26. Rahman F, Nguyen T-M, Adekoya O, Campestre C, Tortorella P, Sylte I, et al. Inhibition of bacterial and human zinc-metalloproteases by bisphosphonate- and catechol-containing compounds. Journal of Enzyme Inhibition and Medicinal Chemistry. 2021;36:819-30.
27. López-Otín C, Bond JS. Proteases: multifunctional enzymes in life and disease. The Journal of biological chemistry. 2008;283(45):30433-7.
28. King JV, Liang WG, Scherpelz KP, Schilling AB, Meredith SC, Tang WJ. Molecular basis of substrate recognition and degradation by human presequence protease. Structure. 2014;22(7):996-1007.
29. Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. Nucleic Acids Research. 2018;46(D1):D624-D32.
30. De Groef L, Van Hove I, Dekeyster E, Stalmans I, Moons L. MMPs in the neuroretina and optic nerve: modulators of glaucoma pathogenesis and repair? Invest Ophthalmol Vis Sci. 2014;55(3):1953-64.
31. Wolberg AS, Mast AE. Tissue factor and factor VIIa--hemostasis and beyond. Thromb Res. 2012;129 Suppl 2(Suppl 2):S1-4.
32. Quirós PM, Langer T, López-Otín C. New roles for mitochondrial proteases in health, ageing and disease. Nat Rev Mol Cell Biol. 2015;16(6):345-59.
33. Hadler-Olsen E, Fadnes B, Sylte I, Uhlin-Hansen L, Winberg JO. Regulation of matrix metalloproteinase activity in health and disease. Febs j. 2011;278(1):28-45.
34. Overall CM, Blobel CP. In search of partners: linking extracellular proteases to substrates. Nat Rev Mol Cell Biol. 2007;8(3):245-57.
35. Bode W, Gomis-Rüth FX, Stöckler W. Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. FEBS Lett. 1993;331(1-2):134-40.
36. Rahman F, Nguyen T-M, Adekoya OA, Campestre C, Tortorella P, Sylte I, et al. Inhibition of bacterial and human zinc-metalloproteases by bisphosphonate- and catechol-

containing compounds. Journal of Enzyme Inhibition and Medicinal Chemistry. 2021;36(1):819-30.
37. Sabeh F, Li X-Y, Saunders TL, Rowe RG, Weiss SJ. Secreted Versus Membrane-anchored Collagenases: RELATIVE ROLES IN FIBROBLAST-DEPENDENT COLLAGENOLYSIS AND INVASION*. Journal of Biological Chemistry. 2009;284(34):23001-11.
38. Snyman C, Niesler CU. MMP-14 in skeletal muscle repair. J Muscle Res Cell Motil. 2015;36(3):215-25.
39. Häse CC, Finkelstein RA. Bacterial extracellular zinc-containing metalloproteases. Microbiol Rev. 1993;57(4):823-37.
40. Hooper NM. Families of zinc metalloproteases. FEBS Lett. 1994;354(1):1-6.
41. Adekoya OA, Sylte I. The thermolysin family (M4) of enzymes: therapeutic and biotechnological potential. Chem Biol Drug Des. 2009;73(1):7-16.
42. Gomis-Rüth FX, Botelho TO, Bode W. A standard orientation for metallopeptidases. Biochim Biophys Acta. 2012;1824(1):157-63.
43. Wang Y, Liu B-X, Cheng J-H, Su H-N, Sun H-M, Li C-Y, et al. Characterization of a New M4 Metalloprotease With Collagen-Swelling Ability From Marine Vibrio pomeroyi Strain 12613. Frontiers in Microbiology. 2020;11(1868).
44. Ooshima H, Mori H, Harano Y. Synthesis of aspartame precursor by solid thermolysin in organic solvent. Biotechnology Letters. 1985;7(11):789-92.
45. Jurado-Martín I, Sainz-Mejías M, McClean S. Pseudomonas aeruginosa: An Audacious Pathogen with an Adaptable Arsenal of Virulence Factors. International Journal of Molecular Sciences. 2021;22(6):3128.
46. Kessle E, Ohman DE. 102 - Pseudolysin. In: Barrett AJ, Rawlings ND, Woessner JF, editors. Handbook of Proteolytic Enzymes (Second Edition). London: Academic Press; 2004. p. 401-9.
47. Yeats C, Rawlings ND, Bateman A. The PepSY domain: a regulator of peptidase activity in the microbial environment? Trends Biochem Sci. 2004;29(4):169-72.
48. Adekoya OA, Sylte I. The Thermolysin Family (M4) of Enzymes: Therapeutic and Biotechnological Potential. Chemical Biology & Drug Design. 2009;73(1):7-16.
49. Heinrikson RL. Applications of thermolysin in protein structural analysis. Methods Enzymol. 1977;47:175-89.
50. Ligné T, Pauthe E, Monti JP, Gacel G, Larreta-Garde V. Additional data about thermolysin specificity in buffer- and glycerol-containing media. Biochim Biophys Acta. 1997;1337(1):143-8.
51. Potempa J, Travis J. 96 - Aureolysin. In: Barrett AJ, Rawlings ND, Woessner JF, editors. Handbook of Proteolytic Enzymes (Second Edition). London: Academic Press; 2004. p. 389-93.
52. Galloway DR. Pseudomonas aeruginosa elastase and elastolysis revisited: recent developments. Mol Microbiol. 1991;5(10):2315-21.
53. Thayer MM, Flaherty KM, McKay DB. Three-dimensional structure of the elastase of Pseudomonas aeruginosa at 1.5-A resolution. J Biol Chem. 1991;266(5):2864-71.
54. Matthews BW. Structural basis of the action of thermolysin and related zinc peptidases. Accounts of Chemical research. 1988;21(9):333-40.
55. Banbula A, Potempa J, Travis J, Fernandez-Catalén C, Mann K, Huber R, et al. Amino-acid sequence and three-dimensional structure of the Staphylococcus aureus metalloproteinase at 1.72 å resolution. Structure. 1998;6(9):1185-93.

56. Calabrese B. Cloud-Based Molecular Modeling Systems. In: Ranganathan S, Gribskov M, Nakai K, Schönbach C, editors. Encyclopedia of Bioinformatics and Computational Biology. Oxford: Academic Press; 2019. p. 261-4.
57. Silakari O, Singh PK. Chapter 6 - Molecular docking analysis: Basic technique to predict drug-receptor interactions. In: Silakari O, Singh PK, editors. Concepts and Experimental Protocols of Modelling and Informatics in Drug Design: Academic Press; 2021. p. 131-55.
58. Kitchen DB, Decornez H, Furr JR, Bajorath J. Docking and scoring in virtual screening for drug discovery: methods and applications. Nat Rev Drug Discov. 2004;3(11):935-49.
59. Wei BQ, Weaver LH, Ferrari AM, Matthews BW, Shoichet BK. Testing a flexible-receptor docking algorithm in a model binding site. J Mol Biol. 2004;337(5):1161-82.
60. Meng X-Y, Zhang H-X, Mezei M, Cui M. Molecular docking: a powerful approach for structure-based drug discovery. Curr Comput Aided Drug Des. 2011;7(2):146-57.
61. Venhorst J, ter Laak AM, Commandeur JN, Funae Y, Hiroi T, Vermeulen NP. Homology modeling of rat and human cytochrome P450 2D (CYP2D) isoforms and computational rationalization of experimental ligand-binding specificities. J Med Chem. 2003;46(1):74-86.
62. Boehm HJ, Boehringer M, Bur D, Gmuender H, Huber W, Klaus W, et al. Novel inhibitors of DNA gyrase: 3D structure based biased needle screening, hit validation by biophysical methods, and 3D guided optimization. A promising alternative to random screening. J Med Chem. 2000;43(14):2664-74.
63. Zhang X, Wong SE, Lightstone FC. Message passing interface and multithreading hybrid for parallel molecular docking of large databases on petascale high performance computing machines. J Comput Chem. 2013;34(11):915-27.
64. Plewczynski D, Łaźniewski M, Augustyniak R, Ginalski K. Can we trust docking results? Evaluation of seven commonly used programs on PDBbind database. J Comput Chem. 2011;32(4):742-55.
65. Rastelli G, Del Rio A, Degliesposti G, Sgobba M. Fast and accurate predictions of binding free energies using MM-PBSA and MM-GBSA. J Comput Chem. 2010;31(4):797-810.
66. Hou T, Wang J, Li Y, Wang W. Assessing the performance of the molecular mechanics/Poisson Boltzmann surface area and molecular mechanics/generalized Born surface area methods. II. The accuracy of ranking poses generated from docking. J Comput Chem. 2011;32(5):866-77.
67. Beveridge DL, DiCapua FM. Free energy via molecular simulation: applications to chemical and biomolecular systems. Annu Rev Biophys Biophys Chem. 1989;18:431-92.
68. Mysinger MM, Carchia M, Irwin JJ, Shoichet BK. Directory of useful decoys, enhanced (DUD-E): better ligands and decoys for better benchmarking. J Med Chem. 2012;55(14):6582-94.
69. Zhang X, Perez-Sanchez H, Lightstone FC. A Comprehensive Docking and MM/GBSA Rescoring Study of Ligand Recognition upon Binding Antithrombin. Curr Top Med Chem. 2017;17(14):1631-9.
70. Zhang X, Wong SE, Lightstone FC. Toward fully automated high performance computing drug discovery: a massively parallel virtual screening pipeline for docking and molecular mechanics/generalized Born surface area rescoring to improve enrichment. J Chem Inf Model. 2014;54(1):324-37.
71. Eble JA. Matrix biology meets toxinology. Matrix Biol. 2010;29(4):239-47.

72. Cleland WW. The kinetics of enzyme-catalyzed reactions with two or more substrates or products: I. Nomenclature and rate equations. Biochimica et Biophysica Acta (BBA) - Specialized Section on Enzymological Subjects. 1963;67:104-37.
73. Henderson PJ. A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors. Biochem J. 1972;127(2):321-33.
74. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol. 1973;22(23):3099-108.
75. Saboury AA. Enzyme inhibition and activation: A general theory. Journal of the Iranian Chemical Society. 2009;6(2):219-29.
76. Williams JW, Morrison JF. The kinetics of reversible tight-binding inhibition. Methods Enzymol. 1979;63:437-67.
77. Cha S. Tight-binding inhibitors-I. Kinetic behavior. Biochem Pharmacol. 1975;24(23):2177-85.
78. Cleland WW. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. Biochim Biophys Acta. 1963;67:104-37.
79. Krippendorff B-F, Neuhaus R, Lienau P, Reichel A, Huisinga W. Mechanism-Based Inhibition: Deriving KI and kinact Directly from Time-Dependent IC50 Values. Journal of Biomolecular Screening. 2009;14(8):913-23.
80. DeLean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. American Journal of Physiology-Endocrinology and Metabolism. 1978;235(2):E97.
81. Morrison JF. Kinetics of the reversible inhibition of enzyme-catalysed reactions by tight-binding inhibitors. Biochim Biophys Acta. 1969;185(2):269-86.
82. Kinetics of Two-Substrate Enzymatic Reactions. Kinetics of Enzyme Action2011. p. 141-68.
83. Cornish-Bowden A. One hundred years of Michaelis–Menten kinetics. Perspectives in Science. 2015;4:3-9.
84. Atkins GL, Nimmo IA. The reliability of Michaelis constants and maximum velocities estimated by using the integrated Michaelis-Menten equation. Biochem J. 1973;135(4):779-84.
85. Sylte I, Dawadi R, Malla N, von Hofsten S, Nguyen T-M, Solli AI, et al. The selectivity of galardin and an azasugar-based hydroxamate compound for human matrix metalloproteases and bacterial metalloproteases. PLoS One. 2018;13(8):e0200237.
86. Sjøli S, Nuti E, Camodeca C, Bilto I, Rossello A, Winberg JO, et al. Synthesis, experimental evaluation and molecular modelling of hydroxamate derivatives as zinc metalloproteinase inhibitors. Eur J Med Chem. 2016;108:141-53.
87. Cui N, Hu M, Khalil RA. Biochemical and Biological Attributes of Matrix Metalloproteinases. Prog Mol Biol Transl Sci. 2017;147:1-73.
88. Hadler-Olsen E, Winberg JO, Uhlin-Hansen L. Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets. Tumour Biol. 2013;34(4):2041-51.
89. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science. 2002;295(5564):2387-92.
90. Leiris S, Davies DT, Sprynski N, Castandet J, Beyria L, Bodnarchuk MS, et al. Virtual Screening Approach to Identifying a Novel and Tractable Series of Pseudomonas aeruginosa Elastase Inhibitors. ACS Medicinal Chemistry Letters. 2021;12(2):217-27.

