International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

In silico Analysis of Molecules for the Inhibition of Trypsin, and its Inhibition Assay using Trilobatin

Arenza Sandra Rai [1], *Arjun Rao Isukapatla* [1*]

[1] *Department of Life Sciences, CHRIST University, Bangalore, Karnataka, India*

Abstract

Trypsin is a serine protease enzyme that has a wide range of functions in physiological and pathological processes. Although most of its functions are often associated with positive effects, there is still a need for its inhibition, because its overexpression has been associated with various forms of cancers and poses to be fatal if left unaddressed. Early detection of elevated trypsin levels can help diagnose cancer, and its inhibition can prevent its progression. Therefore, Molecular Docking was performed for four compounds, allantoin, barbituric acid, azoindole, and trilobatin, to test their inhibition potential against human trypsin. The binding affinities and the interacting residues were predicted using AutoDock. Azoindole, out of the four compounds, showed the highest binding affinity, followed by trilobatin. Moreover, the trypsin inhibition assay was performed using trilobatin as the inhibitor. A constant concentration of trypsin was maintained at 0.013mg/mL. To assess the use of trilobatin as a drug, SwissADME was employed. Its use as a potential oral drug was discovered.

Keywords: Trypsin, inhibition, molecular docking, AutoDock, Trilobatin, Azoindole, SwissADME

Full length article*Corresponding Author*, e-mail: *arjun.rao@christuniversity.in* *arjun4n6@gmail.com,* Doi # https://doi.org/10.62877/25-IJCBS-25-27-21-25

Submitted:28-05-2025; Accepted: 10-08-2025; Published: 13-08-2025

1. Introduction

Trypsin is a serine protease first secreted in its inactive form, trypsinogen, released into the intestinal lumen by the exocrine cells of the pancreas, which is later activated into its active form upon the action of the enzyme enterokinase [1]. Besides the lumen of the small intestine, it is also secreted in the oesophagus, stomach, and even the epithelial cells. [2]. Apart from its proteolytic functions, it also helps in the proper maintenance of the immune system, nervous system, signalling, and apoptosis. A signalling molecule, Cholecystokinin (CCK), helps in the secretion of pancreatic enzymes and bile, and is also dependent on the concentration of trypsin. Therefore, trypsin provides negative feedback by degradation of peptides to inhibit the release of CCK when the level of digestive enzymes is adequate [3]. Trypsin shows high specificity for peptide bonds at the carboxyl side of basic amino acids, specifically lysine and arginine. The negatively charged aspartate residue at the S1 pocket of trypsin shows affinity for the positively charged side chains of the substrate.

This specificity can, however, harbour negative impacts by leading to the unwanted cleavage of non-target proteins under uncontrolled conditions. The auto-digestion property of trypsin hinders its stability in the absence of inhibitors or stabilizing agents like calcium ions; a proper balance in its activity should be maintained, else the cells can be prone to proteolytic damage to their cellular components [4]. In normal cases, trypsinogen should be activated in the duodenum; however, in abnormal situations, it gets prematurely activated to trypsin in the pancreas, leading to pancreatitis. This happens due to activation of zymogens by trypsin, leading to proteolytic digestion of pancreatic cells and their components. This can be accounted due to the mutations in the PRSS1 gene leading to insufficient secretion of trypsin inhibitors like PSTI/SPINK1 [5]. Trypsin is employed widely in cell and tissue culture for the removal of dead and damaged cells while allowing tissue regeneration and healing. Nonetheless, uncontrolled reactions may pose a likely threat to normal cells [6].

