

**Evaluation of Vanillin–4-Methylthiosemicarbazone as
a Potential Inhibitor of SARS-CoV-2 3CL-protease:
Enzymatic and Computational Study**

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**This Thesis was Submitted in Fulfillment of the Requirements
for the Master's Degree in Pharmaceutical Sciences
at Middle East University**

December, 2025

تقييم الفانيلين-4-ميثيلثايوسيميكاربازون كمشبط محتمل لبروتياز 3 CL
لفيروس كورونا المستجد دراسة إنزيمية وحاسوبية

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قدّمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الماجستير في
العلوم الصيدلانية في جامعة الشرق الأوسط

ديسمبر، 2025

Thesis Committee Decision

This thesis, titled “Evaluation of Vanillin-4-Methylthiosemicarbazone as a Potential Inhibitor of SARS-CoV-2 3CL-protease: Enzymatic and Computational Study” by researcher Fahad Hazaa Mahmoud Khateri and was successfully defended and approved on 30-12-2025

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A handwritten signature in blue ink, appearing to be 'Fahad', written in a cursive style.

Acknowledgement

At the beginning, I thank **Allah**, who granted me the strength and opportunity to live, learn, and complete this work.

Gratefully, I would like to thank **Dr. Mohamed Saadh**, Associate Professor of Biochemistry and Molecular Biology, Faculty of Pharmacy, Middle East University, Jordan, for not only suggesting the research topic and providing me with all the laboratory facilities, but also for his invaluable supervision, constructive revision, continuous advice, and expert guidance throughout this work. I am deeply grateful for his support.

I would also like to express my sincere appreciation to the members of the thesis committee for their valuable time, insightful comments, and constructive suggestions, which greatly strengthened this thesis.

My heartfelt thanks go to the staff of the laboratory for their technical support and assistance, and to my colleagues and lab mates for their cooperation, encouragement, and the positive research environment they helped create.

Finally, I extend my deepest gratitude to my family for their patience, love, and unwavering support throughout this journey. Their prayers and encouragement were a constant source of strength.

Fahad Khateri
2025

Dedication

I dedicate this work to...

To my beloved **father**, my first pillar of strength and the man who continues to teach me perseverance with quiet dignity. Your guidance, patience, and unwavering belief in me have shaped every step of this journey, and your presence in my life remains my greatest source of support and confidence.

To my beloved **mother**, the heart of our home and the source of endless compassion. Your prayers, encouragement, and gentle reassurance have carried me through every moment of doubt and fatigue. I feel your love surrounding me and guiding my steps every day. I dedicate this work to you as a small tribute to a love that gives without ever asking for anything in return.

My loving **wife**, my true partner in every season of life. You stood by me with remarkable patience, understanding, and constant support, turning difficult days into manageable ones. This dedication is for you—because your presence made this journey possible and meaningful.

To my **dear daughters**, the source of my happiness and my strongest motivation. You remind me of the importance of hard work and the value of dreams. I dedicate this work to you, hoping it will inspire you to believe in yourselves and to pursue your ambitions with courage and integrity.

My dear brothers, my lifelong companions and steady support. Your encouragement, generosity, and presence have been a source of comfort and strength throughout this journey. I dedicate this achievement to you with heartfelt appreciation and love.

Fahad Khateri
2025

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List of Abbreviations

Abbreviation	Full Term
3CL-protease	3-Chymotrypsin-Like Protease (main protease of SARS-CoV-2)
4PL	Four-Parameter Logistic (model)
ATR	Attenuated Total Reflectance
COVID-19	Coronavirus Disease 2019
DMSO	Dimethyl Sulfoxide
FRET	Förster (Fluorescence) Resonance Energy Transfer
FT-IR	Fourier-Transform Infrared (spectroscopy)
GC-376	GC-376, a 3CL-protease inhibitor used as reference/positive control
IC ₅₀	Half-Maximal Inhibitory Concentration
PDB	Protein Data Bank
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SBDD	Structure-Based Drug Design
VTSC	Vanillin–Thiosemicarbazone