91. Henke HA, Eppendorf A. Five Challenges in Plate Assays that Can Be Mastered by the Right Choice of Pipetting Tool.
92. Khastsaev BD. Single channel and multichannel adjustable micropipettes. Biomedical Engineering. 1993;27(5):285-91.
93. Ewald K, Eppendorf A. Impact of pipetting techniques on precision and accuracy. Eppendorf Userguide. 2015(20):1-4.
94. Nagase H, Barrett AJ, Woessner JF, Jr. Nomenclature and glossary of the matrix metalloproteinases. Matrix Suppl. 1992;1:421-4.
95. Clark IM, Cawston TE. Fragments of human fibroblast collagenase. Purification and characterization. Biochem J. 1989;263(1):201-6.
96. Levin M, Udi Y, Solomonov I, Sagi I. Next generation matrix metalloproteinase inhibitors — Novel strategies bring new prospects. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2017;1864(11, Part A):1927-39.
97. Sela-Passwell N, Rosenblum G, Shoham T, Sagi I. Structural and functional bases for allosteric control of MMP activities: can it pave the path for selective inhibition? Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2010;1803(1):29-38.
98. Bhowmick M, Stawikowska R, Tokmina-Roszyk D, Fields GB. Matrix metalloproteinase inhibition by heterotrimeric triple-helical peptide transition state analogs. Chembiochem: a European journal of chemical biology. 2015;16(7):1084.
99. Laskowski RA, Gerick F, Thornton JM. The structural basis of allosteric regulation in proteins. FEBS letters. 2009;583(11):1692-8.
100. Udi Y, Fragai M, Grossman M, Mitternacht S, Arad-Yellin R, Calderone V, et al. Unraveling hidden regulatory sites in structurally homologous metalloproteases. Journal of molecular biology. 2013;425(13):2330-46.
101. Sela-Passwell N, Kikkeri R, Dym O, Rozenberg H, Margalit R, Arad-Yellin R, et al. Antibodies targeting the catalytic zinc complex of activated matrix metalloproteinases show therapeutic potential. Nature medicine. 2012;18(1):143-7.
102. Moss ML, Bomar M, Liu Q, Sage H, Dempsey P, Lenhart PM, et al. The ADAM10 prodomain is a specific inhibitor of ADAM10 proteolytic activity and inhibits cellular shedding events. Journal of Biological Chemistry. 2007;282(49):35712-21.
103. Levin M, Amar D, Aharoni A. Employing directed evolution for the functional analysis of multi-specific proteins. Bioorganic & medicinal chemistry. 2013;21(12):3511-6.
104. Rabinovich E, Heyne M, Bakhman A, Kosloff M, Shifman JM, Papo N. Identifying residues that determine SCF molecular-level interactions through a combination of experimental and in silico analyses. Journal of molecular biology. 2017;429(1):97-114.
105. Amar D, Berger I, Amara N, Tafa G, Meijler MM, Aharoni A. The transition of human estrogen sulfotransferase from generalist to specialist using directed enzyme evolution. Journal of molecular biology. 2012;416(1):21-32.
106. Mehner C, Batra J, Robinson J, Radisky DC, Radisky ES. Engineering TIMP-1 for selective MMP inhibition and future use as a protein therapeutic. AACR; 2013.
107. Mohan V, Talmi-Frank D, Arkadash V, Papo N, Sagi I. Matrix metalloproteinase protein inhibitors: highlighting a new beginning for metalloproteinases in medicine. Metalloproteinases in medicine. 2016;3:31-47.
108. Rouanet-Mehouas C, Czarny B, Beau F, Cassar-Lajeunesse E, Stura EA, Dive V, et al. Zinc–Metalloproteinase Inhibitors: Evaluation of the Complex Role Played by the Zinc-Binding Group on Potency and Selectivity. Journal of Medicinal Chemistry. 2017;60(1):403-14.

# Appendix

## 1.Calculation of inhibition activity of the compounds (table 1) against TLN

compound 1

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 10.88 | 0.850665 | 11.82 | 10.8 | 0.913706 | 11.75 | 10.26 | 0.873191 |
| 12.41 | 11.06 | 0.891217 | 10.93 | 11.6 | 1.061299 | 12.52 | 11.68 | 0.932907 |
| 12.18 | 10.33 | 0.848112 | 9.57 | 12.23 | 1.277952 | 12.23 | 12.4 | 1.0139 |
| 11.33 | 10.42 | 0.919682 | 12.59 | 10 | 0.794281 | 12.79 | 11.6 | 0.906959 |
| | | | | | | | | |
| mean | | 0.877419 | | | 1.011809 | | | 0.931739 |
| SD | | 0.029796 | | | 0.180433 | | | 0.051946 |

compound 2

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 11.68 | 0.913213 | 11.82 | 11.3 | 0.956007 | 11.75 | 10.64 | 0.905532 |
| 12.41 | 10.68 | 0.860596 | 10.93 | 11.19 | 1.023788 | 12.52 | 11.23 | 0.896965 |
| 12.18 | 11.46 | 0.940887 | 9.57 | 11.14 | 1.164054 | 12.23 | 11.36 | 0.928863 |
| 11.33 | 10.81 | 0.954104 | 12.59 | 10.91 | 0.866561 | 12.79 | | |
| | | | | | | | | |
| mean | | 0.9172 | | | 1.002602 | | | 0.910453 |
| SD | | 0.035857 | | | 0.108621 | | | 0.01348 |

compound 3

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 11.9 | 0.930414 | 11.82 | 11.27 | 0.953469 | 11.75 | 11.94 | 1.01617 |
| 12.41 | 11.28 | 0.908944 | 10.93 | 13.39 | 1.225069 | 12.52 | 11.82 | 0.944089 |
| 12.18 | 11.28 | 0.926108 | 9.57 | 12.41 | 1.296761 | 12.23 | 13.31 | 1.088307 |
| 11.33 | 11.63 | 1.026478 | 12.59 | 12.7 | 1.008737 | 12.79 | 14.3 | 1.118061 |
| | | | | | | | | |
| mean | | 0.947986 | | | 1.121009 | | | 1.041657 |
| SD | | 0.046024 | | | 0.14352 | | | 0.067422 |

compound 4

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 12.18 | 0.952306 | 11.82 | 14.35 | 1.214044 | 11.75 | 14.4 | 1.225532 |
| 12.41 | 13.76 | 1.108783 | 10.93 | 13.51 | 1.236048 | 12.52 | 14.26 | 1.138978 |
| 12.18 | 13.41 | 1.100985 | 9.57 | 11.78 | 1.23093 | 12.23 | 14.66 | 1.198692 |
| 11.33 | 13.78 | 1.21624 | 12.59 | 14.13 | 1.122319 | 12.79 | 12.79 | 1 |
| | | | | | | | | |
| mean | | 1.094579 | | | 1.200835 | | | 1.1408 |
| SD | | 0.093922 | | | 0.046057 | | | 0.087119 |

compound 5

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 6.81 | 0.532447 | 11.82 | 6.58 | 0.556684 | 11.75 | 6.96 | 0.59234 |
| 12.41 | 7.18 | 0.578566 | 10.93 | 5.8 | 0.53065 | 12.52 | 6.82 | 0.544728 |
| 12.18 | 6.82 | 0.559934 | 9.57 | 7.23 | 0.755486 | 12.23 | 7 | 0.572363 |
| 11.33 | 8.64 | 0.762577 | 12.59 | 7.69 | 0.610802 | 12.79 | 8.03 | 0.627834 |
| | | | | | | | | |
| mean | | 0.608381 | | | 0.613405 | | | 0.584317 |
| SD | | 0.090524 | | | 0.086976 | | | 0.030283 |

compound 6

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 11.46 | 0.896013 | 11.82 | 11.65 | 0.985618 | 11.75 | 11.89 | 1.011915 |
| 12.41 | 10.69 | 0.861402 | 10.93 | 10.28 | 0.940531 | 12.52 | 10.93 | 0.873003 |
| 12.18 | 10.64 | 0.873563 | 9.57 | 11.89 | 1.242424 | 12.23 | 11.43 | 0.934587 |
| 11.33 | 10.4 | 0.917917 | 12.59 | 11.25 | 0.893566 | 12.79 | 11.68 | 0.913213 |
| | | | | | | | | |
| mean | | 0.887224 | | | 1.015535 | | | 0.93318 |
| SD | | 0.021637 | | | 0.134978 | | | 0.05055 |

compound 7

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.79 | 11.26 | 0.880375 | 11.82 | 9.58 | 0.810491 | 11.75 | 8.64 | 0.735319 |
| 12.41 | 9.35 | 0.753425 | 10.93 | 8.76 | 0.801464 | 12.52 | 9.38 | 0.749201 |
| 12.18 | 10.93 | 0.897373 | 9.57 | 9.35 | 0.977011 | 12.23 | 9.96 | 0.814391 |
| 11.33 | | | 12.59 | 9.86 | 0.783161 | 12.79 | | |
| | | | | | | | | |
| mean | | 0.843724 | | | 0.843032 | | | 0.766304 |
| SD | | 0.064227 | | | 0.077977 | | | 0.034472 |

compound 8

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 8.44 | 6 | 0.7109 | 7.71 | 7.19 | 0.932555 | 9.06 | 6.78 | 0.748344 |
| 8.04 | 8.03 | 0.998756 | 8.24 | 7.73 | 0.938107 | 7.53 | 7.12 | 0.945551 |
| 8.09 | 7.3 | 0.902349 | 9.32 | 8.34 | 0.89485 | 7.71 | 6.72 | 0.871595 |
| 7.51 | 7.12 | 0.948069 | | 7.31 | 0.887136 | | 6.89 | 0.893645 |
| | | | | | | | | |
| mean | | 0.890019 | | | 0.913162 | | | 0.864784 |
| SD | | 0.108891 | | | 0.022422 | | | 0.072389 |

## compound 9

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 8.44 | 8.16 | 0.966825 | 7.71 | 7.96 | 1.032425 | 9.06 | 7.79 | 0.859823 |
| 8.04 | 7.28 | 0.905473 | 8.24 | 9.33 | 1.132282 | 7.53 | 9.21 | 1.223108 |
| 8.09 | | 0.899876 | 9.32 | 8.41 | 0.902361 | 7.71 | 7.21 | 0.935149 |
| 7.51 | | 1.008653 | | 8.23 | 0.998786 | | 7.76 | 1.030544 |
| | | | | | | | | |
| mean | | 0.945207 | | | 1.016463 | | | 1.012156 |
| SD | | 0.045073 | | | 0.082159 | | | 0.135991 |