1.1. Carcinogenesis and trypsin

Overexpression of proteases leads to metastasis in cancer cells due to the degradation of the extracellular matrix components of the cells, allowing invasion and proliferation of cancer cells. Trypsin is one of the major proteases known to cause this [7]. PAR-2, a protease-associated receptor that is activated by trypsin, is found in various human cells, such as nerve cells, intestinal cells, and cells of the vascular tissues. The expression of these receptors, especially PAR-1 and PAR-2, is observed in various cancer cell lines such as melanoma and sarcoma [8]. Matrix proteases such as matrix serine proteases (MSPs) and matrix metalloproteinases (MMPs) are crucial for the metastasis and tumour invasion to occur because they help in degrading the extracellular matrix components, allowing tumor cells to affect adjacent cells [9]. Trypsin can hydrolyze a wide variety of proteins, including extracellular matrix, and is therefore involved in invasion of cancer cells in tissues. Trypsin activates pro-enzymes,

including important matrix metalloproteinases (MMPs) such as matrilysin (MMP-7), thereby leading to large degradation of extracellular matrix components along with basement membranes. This allows easy migration of tumour cells, leading to cancer progression and metastasis. Elevated trypsin levels are frequently associated with faster cancer progression, and their effective monitoring is crucial in cancer patients and in the prediction of the recurrence of colorectal cancer [10].

Trypsin also plays a significant role in oesophageal cancer [11]. A need is therefore observed to carry out all of these activities in a controlled fashion. A good inhibiting agent for trypsin would help us address problems of the uncontrolled reaction of trypsin. α1-Antitrypsin activated by plant-based inhibitors of trypsin, helps in the inhibition of trypsin. This AAT plays some role in immunity, inflammation, proteostasis, and apoptosis. When combined in vivo, these mechanisms maintain the lungs and protect it in face of an ongoing attack of pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) brought on by infections, pollutants, or cigarette smoke [12]. Similarly, pancreatic secretory trypsin inhibitor (PSTI) prevents premature trypsin activation in the pancreas and pancreatic duct, leading to a reduced risk of pancreatitis. In serum, it acts as an acute-phase reactant and increases remarkably in number in response to stress or invasion [13]. Plant-derived inhibitors such as Bowman-Birk inhibitors (BBIs) and Kunitz-type inhibitors, found in soybeans and legumes, also work as inhibitors of proteolytic activity of trypsin. It was discovered that Bowman-Birk inhibitor (BBI) is a protein molecule with an affinity for self-association made up of a chain of 71 amino acids 9 cross-linked by seven disulfide linkages [14]. The biological significance of these inhibitors is crucial in enzymatic regulation, drug development, and therapeutic applications.

2. Materials and Methods

2.1. Docking

AutoDock Vina, a molecular docking software, was used to carry out molecular docking simulations of the enzyme Human Trypsin 1 (PDB id: TRN1) with the desired compounds - Barbituric acid (CID 6211), Allantoin (CID 204), Azoindole (CID 136081926), and Trilobatin (CID 6451798). The protein structure was obtained from the Protein Data Bank as PDB files. The 3-D structures of the chemical compounds were obtained from PubChem as SDF files, which were converted into PDB format using the CACTUS Online SMILES Translator. The protein was added to the software, followed by the removal of water molecules, the addition of Kollman Charges, and hydrogens. After preparing the ligands and converting both the protein and the ligand to PDBQT format, grid was prepared, and the docking was initiated by running code in Command Prompt. All three ligands were docked in the active sites of trypsin, and the docking results were evaluated using binding energy values.

2.2. Visualization of the Docking results

PyMOL Molecular Graphics System 3.0.5, Schrödinger, LLC, was used to visualize and analyze the docking results. The protein–ligand complex structures obtained from molecular docking using AutoDock Vina were loaded into PyMOL in PDB format. The protein was highlighted using the Cartoon format for structural clarity, and the ray-traced images of the simulations were obtained in order to assess the binding site of the ligands and their distances from the protein.

2.3. Sample Reaction (With trilobatin)

0.5mL of trilobatin with varying concentrations, 0.5mL of BAPA working solution, and 1mL of distilled water were taken in a series of test tubes. The mixture was incubated at 37 °C for 10 minutes. 0.5mL of trypsin working solution was added. Incubation was done again at 37 °C for 10 minutes. The reaction was stopped by the addition of 0.6mL of 30% acetic acid.