## compound 10

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 5.74 | 0.458466 | 11.99 | 6.48 | 0.54045 | 12.86 | 6.54 | 0.508554 |
| 13.59 | 6.64 | 0.488595 | 12.6 | 7.62 | 0.604762 | 12.44 | 9.49 | 0.762862 |
| 12.92 | 6.36 | 0.49226 | 13.57 | 5.96 | 0.439204 | 12.98 | 8.23 | 0.634052 |
| 12.6 | 6.56 | 0.520635 | 12.18 | | | | | |
| | | | | | | | | |
| mean | | 0.489989 | | | 0.528139 | | | 0.635156 |
| SD | | 0.022022 | | | 0.068147 | | | 0.103824 |

## compound 11

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 8.44 | 7.3 | 0.864929 | 7.71 | 7.53 | 0.913835 | 9.06 | 7.61 | 0.839956 |
| 8.04 | 6.46 | 0.803483 | 8.24 | 6.17 | 0.800259 | 7.53 | 7.69 | 1.021248 |
| 8.09 | 6.13 | 0.757726 | 9.32 | 7.21 | 0.773605 | 7.71 | 7.46 | 0.967575 |
| 7.51 | | 0.816245 | | | | | 6.99 | 0.928287 |
| | | | | | | | | |
| mean | | 0.810596 | | | 0.829233 | | | 0.939266 |
| SD | | 3.792881 | | | 3.667555 | | | #DIV/0! |

## compound 12

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 8.44 | 7.35 | 0.870853 | 7.71 | 7.08 | 0.918288 | 9.06 | 7.01 | 0.773731 |
| 8.04 | 8.54 | 1.062189 | 8.24 | 8.31 | 1.008495 | 7.53 | 6.01 | 0.798141 |
| 8.09 | 6.62 | 0.818294 | 9.32 | 6.96 | 0.902724 | 7.71 | 5.66 | 0.734112 |
| 7.51 | 6.85 | 0.912117 | | | | | 7.4 | 0.816777 |
| | | | | | | | | |
| mean | | 0.915863 | | | 0.943169 | | | 0.78069 |
| SD | | 0.090789 | | | 0.046628 | | | 0.030922 |

## compound 13

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 8.44 | 8.43 | 0.998815 | 7.71 | 6.64 | 0.861219 | 9.06 | 6.6 | 0.728477 |
| 8.04 | 7.76 | 0.965174 | 8.24 | 6.85 | 0.831311 | 7.53 | 6.3 | 0.836653 |
| 8.09 | 6.21 | 0.767614 | 9.32 | 6.73 | 0.722103 | 7.71 | 6.94 | 0.90013 |
| 7.51 | 7.15 | 0.952064 | | | | | | |
| | | | | | | | | |
| mean | | 0.920917 | | | 0.804878 | | | 0.821753 |
| SD | | 0.090137 | | | 0.059791 | | | 0.070865 |

## compound 14

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 8.44 | 7.29 | 0.863744 | 7.71 | 7.27 | 0.942931 | 9.06 | 6.93 | 0.920319 |
| 8.04 | 8.76 | 1.089552 | 8.24 | 6.47 | 0.785194 | 7.53 | 8.26 | 1.096946 |
| 8.09 | 6.13 | 0.757726 | 9.32 | 7.01 | 0.752146 | 7.71 | 8.34 | 1.081712 |
| 7.51 | 6.79 | 0.904128 | | 7.07 | 0.85801 | | | |
| | | | | | | | | |
| mean | | 0.903787 | | | 0.83457 | | | 1.032992 |
| SD | | 0.11984 | | | 0.073354 | | | 0.079914 |

## compound 15

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 17.45 | 17.42 | 0.998281 | 18.23 | 16.9 | 0.927043 | 17.19 | 16.8 | 0.977312 |
| 17.12 | 17.43 | 1.018107 | 18 | 17.59 | 0.977222 | 17.67 | 17.4 | 0.98472 |
| 17.13 | 17.31 | 1.010508 | 18.06 | 16.91 | 0.936323 | 18.06 | 18.09 | 1.001661 |
| 17.57 | | 0.985202 | 18 | | | 17.66 | 17.44 | 0.987542 |
| | | | | | | | | |
| mean | | 1.003025 | | | 0.946863 | | | 0.987809 |
| SD | | 0.012486 | | | 0.021799 | | | 0.008827 |

## compound 16

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 17.45 | 16.34 | 0.93639 | 18.23 | 16.89 | 0.926495 | 17.19 | 16.08 | 0.935428 |
| 17.12 | 16 | 0.934579 | 18 | 16.55 | 0.919444 | 17.67 | 17.54 | 0.992643 |
| 17.13 | 14.82 | 0.865149 | 18.06 | 16.54 | 0.915836 | 18.06 | 16.53 | 0.915282 |
| 17.57 | 16.84 | 0.958452 | 18 | 16.44 | 0.913333 | 17.66 | 16.42 | 0.929785 |
| | | | | | | | | |
| mean | | 0.923642 | | | 0.918777 | | | 0.943284 |
| SD | | 0.035055 | | | 0.004957 | | | 0.029429 |

## compound 17

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 6.7 | 0.535144 | 11.99 | 5.64 | 0.470392 | 12.86 | 7.42 | 0.576983 |
| 13.59 | 5.2 | 0.382634 | 12.6 | 7.3 | 0.579365 | 12.44 | 9.9 | 0.79582 |
| 12.92 | 7.8 | 0.603715 | 13.57 | 7.82 | 0.576271 | 12.98 | 8.35 | 0.643297 |
| 12.6 | 6.45 | 0.511905 | 12.18 | 6.55 | 0.537767 | | 7.62 | 0.61254 |
| | | | | | | | | |
| mean | | 0.508349 | | | 0.540949 | | | 0.65716 |
| SD | | 0.080046 | | | 0.043909 | | | 0.083424 |

## compound 18

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 17.45 | 15.97 | 0.915186 | 18.23 | 14.13 | 0.775096 | 17.19 | 13.95 | 0.811518 |
| 17.12 | 14.95 | 0.873248 | 18 | 14.78 | 0.821111 | 17.67 | 14.51 | 0.821166 |
| 17.13 | 14.14 | 0.825452 | 18.06 | 13.36 | 0.739756 | 18.06 | 14.26 | 0.78959 |
| 17.57 | 14.79 | 0.841776 | 18 | 13.72 | 0.762222 | 17.66 | | |
| | | | | | | | | |
| mean | | 0.863916 | | | 0.774546 | | | 0.807425 |
| SD | | 0.034225 | | | 0.02971 | | | 0.013212 |

## compound 19

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 17.45 | 15.83 | 0.907163 | 18.23 | 15.54 | 0.852441 | 17.19 | 15.93 | 0.926702 |
| 17.12 | 14.8 | 0.864486 | 18 | 15.82 | 0.878889 | 17.67 | 14.94 | 0.845501 |
| 17.13 | 16.47 | 0.961471 | 18.06 | 14.72 | 0.815061 | 18.06 | 15.19 | 0.841085 |
| 17.57 | 16.64 | 0.947069 | 18 | | | 17.66 | 17.04 | 0.964892 |
| | | | | | | | | |
| mean | | 0.920047 | | | 0.848797 | | | 0.894545 |
| SD | | 0.037746 | | | 0.026185 | | | 0.053024 |

## compound 20

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 17.45 | 17.73 | 1.016046 | 18.23 | 17.63 | 0.967087 | 17.19 | 16.84 | 0.979639 |
| 17.12 | 17.99 | 1.050818 | 18 | 16.86 | 0.936667 | 17.67 | 17.18 | 0.972269 |
| 17.13 | 17.12 | 0.999416 | 18.06 | 18.23 | 1.009413 | 18.06 | 17.27 | 0.956257 |
| 17.57 | 18.28 | 1.04041 | 18 | 16.23 | 0.901667 | 17.66 | 17.58 | 0.99547 |
| | | | | | | | | |
| mean | | 1.026672 | | | 0.953708 | | | 0.975909 |
| SD | | 0.020171 | | | 0.039626 | | | 0.014107 |

## compound 21

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 17.45 | 19.81 | 1.135244 | 18.23 | 19.71 | 1.081185 | 17.19 | 18.39 | 1.069808 |
| 17.12 | 18.22 | 1.064252 | 18 | 17.69 | 0.982778 | 17.67 | 18.22 | 1.031126 |
| 17.13 | 18.56 | 1.083479 | 18.06 | 15.22 | 0.842746 | 18.06 | 17.44 | 0.96567 |
| 17.57 | 19.22 | 1.09391 | 18 | 17.86 | 0.992222 | 17.66 | | |
| | | | | | | | | |
| mean | | 1.094221 | | | 0.974733 | | | 1.022201 |
| SD | | 0.025964 | | | 0.085327 | | | 0.04298 |

## compound 22

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 11.7 | 0.934505 | 11.99 | 11.76 | 0.980817 | 12.86 | 10.78 | 0.838258 |
| 13.59 | 11.03 | 0.811626 | 12.6 | 11.57 | 0.918254 | 12.44 | 11.77 | 0.946141 |
| 12.92 | 12.16 | 0.941176 | 13.57 | 11.96 | 0.881356 | 12.98 | 12.4 | 0.955316 |
| 12.6 | 12.29 | 0.975397 | 12.18 | | 0.981938 | | | |
| | | | | | | | | |
| mean | | 0.915676 | | | 0.940591 | | | 0.913239 |
| SD | | 0.062044 | | | 0.042824 | | | 0.053151 |

## compound 23

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 11.35 | 0.90655 | 11.99 | 11.4 | 0.950792 | 12.86 | 12.46 | 0.968896 |
| 13.59 | 10.61 | 0.780721 | 12.6 | 12.42 | 0.985714 | 12.44 | 13.25 | 1.065113 |
| 12.92 | 11.92 | 0.922601 | 13.57 | 11.84 | 0.872513 | 12.98 | 11.896 | 0.916487 |
| 12.6 | 10.47 | 0.830952 | 12.18 | 11.6 | 0.952381 | | 12.77 | 1.026527 |
| | | | | | | | | |
| mean | | 0.860206 | | | 0.94035 | | | 0.994256 |
| SD | | 0.057477 | | | 0.041574 | | | 0.056465 |

## compound 24

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 10.65 | 0.850639 | 11.99 | 9.72 | 0.810676 | 12.86 | 11.68 | 0.908243 |
| 13.59 | 12.28 | 0.903606 | 12.6 | 11.92 | 0.946032 | 12.44 | 10.35 | 0.831994 |
| 12.92 | 9.83 | 0.760836 | 13.57 | 11.3 | 0.832719 | 12.98 | 14.35 | 1.105547 |
| 12.6 | 10.74 | 0.852381 | 12.18 | 9.96 | 0.817734 | | | |
| | | | | | | | | |
| mean | | 0.841865 | | | 0.85179 | | | 0.948594 |
| SD | | 0.051394 | | | 0.05499 | | | 0.115265 |

## compound 25

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 10.4 | 0.830671 | 11.99 | 10.76 | 0.897415 | 12.86 | 10.71 | 0.832815 |
| 13.59 | 9.8 | 0.721118 | 12.6 | 10.84 | 0.860317 | 12.44 | 10.34 | 0.83119 |
| 12.92 | 12.85 | 0.994582 | 13.57 | 11.85 | 0.87325 | 12.98 | 10.87 | 0.837442 |
| 12.6 | 10.55 | 0.837302 | 12.18 | | | | 12.08 | 0.971061 |
| | | | | | | | | |
| mean | | 0.845918 | | | 0.876994 | | | 0.868127 |
| SD | | 0.097446 | | | 0.015374 | | | 0.059473 |

## compound 26

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 12.52 | 10.34 | 0.825879 | 11.99 | 10.2 | 0.850709 | 12.86 | 9.57 | 0.744168 |
| 13.59 | 10.81 | 0.795438 | 12.6 | 9.65 | 0.765873 | 12.44 | 11.99 | 0.963826 |
| 12.92 | 10.29 | 0.79644 | 13.57 | 10.44 | 0.769344 | 12.98 | 9.66 | 0.744222 |
| 12.6 | | | 12.18 | 9.28 | 0.761905 | | 9.81 | 0.755778 |
| | | | | | | | | |
| mean | | 0.805919 | | | 0.786958 | | | 0.801999 |
| SD | | 0.01412 | | | 0.036901 | | | 0.093551 |

## 2.Calculation of inhibition activity of the compounds (table 1) against PLN

### compound 1

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 44.19 | | | 55.58 | 44.58 | 0.802087 | 48.44 | 47.57 | 0.98204 |
| 57.44 | 44.85 | 0.780815 | 48.19 | 43.34 | 0.899357 | 49.56 | 42.97 | 0.86703 |
| 50.2 | 41.8 | 0.832669 | 47.46 | 40.55 | 0.854404 | 49.16 | 42.65 | 0.867575 |
| 50.13 | 43.13 | 0.860363 | | 40.21 | 0.84724 | 52.56 | 45.39 | 0.863584 |
| | | | | | | | | |
| mean | | 0.824616 | | | 0.850772 | | | 0.895057 |
| SD | | 0.032971 | | | 0.034483 | | | 0.050243 |

### compound 3

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 35.7 | 45.8 | 1.191467 | 42.79 | 47.36 | 1.106801 | 64.69 | 63.3 | 0.978513 |
| 37.28 | 41.29 | 1.107564 | 40.46 | 41.41 | 1.02348 | 58.21 | 63.15 | 1.084865 |
| 38.44 | 39.05 | 1.015869 | 38.71 | 44.95 | 1.161199 | 52.03 | | |
| | | | | | | 47.16 | | 1.084865 |
| | | | | | | | | |
| mean | | 1.104967 | | | 1.09716 | | | 1.049414 |
| SD | | 0.071711 | | | 0.056635 | | | 0.050135 |

### compound 2

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 33.68 | 32.33 | 0.959917 | 36.51 | 32.99 | 0.903588 | 34.96 | 35 | 1.001144 |
| 33.13 | 30.38 | 0.916994 | 34.64 | 32.55 | 0.939665 | 34.69 | 32.78 | 0.944941 |
| 31.72 | 32.33 | 1.019231 | 31.97 | 29.95 | 0.936816 | 33.55 | 33.21 | 0.989866 |
| | 36.33 | 1.096589 | | | | | | |
| | | | | | | | | |
| mean | | 0.998183 | | | 0.92669 | | | 0.97865 |
| SD | | 0.067422 | | | 0.016377 | | | 0.024277 |

### compound 4

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 48 | | | 44.34 | | | 46 | 48.08 | 1.045217 |
| 47.85 | 47.95 | 1.00209 | 50.59 | 49.02 | 0.968966 | 46.59 | 48.29 | 1.036489 |
| 45.96 | 46.39 | 1.009356 | 50.94 | 50.12 | 0.983903 | 44.12 | 43.05 | 0.975748 |
| 45.99 | 46.09 | 1.002174 | 53.57 | 48.04 | 0.896771 | 44.83 | 46.88 | 1.045728 |
| | | | | | | | | |
| mean | | 1.00454 | | | 0.94988 | | | 1.025796 |
| SD | | 0.003406 | | | 0.038046 | | | 0.029127 |

### compound 5

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 27.48 | 22.69 | 0.825691 | 34.28 | 33.12 | 0.966161 | 33.12 | 38.67 | 1.167572 |
| 31 | 27.76 | 0.895484 | 36.16 | 32.89 | 0.909569 | 32.89 | 37.69 | 1.145941 |
| | | | | 28.56 | 0.833139 | 28.56 | | |
| | | | | 33.69 | 0.931692 | 33.69 | | 1.11873 |
| | | | | | | | | |
| mean | | 0.860588 | | | 0.91014 | | | 1.144081 |
| SD | | 0.034896 | | | 0.048817 | | | 0.019983 |

### compound 7

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.67 | 37.23 | 0.87251 | 44.55 | 36.78 | 0.825589 | 44.74 | 38.68 | 0.864551 |
| 42.63 | 38.1 | 0.893737 | 46.41 | 38.44 | 0.82827 | 45.16 | 39.32 | 0.870682 |
| 41.71 | 37.23 | 0.892592 | 43.17 | 38.21 | 0.885105 | 45.65 | 40.68 | 0.891128 |
| 42.49 | | | 4426 | | | 43.39 | 43.21 | 0.995852 |
| | | | | | | | | |
| mean | | 0.886279 | | | 0.846321 | | | 0.905553 |
| SD | | 0.009748 | | | 0.027446 | | | 0.053054 |

### compound 6

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.36 | 42.04 | 0.992446 | 44.03 | 38.34 | 0.87077 | 41.44 | 32.83 | 0.79223 |
| 49.01 | 39.46 | 0.805142 | 44.27 | 44.15 | 0.997289 | 35.2 | 28.29 | 0.803693 |
| 39.77 | 37.14 | 0.93387 | 39.57 | 36.69 | 0.927218 | 38.65 | 27.7 | 0.716688 |
| 47.92 | 38.06 | 0.79424 | 35.35 | 31.04 | 0.878076 | | | |
| | | | | | | | | |
| mean | | 0.881424 | | | 0.918338 | | | 0.77087 |
| SD | | 0.084404 | | | 0.050487 | | | 0.038597 |

### compound 8

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.43 | 36.76 | 0.909226 | 42.45 | 35.12 | 0.900744 | 43.2 | 36.39 | 0.842361 |
| 41.97 | 34.62 | 0.824875 | 37.93 | 38.16 | 1.006064 | 41 | 35.77 | 0.872439 |
| 31.43 | 34.8 | 1.107222 | 38.99 | 36.35 | 0.93229 | 44.13 | 33.5 | 0.759121 |
| 35.47 | 30.04 | 0.846913 | 39.72 | 36.18 | 0.910876 | | 33.75 | 0.78125 |
| | | | | | | | | |
| mean | | 0.922059 | | | 0.937494 | | | 0.813793 |
| SD | | 0.11129 | | | 0.041195 | | | 0.045562 |

## compound 9

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.67 | 40.72 | 0.9543 | 44.55 | 39.33 | 0.882828 | 44.74 | 39.27 | 0.877738 |
| 42.63 | 39.12 | 0.917664 | 46.41 | 41.18 | 0.887309 | 45.16 | 41.62 | 0.921612 |
| 41.71 | 42.72 | 1.024215 | 43.17 | 41.48 | 0.960852 | 45.65 | 40.86 | 0.895071 |
| 42.49 | 38.84 | 0.914097 | 44.26 | 40.56 | 0.916403 | 43.39 | 39.33 | 0.90643 |
| | | | | | | | | |
| mean | | 0.952569 | | | 0.911848 | | | 0.900213 |
| SD | | 0.044257 | | | 0.031091 | | | 0.016032 |

## compound 10

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.67 | 28.69 | 0.672369 | 44.55 | 32.5 | 0.729517 | 44.74 | 33.25 | 0.743183 |
| 42.63 | 27.17 | 0.637345 | 46.41 | 31.24 | 0.673131 | 45.16 | 34.05 | 0.753986 |
| 41.71 | 34.11 | 0.817789 | 43.17 | 31.59 | 0.731758 | 45.65 | 33.45 | 0.732749 |
| 42.49 | 34.12 | 0.803012 | 44.26 | 32.02 | 0.723452 | 43.39 | 35.91 | 0.82761 |
| | | | | | | | | |
| mean | | 0.732629 | | | 0.714465 | | | 0.764382 |
| SD | | 0.078925 | | | 0.024057 | | | 0.037269 |

## compound 11

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 36.94 | 37.18 | 1.006497 | 37 | | | 39.66 | 37.22 | 0.938477 |
| 37.16 | 37.82 | 1.017761 | 36.37 | 37.68 | | | 30.25 | 0.762733 |
| 28.18 | | | 45.5 | 36.65 | 0.805495 | 37.68 | 32.96 | 0.874735 |
| | 30.88 | 1.095813 | | 36.69 | 0.806374 | | | |
| | | | | | | | | |
| mean | | 1.040024 | | | 0.805934 | | | 0.858648 |
| SD | | 0.039716 | | | 0.00044 | | | 0.072643 |

## compound 12

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 35.13 | 26.2 | 0.745801 | 32.17 | 32.26 | | 31.33 | 30.39 | 0.969997 |
| 24.64 | | | 30.93 | 23.6 | 0.763013 | 30 | 29.47 | 0.982333 |
| 31.37 | 26.71 | 0.85145 | 31.05 | 28.7 | 0.924316 | 31.31 | 28.75 | 0.918237 |
| | 26.44 | 0.842843 | | 27.35 | 0.880837 | | | |
| | | | | | | | | |
| mean | | 0.813365 | | | 0.856055 | | | 0.956856 |
| SD | | 0.047904 | | | 0.068143 | | | 0.027768 |

## compound 13

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 53.11 | 43.16 | 0.812653 | 55.54 | 48.33 | 0.870184 | 48.72 | 41.03 | 0.842159 |
| 35.63 | | | 55.46 | 46.26 | 0.834115 | 47.17 | 41.47 | 0.87916 |
| 55.62 | 45.22 | 0.813017 | 55.49 | 42.94 | 0.773833 | 39.97 | 42.24 | |
| | 45.8 | 0.823445 | | | | | | |
| | | | | | | | | |
| mean | | 0.816372 | | | 0.826044 | | | 0.86066 |
| SD | | 0.005004 | | | 0.039747 | | | 0.018501 |

## compound 14

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 38.13 | 29.12 | 0.763703 | 36.8 | 36.37 | 0.988315 | 33.65 | 31.35 | 0.931649 |
| 33.44 | 32.72 | 0.978469 | 32.84 | 26.36 | 0.80268 | 32.25 | 32.33 | 1.002481 |
| 26.05 | 27.6 | | 34.48 | 27.81 | 0.806555 | 29 | 32.81 | 1.131379 |
| | 28.67 | 0.857356 | | | | | | |
| | | | | | | | | |
| MEAN | | 0.866509 | | | 0.86585 | | | 1.021836 |
| SD | | 0.087916 | | | 0.086611 | | | 0.08268 |

## compound 15

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 33.12 | 39.49 | 1.096031 | 35.23 | 30.07 | 0.853534 | 33.23 | 36.15 | 1.087872 |
| 32.33 | 33.79 | 1.045159 | 31.25 | 30.16 | 0.96512 | 30.16 | 33.01 | 1.094496 |
| 36.03 | 30.59 | 0.94618 | 33.01 | 33.16 | 1.004544 | 30.07 | 31.25 | 1.039242 |
| | 33.18 | 1.026291 | 36.15 | | 0.917289 | | 35.231 | 1.171633 |
| | | | | | | | | |
| | | | | | | | | |
| MEAN | | 1.028415 | | | 0.935122 | | | 1.098311 |
| SD | | 0.053897 | | | 0.056334 | | | 0.047405 |