2.4. Reference Reaction (No Inhibitor)

0.5mL of distilled water, 0.5 mL of BAPA working solution, and 1mL of distilled water were taken in a test tube and mixed. 0.5mL of trypsin working standard was added. Incubation was done at 37 °C for 10 minutes. The reaction was stopped upon the addition of 0.5mL 30% acetic acid.
Sample blank:
0.5 mL trilobatin, 0.5mL of BAPA, and 1mL of water were added and mixed together.
Reference blank (abr):
0.5 mL of BAPA and, 2mL of distilled water were added and mixed together.

2.5. Absorbance Measurement and Calculation

The absorbance of all reaction mixtures was measured at 410 nm using a UV–Vis spectrophotometer. Trypsin inhibitor activity (%) was calculated using the following equation:
TIA(%)=[(Ar−Abr)−(As−Abs)Ar−Abr]×100\text{TIA} (\%) = \left[\frac{(A_r - A_{br}) - (A_s - A_{bs})}{A_r - A_{br}} \right] \times 100TIA(%)=[Ar−Abr(Ar−Abr)−(As −Abs)]×100
Where:

- ArA_rAr = absorbance of reference sample (no inhibitor)
- AbrA_{br}Abr = absorbance of the reference blank
- AsA_sAs = absorbance of sample (with inhibitor)
- AbsA_{bs}Abs = absorbance of the sample blank

ADMET: SwissADME was utilized to check the usability of Trilobatin as an oral drug.

3. Results and discussion

3.1. Results

3.1.1. Result for the inhibition assay

No colour change was observed in the test samples. The values obtained were negligible.

3.2. Discussion

Using molecular docking analysis, we explored the binding interactions of four screened ligands—Allantoin, Barbituric Acid, Azoindole, and Trilobatin—against the serine protease enzyme Trypsin. Docking studies demonstrated the compounds' differing binding affinities and indicated their potential as trypsin inhibitors. Of the four compounds, Trilobatin showed a binding affinity of –7.2 kcal/mol, suggesting a strong interaction with Trypsin's active site. This is facilitated by presence of a hydroxyl group that can interact with the amino acid residues of trypsin and for hydrogen bonds with it. The second was azoindole, which had a binding affinity of -7.8 kcal/mol.