## compound 16

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.67 | 38.35 | 0.898758 | 44.55 | 37.13 | 0.833446 | 44.74 | 38.67 | 0.864327 |
| 42.63 | 31.14 | 0.730471 | 46.41 | 39.17 | 0.843999 | 45.16 | 40.36 | 0.893711 |
| 41.71 | 38.84 | 0.931192 | 43.17 | 38.24 | 0.8858 | 45.65 | 39.07 | 0.85586 |
| 42.49 | 35.37 | 0.832431 | 44.26 | 35.27 | 0.796882 | 43.39 | 38.56 | 0.888684 |
| | | | | | | | | |
| mean | | 0.848213 | | | 0.840032 | | | 0.875646 |
| SD | | 0.076734 | | | 0.031685 | | | 0.015937 |

## compound 17

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 28.15 | 0.784341 | 34.88 | 27 | 0.774083 | 35.65 | 25.9 | 0.726508 |
| 34.53 | 31.33 | 0.907327 | 35.88 | 29.15 | 0.81243 | 35.5 | 27.73 | 0.781127 |
| 35.08 | 28.59 | 0.814994 | 33.72 | 26.77 | 0.793891 | 35.04 | 29.94 | 0.854452 |
| 34.39 | 26.46 | 0.76941 | 33.56 | 28.15 | 0.838796 | 39.65 | 30.7 | 0.774275 |
| | | | | | | | | |
| mean | | 0.819018 | | | 0.8048 | | | 0.78409 |
| SD | | 0.053568 | | | 0.023857 | | | 0.045748 |

## compound 18

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 29.87 | 0.832265 | 34.88 | 30.13 | 0.863819 | 35.65 | 30.67 | 0.860309 |
| 34.53 | 31.17 | 0.902693 | 35.88 | 30.12 | 0.839465 | 35.5 | 29.95 | 0.843662 |
| 35.08 | 31 | 0.883694 | 33.72 | 31.09 | 0.922005 | 35.04 | 29.86 | 0.852169 |
| 34.39 | 29.96 | 0.871183 | 33.56 | 31.68 | 0.943981 | 39.65 | 30.35 | 0.765448 |
| | | | | | | | | |
| mean | | 0.872459 | | | 0.892317 | | | 0.830397 |
| SD | | 0.025776 | | | 0.042297 | | | 0.037958 |

## compound 19

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 33.35 | 0.929228 | 34.88 | 33.21 | 0.952122 | 35.65 | 34.35 | 0.963534 |
| 34.53 | 34.54 | 1.00029 | 35.88 | 29.74 | 0.828874 | 35.5 | 33.84 | 0.953239 |
| 35.08 | 29.54 | 0.842075 | 33.72 | 33.04 | 0.979834 | 35.04 | 35.4 | 1.010274 |
| 34.39 | 30.24 | 0.879325 | 33.56 | 31.61 | 0.941895 | | | |
| | | | | | | | | |
| mean | | 0.91273 | | | 0.925681 | | | 0.975683 |
| SD | | 0.05926 | | | 0.057589 | | | 0.024818 |

## compound 20

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 36.12 | 1.006408 | 34.88 | 32.79 | 0.94008 | 35.65 | 36.18 | 1.014867 |
| 34.53 | 36.89 | 1.068346 | 35.88 | 32.25 | 0.898829 | 35.5 | 36.27 | 1.02169 |
| 35.08 | 33.92 | 0.966933 | 33.72 | 35.08 | 1.040332 | 35.04 | 36.69 | 1.047089 |
| 34.39 | 35.94 | 1.045071 | 33.56 | 34.24 | 1.020262 | 39.65 | 36.34 | 0.91652 |
| | | | | | | | | |
| mean | | 1.02169 | | | 0.974876 | | | 1.000041 |
| SD | | 0.038586 | | | 0.057746 | | | 0.049694 |

## compound 21

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 30.88 | 0.860407 | 34.88 | 32.55 | 0.9332 | 35.65 | 30.49 | 0.855259 |
| 34.53 | 32.22 | 0.933102 | 35.88 | 29.07 | 0.810201 | 35.5 | 31.42 | 0.88507 |
| 35.08 | 26.34 | 0.750855 | 33.72 | 31.28 | 0.927639 | 35.04 | 34.62 | 0.988014 |
| 34.39 | 29.96 | 0.871183 | 33.56 | 35.1 | 1.045888 | 39.65 | | |
| | | | | | | | | |
| mean | | 0.853887 | | | 0.929232 | | | 0.909448 |
| SD | | 0.065636 | | | 0.08336 | | | 0.056872 |

## compound 22

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 33.99 | 0.94706 | 34.88 | 30.63 | 0.878154 | 35.65 | 33.07 | 0.92763 |
| 34.53 | 34.91 | 1.011005 | 35.88 | 32.02 | 0.892419 | 35.5 | 35.28 | 0.993803 |
| 35.08 | 31.94 | 0.91049 | 33.72 | 33.93 | 1.006228 | 35.04 | 33.48 | 0.955479 |
| 34.39 | 31.78 | 0.924106 | 33.56 | 22.91 | 0.682658 | 39.65 | 33.12 | 0.835309 |
| | | | | | | | | |
| mean | | 0.948165 | | | 0.864865 | | | 0.928055 |
| SD | | 0.038563 | | | 0.116317 | | | 0.058474 |

## compound 23

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 35.89 | 34.43 | 0.95932 | 34.88 | 32.93 | 0.944094 | 35.65 | 31.41 | 0.881066 |
| 34.53 | 34.06 | 0.986389 | 35.88 | 30.25 | 0.843088 | 35.5 | 31.82 | 0.896338 |
| 35.08 | 33.25 | 0.947834 | 33.72 | 30.92 | 0.916963 | 35.04 | 35.63 | 1.016838 |
| 34.39 | 34.84 | 1.013085 | 33.56 | 32.81 | 0.977652 | | | |
| | | | | | | | | |
| mean | | 0.976657 | | | 0.920449 | | | 0.931414 |
| SD | | 0.025264 | | | 0.049568 | | | 0.060725 |

## compound 24

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without l | with l | VI/V0 | without l | with l | VI/V0 | without l | with l | VI/V0 |
| 42.67 | 36.8 | 0.862433 | 44.55 | 35.53 | 0.797531 | 44.74 | 36.2 | 0.809119 |
| 42.63 | 36.02 | 0.844945 | 46.41 | 37.78 | 0.814049 | 45.16 | 35.73 | 0.791187 |
| 41.71 | 39.36 | 0.943659 | 43.17 | 35.93 | 0.832291 | 45.65 | 37.16 | 0.81402 |
| 42.49 | 38.32 | 0.901859 | 44.26 | 34.51 | 0.779711 | 43.39 | 40.73 | 0.938696 |
| | | | | | | | | |
| mean | | 0.888224 | | | 0.805895 | | | 0.838255 |
| SD | | 0.03807 | | | 0.019486 | | | 0.058609 |

compound 25

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.67 | 30.68 | 0.719006 | 44.55 | 36.67 | 0.82312 | 44.74 | 40.21 | 0.898748 |
| 42.63 | 33.2 | 0.778794 | 46.41 | 36.66 | 0.789916 | 45.16 | 37.81 | 0.837245 |
| 41.71 | 33.89 | 0.812515 | 43.17 | 35 | 0.810748 | 45.65 | 37.22 | 0.815334 |
| 42.49 | 37.77 | 0.888915 | 44.26 | | | 43.39 | 36.51 | 0.841438 |
| | | | | | | | | |
| mean | | 0.799808 | | | 0.807928 | | | 0.848191 |
| SD | | 0.061384 | | | 0.013701 | | | 0.030826 |

compound 26

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 42.67 | 35.42 | 0.830091 | 44.55 | 31.61 | 0.70954 | 44.74 | 37.5 | 0.838176 |
| 42.63 | 34.86 | 0.817734 | 46.41 | 38.55 | 0.83064 | 45.16 | 38.46 | 0.851639 |
| 41.71 | 35.78 | 0.857828 | 43.17 | 37.55 | 0.869817 | 45.65 | 37.06 | 0.811829 |
| 42.49 | 33.53 | 0.789127 | 44.26 | | | 43.39 | | |
| | | | | | | | | |
| mean | | 0.823695 | | | 0.803332 | | | 0.833881 |
| SD | | 0.02468 | | | 0.068223 | | | 0.016533 |

## 3. Calculation of inhibition activity of the compounds (table 1) against ALN

compound 1

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 15.45 | 0.818326 | 18.86 | 16.73 | 0.887063 | 17.06 | 15.24 | 0.893318 |
| 18.18 | 17.07 | 0.938944 | 18.43 | 17.36 | 0.941942 | 16.77 | 15.84 | 0.944544 |
| 18.66 | 17.48 | 0.936763 | 18.3 | 17.64 | 0.963934 | 18.11 | 16.52 | 0.912203 |
| 17.47 | 15.9 | 0.910132 | 16.79 | 17.28 | 1.029184 | 17.45 | 16.6 | 0.951289 |
| | | | | | | | | |
| mean | | 0.901041 | | | 0.955531 | | | 0.925339 |
| SD | | 0.049084 | | | 0.050912 | | | 0.023665 |

compound 3

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 17.01 | 0.900953 | 18.86 | 17.57 | 0.931601 | 17.06 | 16.31 | 0.956038 |
| 18.18 | 17.4 | 0.957096 | 18.43 | 16.68 | 0.905046 | 16.77 | 15.32 | 0.913536 |
| 18.66 | 17.6 | 0.943194 | 18.3 | 16.2 | 0.885246 | 18.11 | 17.04 | 0.940917 |
| 17.47 | 18.65 | 1.067544 | 16.79 | 16 | 0.952948 | 17.45 | 17.36 | 0.994842 |
| | | | | | | | | |
| mean | | 0.967197 | | | 0.91871 | | | 0.951333 |
| SD | | 0.061514 | | | 0.025715 | | | 0.029378 |

compound 2

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 16.66 | 0.882415 | 18.86 | 16.95 | 0.898727 | 17.06 | 17.25 | 1.011137 |
| 18.18 | 15.65 | 0.860836 | 18.43 | 17.81 | 0.966359 | 16.77 | 15.06 | 0.898032 |
| 18.66 | 16.64 | 0.891747 | 18.3 | 17.41 | 0.951366 | 18.11 | 16.93 | 0.934843 |
| 17.47 | 15.36 | 0.879222 | 16.79 | 15.74 | 0.937463 | 17.45 | | 0.970201 |
| | | | | | | | | |
| mean | | 0.878555 | | | 0.938479 | | | 0.953553 |
| SD | | 0.011218 | | | 0.025123 | | | 0.04191 |

compound 4

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 17.98 | 0.952331 | 18.86 | 19.09 | 1.012195 | 17.06 | 19 | 1.113716 |
| 18.18 | 17.6 | 0.968097 | 18.43 | 18.09 | 0.981552 | 16.77 | 19.72 | 1.175909 |
| 18.66 | 15.73 | 0.84298 | 18.3 | 19.92 | 1.088525 | 18.11 | 19.15 | 1.057427 |
| 17.47 | | | 16.79 | 19.23 | 1.145325 | 17.45 | | |
| | | | | | | | | |
| mean | | 0.921136 | | | 1.056899 | | | 1.115684 |
| SD | | 0.055638 | | | 0.044979 | | | 0.04839 |

## compound 5

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 10.71 | 0.567267 | 18.86 | 12.48 | 0.661718 | 17.06 | 12.09 | 0.708675 |
| 18.18 | 12.03 | 0.661716 | 18.43 | 12.37 | 0.671188 | 16.77 | 11.02 | 0.657126 |
| 18.66 | 11.9 | 0.637728 | 18.3 | 12.62 | 0.689617 | 18.11 | 11.7 | 0.646052 |
| 17.47 | 11.36 | 0.650258 | 16.79 | 12.44 | 0.740917 | 17.45 | 12.56 | 0.719771 |
| | | | | | | | | |
| mean | | 0.629242 | | | 0.69086 | | | 0.682906 |
| SD | | 0.036773 | | | 0.030592 | | | 0.031804 |

## compound 6

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 16.06 | 0.850636 | 18.86 | 16.33 | 0.865854 | 17.06 | 15.89 | 0.931419 |
| 18.18 | 15.33 | 0.843234 | 18.43 | 16.2 | 0.879002 | 16.77 | 15 | 0.894454 |
| 18.66 | 15.35 | 0.822615 | 18.3 | 17.35 | 0.948087 | 18.11 | 15.1 | 0.833793 |
| 17.47 | 15.39 | 0.880939 | 16.79 | 15.35 | 0.914235 | 17.45 | 17.23 | 0.987393 |
| | | | | | | | | |
| mean | | 0.849356 | | | 0.901794 | | | 0.911765 |
| SD | | 0.020926 | | | 0.032051 | | | 0.055868 |

## compound 7

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 18.88 | 15.43 | 0.817267 | 18.86 | 14.53 | 0.770414 | 17.06 | 18.08 | 1.059789 |
| 18.18 | 14.147 | 0.778163 | 18.43 | 14.44 | 0.783505 | 16.77 | 18.59 | 1.108527 |
| 18.66 | 14.3 | 0.766345 | 18.3 | 16.7 | 0.912568 | 18.11 | 18 | 0.993926 |
| 17.47 | 15.21 | 0.870635 | 16.79 | 14.54 | 0.865992 | 17.45 | | |
| | | | | | | | | |
| mean | | 0.808103 | | | 0.83312 | | | 1.054081 |
| SD | | 0.040726 | | | 0.058708 | | | 0.04696 |

## compound 8

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 14.25 | 0.990271 | 15.13 | 13.1 | 0.865829 | 15.1 | 14.42 | 0.954967 |
| 14.96 | 12.2 | 0.815508 | 15.19 | 13.04 | 0.85846 | 14.95 | 14.51 | 0.970569 |
| 14.57 | 12.16 | 0.834592 | 15.42 | 13.64 | 0.884565 | 15.65 | 13.85 | 0.884984 |
| 14.97 | | | 14.17 | 13.26 | 0.93578 | 15.24 | | |
| | | | | | | | | |
| mean | | 0.880124 | | | 0.869618 | | | 0.93684 |
| SD | | 0.078275 | | | 0.010989 | | | 0.037217 |

## compound 9

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 13.85 | 0.962474 | 15.13 | 13.98 | 0.923992 | 15.1 | 14.03 | 0.929139 |
| 14.96 | 13.37 | 0.893717 | 15.19 | 13.24 | 0.871626 | 14.95 | 15.3 | 1.023411 |
| 14.57 | 14.5 | 0.995196 | 15.42 | 14.32 | 0.928664 | 15.65 | 15.34 | 0.980192 |
| 14.97 | 11.5 | 0.768203 | 14.17 | 13.61 | 0.96048 | 15.24 | 15.31 | 1.004593 |
| | | | | | | | | |
| mean | | 0.904897 | | | 0.921191 | | | 0.984334 |
| SD | | 0.087005 | | | 0.031875 | | | 0.035359 |

## compound 10

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 8.85 | 0.61501 | 15.13 | 9.15 | 0.604759 | 15.1 | 9.09 | 0.601987 |
| 14.96 | 9.12 | 0.609626 | 15.19 | 7.93 | 0.522054 | 14.95 | 9.33 | 0.62408 |
| 14.57 | 9.87 | 0.677419 | 15.42 | 10.72 | 0.695201 | 15.65 | 10.29 | 0.657508 |
| 14.97 | | | 14.17 | 10.6 | 0.748059 | 15.24 | 9.24 | 0.606299 |
| | | | | | | | | |
| mean | | 0.634018 | | | 0.642518 | | | 0.622469 |
| SD | | 0.030768 | | | 0.086388 | | | 0.021859 |

## compound 11

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 10.88 | 0.756081 | 15.13 | 13.72 | 0.906808 | 15.1 | 13.88 | 0.919205 |
| 14.96 | 12.67 | 0.846925 | 15.19 | 12.68 | 0.83476 | 14.95 | 13.6 | 0.909699 |
| 14.57 | 12.52 | 0.8593 | 15.42 | 12.65 | 0.820363 | 15.65 | 12.67 | 0.809585 |
| 14.97 | 9.86 | 0.658651 | 14.17 | 11.34 | 0.800282 | 15.24 | | |
| | | | | | | | | |
| mean | | 0.780239 | | | 0.840553 | | | 0.879496 |
| SD | | 0.080723 | | | 0.040164 | | | 0.049587 |

## compound 12

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 13.36 | 0.928423 | 15.13 | 13.36 | 0.883014 | 15.1 | 13.82 | 0.915232 |
| 14.96 | 14.42 | 0.963904 | 15.19 | 13.8 | 0.908492 | 14.95 | 13.97 | 0.934448 |
| 14.57 | 13.18 | 0.904598 | 15.42 | 13.58 | 0.880674 | 15.65 | 13.03 | 0.832588 |
| 14.97 | 13.37 | 0.89312 | 14.17 | 14.22 | 1.003529 | 15.24 | 14.28 | 0.937008 |
| | | | | | | | | |
| mean | | 0.922511 | | | 0.918927 | | | 0.904819 |
| SD | | 0.027079 | | | 0.050048 | | | 0.042543 |

## compound 13

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 14.9 | 1.035441 | 15.13 | 13.73 | 0.907469 | 15.1 | 12.05 | 0.798013 |
| 14.96 | 15.06 | 1.006684 | 15.19 | 14.85 | 0.977617 | 14.95 | 13.41 | 0.89699 |
| 14.57 | 13.36 | 0.916953 | 15.42 | 13.15 | 0.852789 | 15.65 | 14.2 | 0.907348 |
| 14.97 | 13.81 | 0.922512 | 14.17 | 13.15 | 0.928017 | 15.24 | 14.98 | 0.98294 |
| | | | | | | | | |
| | | | | | | | | |
| mean | | 0.970398 | | | 0.916473 | | | 0.896323 |
| SD | | 0.051713 | | | 0.044745 | | | 0.065744 |

## compound 14

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 14.39 | 12.8 | 0.889507 | 15.13 | 12.95 | 0.855915 | 15.1 | 12.37 | 0.819205 |
| 14.57 | 11.99 | 0.822924 | 15.19 | 12.44 | 0.81896 | 14.95 | 11.55 | 0.772575 |
| 14.97 | 12.08 | 0.806947 | 15.42 | 13.96 | 0.905318 | 15.65 | 13.31 | 0.850479 |
| | | | 14.17 | 13.56 | 0.956951 | 15.24 | 14.44 | 0.947507 |
| | | | | | | | | |
| mean | | 0.839793 | 14.9775 | 13.2275 | 0.884286 | 15.235 | 12.9175 | 0.847442 |
| SD | | 0.035753 | 0.478611 | 0.579714 | 0.051949 | 0.260624 | 1.077251 | 0.064079 |

## compound 15

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 40.27 | 1.000994 | 43.45 | 38.93 | 0.895972 | 41.6 | 39.83 | 0.957452 |
| 43.66 | 43.63 | 0.999313 | 41.31 | 42.66 | 1.03268 | 40.26 | 37.95 | 0.942623 |
| 43.8 | 36.73 | 0.838584 | 40.82 | 41.07 | 1.077953 | 38.5 | 38.3 | 0.994805 |
| 42.7 | | | 38.1 | 39.17 | 0.959579 | | | |
| | | | | | | | | |
| mean | | 0.946297 | | | 1.002202 | | | 0.96496 |
| SD | | 0.076167 | | | 0.077356 | | | 0.021955 |

## compound 16

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 37.57 | 0.93388 | 43.45 | 36.96 | 0.850633 | 41.6 | 34.31 | 0.82476 |
| 43.66 | 40.83 | 0.935181 | 41.31 | 36.58 | 0.8855 | 40.26 | 36.82 | 0.914555 |
| 43.8 | 39.57 | 0.903425 | 40.82 | 39.34 | 0.963743 | 38.5 | 37.69 | 0.978961 |
| 42.7 | | | 38.1 | | | | | |
| | | | | | | | | |
| mean | | 0.924162 | | | 0.899959 | | | 0.906092 |
| SD | | 0.014673 | | | 0.047295 | | | 0.063236 |

## compound 17

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 29.36 | 0.729804 | 43.45 | 28.06 | 0.6458 | 41.6 | 29.15 | 0.700721 |
| 43.66 | 27.77 | 0.636051 | 41.31 | 28.79 | 0.696926 | 40.26 | 28.69 | 0.712618 |
| 43.8 | 30.49 | 0.696119 | 40.82 | 29.83 | 0.730769 | 38.5 | 30.92 | 0.803117 |
| 42.7 | 29.06 | 0.680562 | 38.1 | 31.84 | 0.835696 | | | |
| | | | | | | | | |
| mean | | 0.685634 | | | 0.727298 | | | 0.738819 |
| SD | | 0.033709 | | | 0.06951 | | | 0.045724 |

## compound 18

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 31.62 | 0.785981 | 43.45 | 40.42 | 0.930265 | 41.6 | 34.14 | 0.820673 |
| 43.66 | 34.26 | 0.7847 | 41.31 | 42.66 | 1.03268 | 40.26 | 33.85 | 0.840785 |
| 43.8 | 29.54 | 0.674429 | 40.82 | 36.81 | 0.901764 | 38.5 | 31.8 | 0.825974 |
| 42.7 | 32.7 | 0.765808 | 38.1 | | | | | |
| | | | | | | | | |
| mean | | 0.752729 | | | 0.954903 | | | 0.829144 |
| SD | | 0.045907 | | | 0.056214 | | | 0.008511 |

## compound 19

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 33.84 | 0.841163 | 43.45 | 29.24 | 0.672957 | 41.6 | 36.75 | 0.883413 |
| 43.66 | 29.59 | 0.677737 | 41.31 | 29.13 | 0.705156 | 40.26 | 30.65 | 0.761302 |
| 43.8 | 33.95 | 0.775114 | 40.82 | 33.19 | 0.813082 | 38.5 | 27.65 | 0.718182 |
| 42.7 | 35.2 | 0.824356 | 38.1 | | | | 30.9 | 0.767511 |
| | | | | | | | | |
| mean | | 0.779593 | | | 0.730398 | | | 0.782602 |
| SD | | 0.063619 | | | 0.059925 | | | 0.061226 |