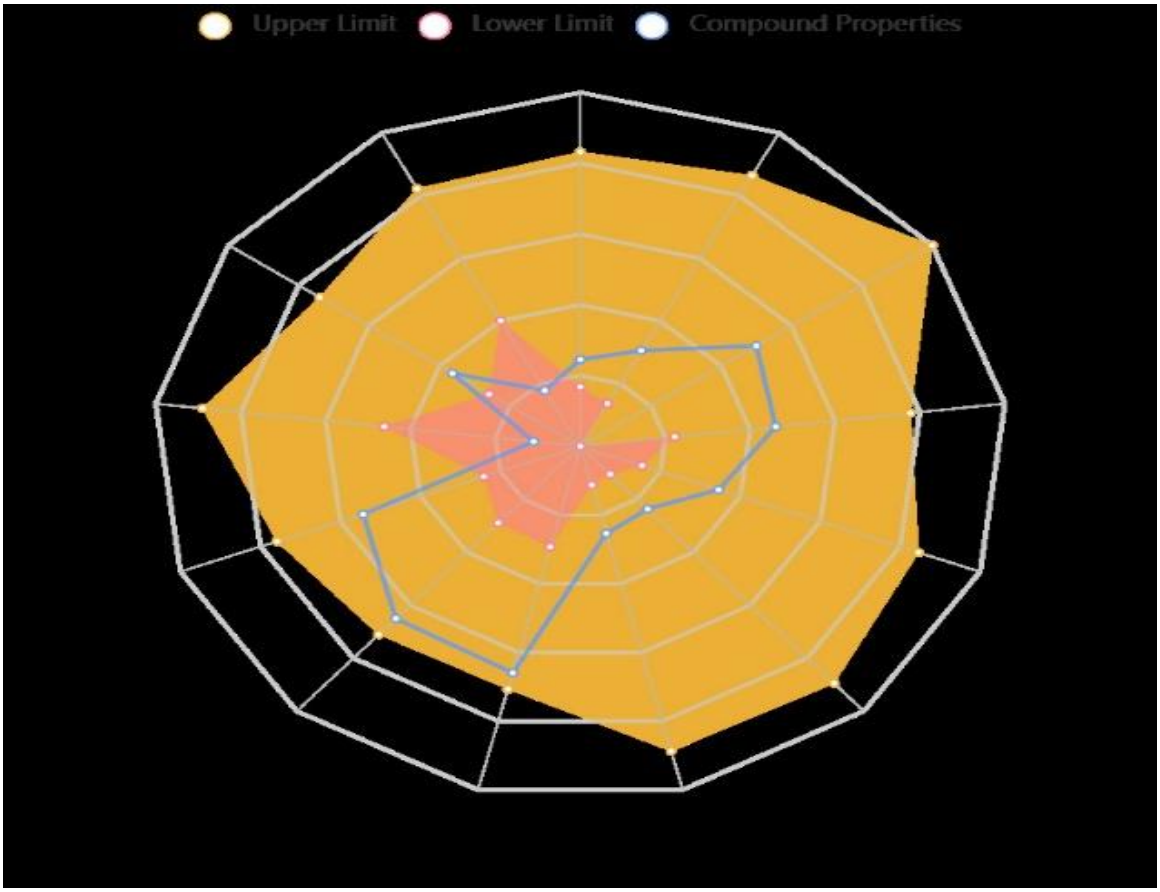

Figure 1: Bioavailability radar

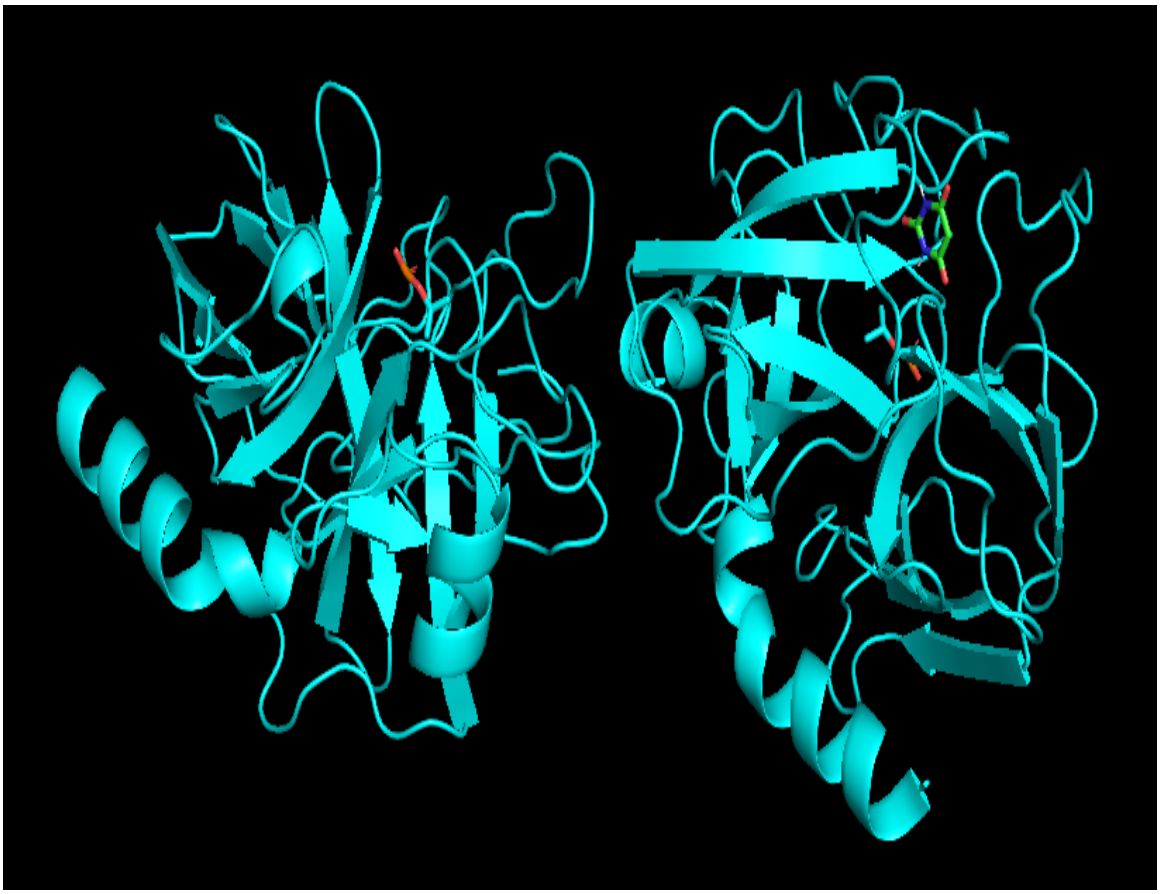

Figure 2: PyMOL visualization of human trypsin-barbituric acid

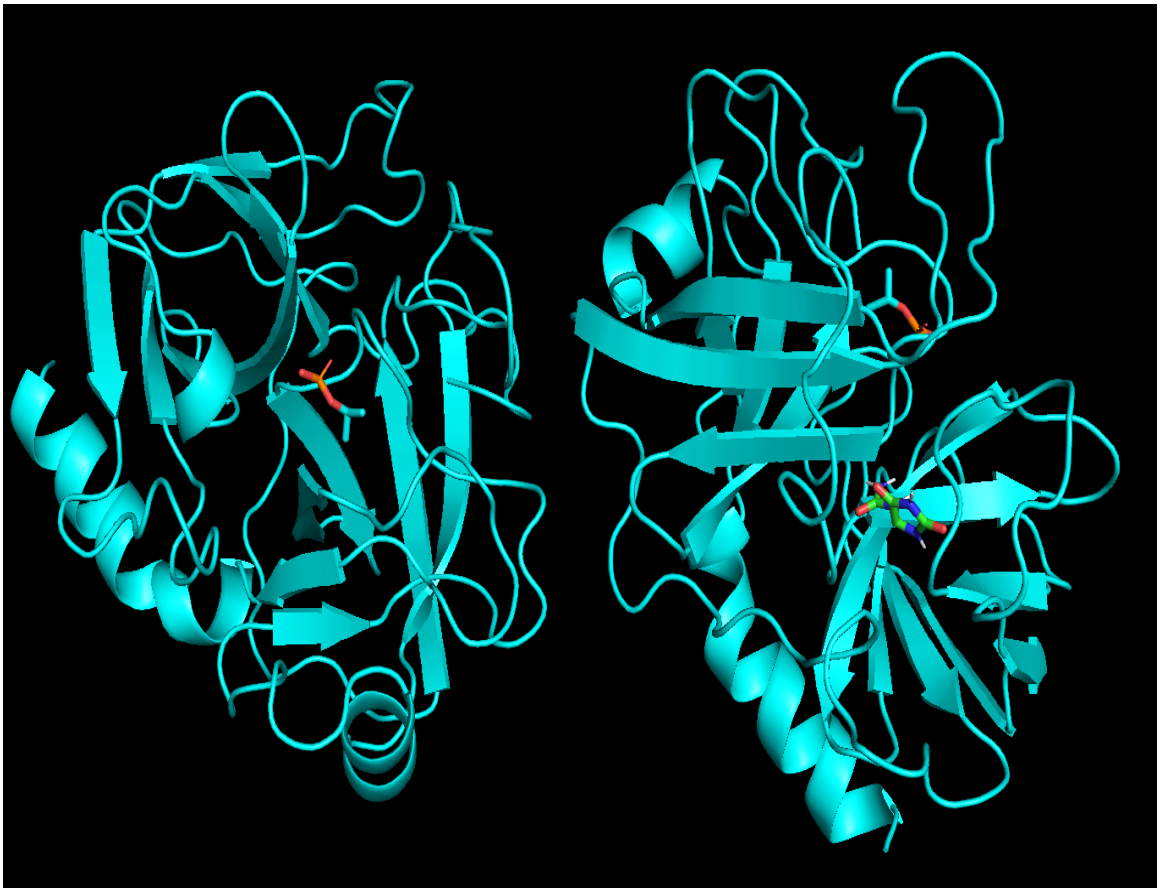

Figure 3: PyMOL visualisation of human trypsin-allantoin

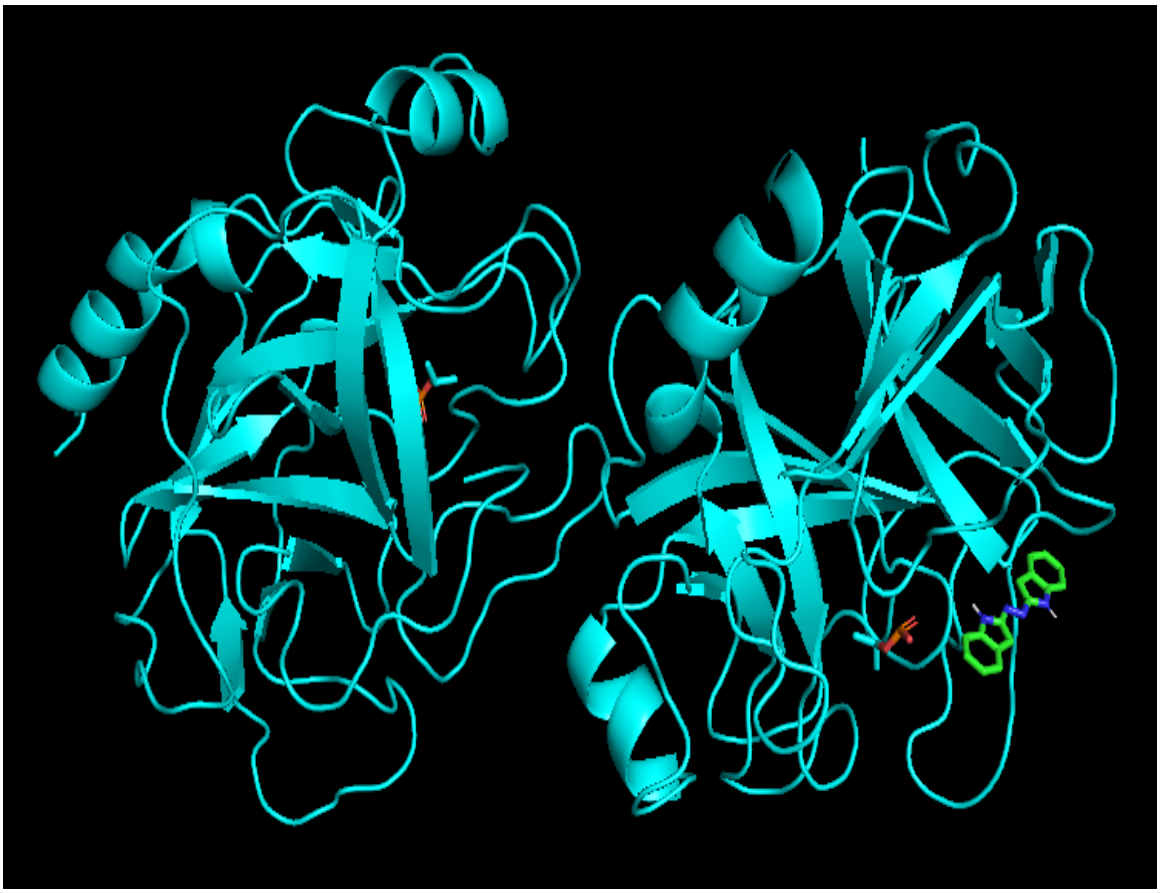

Figure 4: PyMOL visualisation of human trypsin-azoindole

Figure 5: PyMOL visualisation of human trypsin-trilobatin

Table 1: Inhibition Assay of trypsin using trilobatin

Reagent	Final Concentration	Preparation
Trypsin stock solution	0.27 mg/mL	27 mg of trypsin was dissolved in 100 mL of 1 mM HCl containing 5 mM $CaCl_2$.
Trypsin working solution	0.0135 mg/mL	The stock solution was diluted with distilled water (1:20)
BAPA stock solution	1.5 mM	50mg of BAPA was dissolved in 76.67 mL of DMSO.
BAPA working solution	15 µM	The stock solution was diluted (1:100) in 50mM of Tris-HCl (pH 8.2) with5mM $CaCl_2$
Acetic acid	30% (v/v)	This was prepared by diluting the glacial acetic acid.
Trilobatin (stock)	0.5mg/mL	Dissolved in 20 mL of DMSO
Distilled water	—	For dilution and control samples.