## compound 20

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 35.34 | 0.878449 | 43.45 | 49.78 | 1.145685 | 41.6 | 36.65 | 0.88101 |
| 43.66 | 36.95 | 0.846312 | 41.31 | 49.84 | 1.206488 | 40.26 | 35.68 | 0.886239 |
| 43.8 | 37.83 | 0.863699 | 40.82 | 48.12 | 1.178834 | 38.5 | | |
| 42.7 | | | 38.1 | | | | | |
| | | | | | | | | |
| mean | | 0.86282 | | | 1.177002 | | | 0.883625 |
| SD | | 0.013134 | | | 0.024856 | | | 0.002615 |

compound 21

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 40.23 | 32.69 | 0.812578 | 43.45 | 28 | 0.644419 | 41.6 | 31.23 | 0.750721 |
| 43.66 | 29.38 | 0.672927 | 41.31 | 26.98 | 0.653111 | 40.26 | 34.7 | 0.861898 |
| 43.8 | 29.74 | 0.678995 | 40.82 | 28.33 | 0.694023 | 38.5 | 39 | 1.012987 |
| 42.7 | | | 38.1 | 26.01 | 0.682677 | | | |
| | | | | | | | | |
| mean | | 0.7215 | | | 0.663851 | | | 0.875202 |
| SD | | 0.064449 | | | 0.020427 | | | 0.107482 |

compound 22

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 45.52 | 40.09 | 0.880712 | 46.9 | 45.46 | 0.969296 | 50.18 | 45.36 | 0.903946 |
| 46.16 | 44.13 | 0.956023 | 48.27 | 44.89 | 0.929977 | 47.75 | 46.02 | 0.96377 |
| 46.97 | 45.27 | 0.963807 | 48.7 | 47.39 | 0.973101 | 48.84 | 45.78 | 0.937346 |
| 44.9 | 39.66 | 0.883296 | | | | | 46.42 | 0.95045 |
| | | | | | | | | |
| mean | | 0.920959 | | | 0.957458 | | | 0.938878 |
| SD | | 0.039063 | | | 0.019494 | | | 0.022227 |

compound 23

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 45.52 | 44.76 | 0.983304 | 46.9 | 46.29 | 0.986994 | 50.18 | 47.65 | 0.949582 |
| 46.16 | 44.73 | 0.969021 | 48.27 | 44.84 | 0.928941 | 47.75 | 47.69 | 0.998743 |
| 46.97 | 43.53 | 0.926762 | 48.7 | 45.34 | 0.931006 | 48.84 | 49.05 | 1.0043 |
| 44.9 | 38.16 | 0.849889 | | | | | | |
| | | | | | | | | |
| mean | | 0.932244 | | | 0.94898 | | | 0.984208 |
| SD | | 0.051894 | | | 0.026893 | | | 0.02459 |

compound 24

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 45.52 | 38.41 | 0.843805 | 46.9 | 39.23 | 0.836461 | 50.18 | 45.31 | 0.902949 |
| 46.16 | 38.01 | 0.82344 | 48.27 | 38.01 | 0.787446 | 47.75 | 41.36 | 0.866178 |
| 46.97 | 43.67 | 0.929742 | 48.7 | 45.25 | 0.929158 | 48.84 | 41.85 | 0.85688 |
| 44.9 | 44.8 | 0.997773 | | 44.8 | 0.919918 | | | |
| | | | | | | | | |
| mean | | 0.89869 | | | 0.868246 | | | 0.875336 |
| SD | | 0.069743 | | | 0.05899 | | | 0.019891 |

compound 25

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 45.52 | 44.05 | 0.967707 | 46.9 | 45.59 | 0.972068 | 50.18 | 43.72 | 0.871263 |
| 46.16 | 45.62 | 0.988302 | 48.27 | 43.12 | 0.893308 | 47.75 | 44.29 | 0.927539 |
| 46.97 | 44.65 | 0.950607 | 48.7 | 44.69 | 0.917659 | 48.84 | 44.63 | 0.9138 |
| 44.9 | 45.37 | 1.010468 | | 47.54 | 0.976181 | | 47.4 | 0.970516 |
| | | | | | | | | |
| mean | | 0.979271 | | | 0.939804 | | | 0.92078 |
| SD | | 0.022417 | | | 0.035414 | | | 0.035426 |

compound 26

| 0 min | | | 15 min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| without I | with I | VI/V0 | without I | with I | VI/V0 | without I | with I | VI/V0 |
| 45.52 | 42.56 | 0.934974 | 46.9 | 40.54 | 0.864392 | 50.18 | 41.21 | 0.821244 |
| 46.16 | 39.54 | 0.856586 | 48.27 | 43.45 | 0.900145 | 47.75 | 39.41 | 0.82534 |
| 46.97 | 40.67 | 0.865872 | 48.7 | 39.45 | 0.810062 | 48.84 | 38.93 | 0.797093 |
| 44.9 | 40.97 | 0.912472 | | | | | | |
| | | | | | | | | |
| mean | | 0.892476 | | | 0.8582 | | | 0.814559 |
| SD | | 0.032411 | | | 0.037036 | | | 0.012463 |

## 4. Calculation of inhibition activity of the compounds (table 1) against MMP-14

## compound 1

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 16.47 | 0.950375 | 16.47 | 16.8 | 1.020036 | 20.58 | 16.33 | 0.793489 |
| 17.1 | 16.01 | 0.936257 | 18.08 | 15 | 0.829646 | 19.64 | 17.44 | 0.887984 |
| 17.22 | 15.54 | 0.902439 | 17.41 | 16.54 | 0.950029 | 18.92 | 17.98 | 0.950317 |
| 16.71 | 14.31 | 0.856373 | | 16.35 | 0.904314 | 20.15 | 16.39 | 0.8134 |
| | | | | | | | | |
| mean | | 0.911361 | | | 0.926006 | | | 0.861297 |
| SD | | 0.036212 | | | 0.069236 | | | 0.062307 |

## compound 2

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 16.39 | 0.945759 | 16.47 | 17.26 | 1.047966 | 20.58 | 17.28 | 0.83965 |
| 17.1 | 15.46 | 0.904094 | 18.08 | 15.64 | 0.865044 | 19.64 | 16.59 | 0.844705 |
| 17.22 | 14.35 | 0.833333 | 17.41 | 15 | 0.861574 | 18.92 | 16.43 | 0.868393 |
| 16.71 | | | | | | 20.15 | 18.54 | 0.920099 |
| | | | | | | | | |
| mean | | 0.894395 | | | 0.924861 | | | 0.850916 |
| SD | | 0.046407 | | | 0.08706 | | | 0.012529 |

## compound 3

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 17.91 | 1.033468 | 16.47 | 17.25 | 1.047359 | 20.58 | 17.39 | 0.844995 |
| 17.1 | 16.71 | 0.977193 | 18.08 | 15.34 | 0.848451 | 19.64 | 15.95 | 0.812118 |
| 17.22 | 15.95 | 0.926249 | 17.41 | 16.12 | 0.925905 | 18.92 | 16.43 | 0.868393 |
| 16.71 | 15.62 | 0.93477 | | 16.41 | 0.907633 | 20.15 | 17.78 | 0.882382 |
| | | | | | | | | |
| mean | | 0.96792 | | | 0.932337 | | | 0.851972 |
| SD | | 0.042479 | | | 0.072316 | | | 0.026606 |

## compound 4

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 16.53 | 0.953837 | 16.47 | 17.67 | 1.07286 | 20.58 | 18.63 | 0.905248 |
| 17.1 | 16.34 | 0.955556 | 18.08 | 18.3 | 1.012168 | 19.64 | 18.89 | 0.961813 |
| 17.22 | 16.56 | 0.961672 | 17.41 | 17.74 | 1.018955 | 18.92 | 16.83 | 0.889535 |
| 16.71 | 18.59 | 1.112507 | | 18.71 | 1.034845 | 20.15 | 19.57 | 0.971216 |
| | | | | | | | | |
| mean | | 0.995893 | | | 1.034707 | | | 0.931953 |
| SD | | 0.06739 | | | 0.023515 | | | 0.035163 |

## compound 5

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 12.87 | 0.742643 | 16.47 | 12.8 | 0.777171 | 20.58 | 13.92 | 0.676385 |
| 17.1 | 11.66 | 0.681871 | 18.08 | 11.54 | 0.638274 | 19.64 | 13.25 | 0.674644 |
| 17.22 | 12.35 | 0.717189 | 17.41 | 11.95 | 0.686387 | 18.92 | 12.76 | 0.674419 |
| 16.71 | 12.19 | 0.729503 | | 12.1 | 0.669248 | 20.15 | 13.9 | 0.689826 |
| | | | | | | | | |
| mean | | 0.717802 | | | 0.69277 | | | 0.678818 |
| SD | | 0.022613 | | | 0.05169 | | | 0.006401 |

## compound 6

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 17.09 | 0.986151 | 16.47 | 17.02 | 1.033394 | 20.58 | 18.18 | 0.883382 |
| 17.1 | 17.61 | 1.029825 | 18.08 | 17.08 | 0.94469 | 19.64 | 18.55 | 0.944501 |
| 17.22 | 17.3 | 1.004646 | 17.41 | 17.61 | 1.011488 | 18.92 | 17.85 | 0.943446 |
| 16.71 | | | | 17.85 | 1.025273 | 20.15 | | 0.980444 |
| | | | | | | | | |
| mean | | 1.006874 | | | 0.996524 | | | 0.923776 |
| SD | | 0.017899 | | | 0.034964 | | | 0.034844 |

## compound 7

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 17.33 | 16.82 | 0.970571 | 16.47 | 16.23 | 0.985428 | 20.58 | 16.64 | 0.808552 |
| 17.1 | 15.95 | 0.932749 | 18.08 | 15.73 | 0.870022 | 19.64 | 16.28 | 0.828921 |
| 17.22 | 17.57 | 1.020325 | 17.41 | 15.95 | 0.91614 | 18.92 | 17.37 | 0.918076 |
| 16.71 | 16 | 0.95751 | | 16.71 | 0.959793 | 20.15 | | |
| | | | | | | | | |
| | | 0.970289 | | | 0.932846 | | | 0.85185 |
| mean | | 0.031923 | | | 0.043923 | | | 0.047562 |
| SD | | | | | | | | |

## compound 8

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 19.61 | 0.875446 | 22.62 | 19.26 | 0.851459 | 24.95 | 19.8 | 0.793587 |
| 22.9 | 18.92 | 0.826201 | 21.81 | 20.26 | 0.928932 | 24.61 | 20.34 | 0.826493 |
| 20.37 | 18.88 | 0.926853 | 23.06 | 19.43 | 0.842585 | 23.4 | 21.65 | 0.925214 |
| 22.44 | 19.27 | 0.858734 | 22.57 | 18.6 | 0.824103 | 25.26 | 21.84 | 0.864608 |
| | | | | | | | | |
| mean | | 0.871809 | | | 0.861769 | | | 0.852476 |
| SD | | 0.03638 | | | 0.040012 | | | 0.048941 |

## compound 9

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 22.35 | 0.997768 | 22.62 | 21.65 | 0.957118 | 24.95 | 23.12 | 0.926653 |
| 22.9 | 22.82 | 0.996507 | 21.81 | 20.23 | 0.927556 | 24.61 | 22.58 | 0.917513 |
| 20.37 | 20.15 | 0.9892 | 23.06 | 21.12 | 0.915872 | 23.4 | 24.28 | 1.037607 |
| 22.44 | 20.72 | 0.923351 | 22.57 | 22.58 | 1.000443 | 25.26 | | |
| mean | | 0.976706 | | | 0.950247 | | | 0.960591 |
| SD | | 0.030978 | | | 0.032647 | | | 0.054586 |

## compound 10

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 15.36 | 0.685714 | 22.62 | 15.82 | 0.699381 | 24.95 | 15.96 | 0.639679 |
| 22.9 | 14.37 | 0.627511 | 21.81 | 15.21 | 0.697387 | 24.61 | 15.47 | 0.628606 |
| 20.37 | 14.73 | 0.723122 | 23.06 | 15.38 | 0.666956 | 23.4 | 16.87 | 0.72094 |
| 22.44 | 16.6 | 0.73975 | 22.57 | 15.54 | 0.688525 | 25.26 | | |
| mean | | 0.694024 | | | 0.688062 | | | 0.663075 |
| SD | | 0.043101 | | | 0.012853 | | | 0.041166 |