Table 2: ADMET profile of Trilobatin

Class	Criteria	Result	Inference
Drug-likeness	Lipinski's Rule of 5	complied	Good oral bioavailability potential
	Veber Rule	complied	Intestinal permeability is good.
	Ghose, Egan, Muegge	complied	Drug-like according to multiple medicinal filters
Physicochemical	Molecular Weight	Within 200–500 g/mol	Good membrane permeability and absorption.
	LogP (various models)	~2–3	Lipophilicity is balanced and has good membrane permeability and absorption.
	TPSA (Topological Polar Surface Area)	< 90 Å²	Oral absorption is good and shows capacity for blood-brain barrier permeability.
	H-Bond Donors / Acceptors	Within limits	Supports membrane permeability
ADME Profile	GI Absorption	High	Suitable for oral route of administration.
	BBB Permeant	Yes	It can be used as a target drug for CNS-related issues.
	P-gp Substrate	No	Low probability of getting effluxed out of the cell.
	CYP Inhibition (3A4, 2D6, etc.)	No major inhibition predicted	Drug-Drug interaction does not take place.
Water Solubility	Log S (Solubility)	Moderate to good	The dissolution is just enough for dissolution oral administration
Toxicity Alerts	PAINS	None	Low risk of false positives in screening assays
	Brenk Alerts	None	No problematic/toxic substructures
Synthetic Accessibility	SA Score	~2–4	Moderately easy to synthesize

Table 3: Result for molecular docking

PROTEIN NAME	LIGAND	BINDING AFFINITY(kcal/mol)	INTERACTING RESIDUES
Human trypsin	Allantoin	-6.3	GLY18, GLY19, GLN156
Human trypsin	Barbituric acid	-5.1	ASN25, ARG117, GLY69, HIS71, GLU70
Human Trypsin	Azoindole	-7.8	GLY69, ARG117, GLU70, HIS71
Human Trypsin	Trilobatin	-7.2	GLY18, THR144, THR132, GLN165, ALA166

This also indicates a high interaction potential, likely due to its nitrogen-containing aromatic structure which can aid with π-stacking. Barbituric acid and allantoin had binding affinities of –5.1 kcal/mol and –6.3 kcal/mol, respectively. The relatively low interactions suggest that these molecules are unable to engage in hydrogen bonding or stable hydrophobic interactions within the Trypsin active site. These volatile structural characteristics lead to lower binding affinities long-term interactions with essential amino acid residues. The results from molecular docking offer some understanding of the compounds' potential binding capabilities. The findings of molecular docking analyses reveal vital information regarding the ability of the compounds to bind. Additional molecular dynamics simulations along with experimental validation such as enzyme inhibition assays will be needed in order to determine the possible therapeutic value to confirm their inhibitory value against Trypsin. Further optimization of the binding and selectivity of compounds towards Trypsin is suggested as future research. Furthermore, investigating pharmacokinetic and toxicity parameters of these compounds would be critical for drug development. For the failure of the assay, this could be attributed by factors like compound instability, enzyme-substrate instability, and enzyme sensitivity.

4. Conclusion

This study highlights the importance of trypsin inhibition and discusses the role of four compounds as potential trypsin inhibitors. Among the four compounds, azoindole and trilobatin showed the highest binding affinity with trypsin and pose as the upcoming lead for drug development in the field of cancer, where trypsin inhibition is necessary. The use of trilobatin as a drug was analysed using SwissADME, and it showed promising results if taken orally without any side effects. Future drug development and advancement are possible if this is researched widely with more emphasis on the therapeutic potential.