## compound 11

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 20.69 | 0.923661 | 22.62 | 21.07 | 0.931477 | 24.95 | 21.42 | 0.858517 |
| 22.9 | 20.07 | 0.876419 | 21.81 | 20.04 | 0.918845 | 24.61 | 21.48 | 0.872816 |
| 20.37 | 18.28 | 0.897398 | 23.06 | 20.16 | 0.874241 | 23.4 | 21.92 | 0.936752 |
| 22.44 | 19.61 | 0.873886 | 22.57 | 20.7 | 0.917147 | 25.26 | 22.87 | 0.905384 |
| mean | | 0.892841 | | | 0.910427 | | | 0.893367 |
| SD | | 0.019997 | | | 0.021613 | | | 0.030264 |

## compound 12

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 21.28 | 0.95 | 22.62 | 21.44 | 0.947834 | 24.95 | 22.13 | 0.886974 |
| 22.9 | 22.53 | 0.983843 | 21.81 | 21.68 | 0.994039 | 24.61 | 21.8 | 0.885819 |
| 20.37 | 22.17 | 1.088365 | 23.06 | 20.09 | 0.871206 | 23.4 | 20.61 | 0.880769 |
| 22.44 | 19.6 | 0.962199 | | 18.13 | 0.83127 | 25.26 | 25.36 | 1.003959 |
| mean | | 0.996102 | | | 0.911087 | | | 0.884521 |
| SD | | 0.05463 | | | 0.063625 | | | 0.051771 |

## compound 13

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 22.77 | 1.016518 | 22.62 | 23 | 1.016799 | 24.95 | | |
| 22.9 | 22.75 | 0.99345 | 21.81 | 22.16 | 1.016048 | 24.61 | 23.41 | 0.951239 |
| 20.37 | 22.36 | 1.097693 | 23.06 | 24.1 | 1.0451 | 23.4 | 24.3 | 1.038462 |
| 22.44 | 21.75 | 0.969251 | | 21.55 | 0.988079 | 25.26 | 27.46 | 1.087094 |
| mean | | 1.019228 | | | 1.016506 | | | 1.025598 |
| SD | | 0.048286 | | | 0.020162 | | | 0.056203 |

## compound 14

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.4 | 22.3 | 0.995536 | 22.62 | 23.54 | 1.040672 | 24.95 | | |
| 22.9 | 22 | 0.960699 | 21.81 | 23.89 | 1.095369 | 24.61 | 24.08 | 0.978464 |
| 20.37 | 19.56 | 0.960236 | 23.06 | 23.38 | 1.013877 | 23.4 | 25.74 | 1.1 |
| 22.44 | 23.57 | 1.050357 | | 23.17 | 1.00477 | 25.26 | 27.71 | 1.096991 |
| mean | | 0.991707 | | | 1.038672 | | | 1.058485 |
| SD | | 0.036764 | | | 0.035294 | | | 0.056597 |

## compound 15

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 20.23 | 0.911261 | 23.96 | 21.04 | 0.87813 | 23.8 | 22.12 | 0.929412 |
| 22.05 | 19.33 | 0.876644 | 25.57 | 20.97 | 0.888559 | 24.62 | 23.1 | 0.938262 |
| 21.62 | 19.83 | 0.917206 | 23.9 | 21.73 | 0.909205 | 24.63 | 22.76 | 0.924076 |
| 23.16 | 20.1 | 0.867876 | 23.6 | | | 25.12 | 22.34 | 0.889331 |
| mean | | 0.893247 | | | 0.891965 | | | 0.92027 |
| SD | | 0.021319 | | | 0.012913 | | | 0.018567 |

## compound 16

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 19.7 | 0.887387 | 23.96 | 20.22 | 0.843907 | 23.8 | 21.3 | 0.894958 |
| 22.05 | 19.49 | 0.8839 | 25.57 | 20.77 | 0.81228 | 24.62 | 20.78 | 0.844029 |
| 21.62 | 19.23 | 0.889454 | 23.9 | 20.32 | 0.850209 | 24.63 | 19.97 | 0.8108 |
| 23.16 | 18.4 | 0.834467 | 23.6 | 19.85 | 0.841102 | 25.12 | 22.29 | 0.887341 |
| mean | | 0.873802 | | | 0.836874 | | | 0.859282 |
| SD | | 0.022797 | | | 0.014578 | | | 0.034071 |

## compound 17

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 13.58 | 0.611712 | 23.96 | 13.28 | 0.554257 | 23.8 | 14 | 0.588235 |
| 22.05 | 13.37 | 0.606349 | 25.57 | 12.86 | 0.502933 | 24.62 | 12.74 | 0.517465 |
| 21.62 | 11.51 | 0.532377 | 23.9 | 13.72 | 0.574059 | 24.63 | 14.3 | 0.580593 |
| 23.16 | 12.47 | 0.538428 | 23.6 | 13.79 | 0.584322 | 25.12 | | |
| | | | | | | | | |
| mean | | 0.572217 | | | 0.553893 | | | 0.562098 |
| SD | | 0.036925 | | | 0.031343 | | | 0.031714 |

## compound 18

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 18.74 | 0.844144 | 23.96 | 16.39 | 0.684057 | 23.8 | 17.09 | 0.718067 |
| 22.05 | 16.85 | 0.764172 | 25.57 | 17.13 | 0.669926 | 24.62 | 17.09 | 0.694151 |
| 21.62 | 17.62 | 0.814986 | 23.9 | 17.41 | 0.728452 | 24.63 | 17.68 | 0.717824 |
| 23.16 | | | 23.6 | 17.67 | 0.748729 | 25.12 | | |
| | | | | | | | | |
| mean | | 0.807768 | | | 0.694145 | | | 0.710014 |
| SD | | 0.033045 | | | 0.024935 | | | 0.011217 |

## compound 19

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 18.68 | 0.841441 | 23.96 | 17.96 | 0.749583 | 23.8 | 18.75 | 0.787815 |
| 22.05 | 18.43 | 0.835828 | 25.57 | 18.75 | 0.733281 | 24.62 | 18.34 | 0.744923 |
| 21.62 | 18.34 | 0.848289 | 23.9 | 18.96 | 0.793305 | 24.63 | 18.45 | 0.749086 |
| 23.16 | 17.96 | 0.775475 | 23.6 | 18.79 | 0.796186 | 25.12 | | |
| | | | | | | | | |
| mean | | 0.825258 | | | 0.768089 | | | 0.760608 |
| SD | | 0.029079 | | | 0.027292 | | | 0.019313 |

## compound 20

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 19.7 | 0.887387 | 23.96 | 19.96 | 0.833055 | 23.8 | 21.51 | 0.903782 |
| 22.05 | 19.45 | 0.882086 | 25.57 | 20.66 | 0.864435 | 24.62 | 23.16 | 0.940699 |
| 21.62 | 20.12 | 0.93062 | 23.9 | 21.16 | 0.885356 | 24.63 | 23.79 | 0.965895 |
| 23.16 | 20.52 | 0.88601 | 23.6 | 21.65 | 0.917373 | 25.12 | 23.57 | 0.938296 |
| | | | | | | | | |
| mean | | 0.896526 | | | 0.875055 | | | 0.937168 |
| SD | | 0.01978 | | | 0.030715 | | | 0.0221 |

## compound 21

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.2 | 21.3 | 0.959459 | 23.96 | 21.04 | 0.87813 | 23.8 | 22.43 | 0.942437 |
| 22.05 | 20.83 | 0.944671 | 25.57 | 20.71 | 0.866527 | 24.62 | 22.33 | 0.906986 |
| 21.62 | 20.71 | 0.957909 | 23.9 | 22.07 | 0.923431 | 24.63 | 22.74 | 0.923264 |
| 23.16 | | | 23.6 | 21.81 | 0.924153 | | | |
| | | | | | | | | |
| mean | | 0.954013 | | | 0.889363 | | | 0.924229 |
| SD | | 0.006636 | | | 0.024551 | | | 0.014489 |

## compound 22

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.55 | 19.83 | 0.879379 | 23.7 | 21 | 0.886076 | 25.23 | 22.09 | 0.907933 |
| 21.55 | 20.83 | 0.923725 | 24.28 | 21.72 | 0.894563 | 24.33 | 22.57 | 0.927661 |
| 23.19 | 20.06 | 0.865028 | 24.86 | 22.56 | 0.907482 | 23.76 | 23.81 | 0.964358 |
| | | | 24.42 | 20.9 | 0.855856 | 24.69 | | |
| | | | | | | | | |
| mean | | 0.889377 | | | 0.89604 | | | 0.933317 |
| SD | | 0.024984 | | | 0.008801 | | | 0.02338 |

## compound 23

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.55 | 21.09 | 0.935255 | 23.7 | 21.73 | 0.916878 | 25.23 | 22.88 | 0.906857 |
| 21.55 | 21.38 | 0.992111 | 24.28 | 21.7 | 0.89374 | 24.33 | 22.7 | 0.933005 |
| 23.19 | 21.03 | 0.906856 | 24.86 | 21.51 | 0.865245 | 23.76 | 23 | 0.968013 |
| | 20.43 | 0.905987 | 24.42 | 23.57 | 0.965192 | 24.69 | 23.12 | 0.936412 |
| | | | | | | | | |
| mean | | 0.935052 | | | 0.910264 | | | 0.936072 |
| SD | | 0.034984 | | | 0.036608 | | | 0.021699 |

## compound 24

| 0 min | | | 15min | | | 30min | | |
|---|---|---|---|---|---|---|---|---|
| v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| 22.55 | 19.27 | 0.854545 | 23.7 | 19.91 | 0.840084 | 25.23 | 20.53 | 0.813714 |
| 21.55 | 18.19 | 0.844084 | 24.28 | 20.21 | 0.832372 | 24.33 | 18.72 | 0.76942 |
| 23.19 | 19.15 | 0.825787 | 24.86 | 19.96 | 0.802896 | 23.76 | 18.92 | 0.796296 |
| | 19.47 | 0.863415 | 24.42 | 19.76 | 0.809173 | 24.69 | 21.76 | 0.862465 |
| | | | | | | | | |
| mean | | 0.846958 | | | 0.821131 | | | 0.810474 |
| SD | | 0.014008 | | | 0.015501 | | | 0.033912 |

| | compound 25 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 min | | | 15min | | | 30min | | |
| | v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| | 22.55 | 21.38 | 0.948115 | 23.7 | 19.81 | 0.835865 | 25.23 | 23.74 | 0.940943 |
| | 21.55 | 22.03 | 0.949978 | 24.28 | 20.4 | 0.840198 | 24.33 | 23.98 | 0.985614 |
| | 23.19 | 21.15 | 0.912031 | 24.86 | 22.21 | 0.893403 | 23.76 | 24.27 | 1.021465 |
| | | 21.23 | 0.941463 | 24.42 | 23.15 | 0.947993 | 24.69 | 25.61 | 1.037262 |
| | | | | | | | | | |
| mean | | | 0.937897 | | | 0.879365 | | | 0.996321 |
| SD | | | 0.015265 | | | 0.045643 | | | 0.037046 |

| | compound 26 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 min | | | 15min | | | 30min | | |
| | v0 | vi | vi/v0 | v0 | vi | vi/v0 | v0 | vi | vi/v0 |
| | 22.55 | 20.87 | 0.925499 | 23.7 | 21.03 | 0.887342 | 25.23 | 23.15 | 0.917558 |
| | 21.55 | 20.15 | 0.935035 | 24.28 | 21.97 | 0.90486 | 24.33 | 22.58 | 0.928072 |
| | 23.19 | 19.85 | 0.855972 | 24.86 | 21.52 | 0.865648 | 23.76 | 22.88 | 0.962963 |
| | | 20.57 | 0.912195 | 24.42 | 21.34 | 0.873874 | 24.69 | 24.91 | 1.00891 |
| | | | | | | | | | |
| mean | | | 0.907175 | | | 0.882931 | | | 0.954376 |
| SD | | | 0.030655 | | | 0.014842 | | | 0.03569 |