Acknowledgements

I would like to express my heartfelt gratitude to the institute, Christ University, Central Campus, Bengaluru, for providing me with this wonderful opportunity to engage in research work. I would also like to thank the HOD, Dr. Fr. Jobi Xavier, my guide, Dr. Arjun Rao, and my parents, colleagues who have helped and supported me throughout this journey.

References

[1] H.-U. Bergmeyer. (2012). Methods of enzymatic analysis. Academic Press. 19.

[2] N. Koshikawa, S. Hasegawa, Y. Nagashima, K. Mitsuhashi, Y. Tsubota, S. Miyata, Y. Miyagi, H. Yasumitsu, K. Miyazaki. (1998). Expression of trypsin by epithelial cells of various tissues, leukocytes, and neurons in human and mouse. The American journal of pathology. 153(3): 937-944.

[3] K. Miyasaka, D. Guan, R.A. Liddle, G.M. Green. (1989). Feedback regulation by trypsin: evidence for intraluminal CCK-releasing peptide. American Journal of Physiology-Gastrointestinal and Liver Physiology. 257(2): G175-G181.

[4] L. Hedstrom. (2002). Serine protease mechanism and specificity. Chemical reviews. 102(12): 4501-4524.

[5] D.C. Whitcomb. (2004). Mechanisms of disease: advances in understanding the mechanisms leading to chronic pancreatitis. Nature Clinical Practice Gastroenterology & Hepatology. 3(12): 643-52.

[6] W.H. Eaglstein, P.M. Mertz. (1978). New Method for Assessing Epidermal Wound Healing: The Effects of Triamcinolone Acetonide and Polyethelene Film Occlusion. Pharmacology & Therapeutics. 3(3): 313-28.

[7] M.Z. Wojtukiewicz, D. Hempel, E. Sierko, S.C. Tucker, K.V. Honn. (2015). Protease-activated receptors (PARs)—biology and role in cancer invasion and metastasis. Cancer and Metastasis Reviews. 34(4): 775-796.

[8] V.S. Ossovskaya, N.W. Bunnett. (2004). Protease-activated receptors: contribution to physiology and disease. Physiological reviews.

[9] K. Błajecka, M. Marinov, L. Leitner, K. Uth, G. Posern, A. Arcaro. (2012). Phosphoinositide 3-kinase C2β regulates RhoA and the actin cytoskeleton through an interaction with Dbl. PLoS One. 7(9): e44945.

[10] H. Yamamoto, S. Iku, Y. Adachi, A. Imsumran, H. Taniguchi, K. Nosho, Y. Min, S. Horiuchi, M. Yoshida, F. Itoh. (2003). Association of trypsin expression with tumour progression and matrilysin expression in human colorectal cancer. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland. 199(2): 176-184.

[11] H. Yamamoto, S. Iku, F. Itoh, X. Tang, M. Hosokawa, K. Imai. (2001). Association of trypsin expression with recurrence and poor prognosis in human esophageal squamous cell carcinoma. Cancer. 91(7): 1324-1331.

[12] J.J. Enghild, I.B. Thørgersen, S.V. Pizzo, G. Salvesen. (1990). Polypeptide Chain Structure of Inter-α-Trypsin Inhibitor and Pre-α-Trypsin Inhibitor: Evidence for Chain Assembly by Glycan and Comparison with other "Kunin"-Containing Proteins. In Serine Proteases and Their Serpin Inhibitors in the Nervous System: Regulation in Development and in Degenerative and Malignant Disease. Journal of Biological Chemistry. 266(11): 6608-17.

[13] M. Ogawa. (1988). Pancreatic secretory trypsin inhibitor as an acute phase reactant. Clinical biochemistry. 21(1): 19-25.

[14] A. Gitlin-Domagalska, A. Maciejewska, D. Dębowski. (2020). Bowman-Birk inhibitors: Insights into family of multifunctional proteins and peptides with potential therapeutical applications. Pharmaceuticals. MDPI AG. 13: 1-40.