Computational and Experimental Evaluation of Vanillin–Thiosemicarbazone as a Potential Inhibitor of SARS-CoV-2 3CL-protease

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Abstract

Introduction: SARS-CoV-2 3CL-protease represents one of the most essential viral enzymes and is a target for small molecule inhibitors. Vanillin and thiosemicarbazone derivatives have been employed for the demonstration of relevant bioactivity although much research on the combination of vanillin and thiosemicarbazone with 3CLpro to date has been limited. **Aim:** This work aimed at the preparation of Vanillin–4-methylthiosemicarbazone (VMTSC) and comparison of its inhibition against SARS-CoV-2 3CLpro with vanillin and 4-methylthiosemicarbazide (MTSC) through integrated docking and enzyme inhibition studies. **Methods:** Schiff–base condensation of VMTSC was performed and confirmed by FT-IR. Binding affinity and major interactions were evaluated using SARS-CoV-2 3CLpro for molecular docking. Enzyme inhibition was measured using a commercial 3CL protease assay kit that enumerates multiple concentrations of inhibitors. Values IC_{50} from dose–response curves ($n = 3$) were calculated. **Results:** The three compounds inhibited SARS-CoV-2 3CLpro based on a dose-response equation. $IC_{50} = 42.0 \mu\text{M}$ (95% CI: 33–54 μM ; $p < 0.05$) of vanillin and $IC_{50} = 45.0 \mu\text{M}$ (95% CI: 38–53 μM ; $p < 0.05$) of MTSC were observed. The potency results for VMTSC are $IC_{50}=11.3 \mu\text{M}$ (95% CI: 9.8–13.0 μM ; $p < 0.001$). VMTSC was more potent for the most effective decrease of 3CLpro activity, which aligns with a favourable docking score of the VMTSC and a more elaborate interaction network in the catalytic pocket. **Conclusions:** Current study suggest that this compound may represent a promising avenue of research for the design of antiviral drugs against COVID-19.

Keywords: SARS-CoV-2; 3CL-protease; vanillin–4-methylthiosemicarbazone; thiosemicarbazones; IC_{50} ; enzyme inhibition; molecular docking.

التقييم الحاسوبي والتجريبي لمركب فانيلين-ثيوسيميكاربازون كمثبط محتمل لإنزيم البروتياز الرئيسي (3CL-protease) لفيروس كورونا المستجد

إعداد الطالب

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تحت إشراف

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الملخص

المقدمة: يُعدّ البروتياز الرئيسي لفيروس SARS-CoV-2 (3CLpro) هدفاً دوائياً محورياً بسبب دوره الأساسي في معالجة عديدات البروتين الفيروسي وغياب نظير قريب له في الخلايا البشرية. وقد أظهرت مشتقات الفانيلين والثيوسيميكاربازون أنشطة بيولوجية مهمة، إلا أن التقييم التجريبي للمركبات الهجينة المبنية على الفانيلين كمثبطات لهذا الإنزيم ما يزال محدوداً. **الهدف:** هدفت هذه الدراسة إلى تحضير مركب فانيلين-4-مثيل ثيوسيميكاربازون (VMTSC) ودراسة قدرته على تثبيط إنزيم 3CLpro لفيروس SARS-CoV-2، ومقارنة فعاليته مع مركبي الفانيلين و4-مثيل ثيوسيميكاربازيد (MTSC) بالاستناد إلى الدمج بين الإرساء الجزيئي والاختبارات الإنزيمية. **الطريقة:** تم تحضير مركب VMTSC بتفاعل تكاثف شيف بين الفانيلين و4-مثيل ثيوسيميكاربازيد، والتحقق من بنيته باستخدام مطيافية الأشعة تحت الحمراء (FT-IR). أُجريت دراسات الإرساء الجزيئي على إنزيم 3CLpro لفيروس SARS-CoV-2 للتنبؤ بطاقة الارتباط وأنماط التأثير للمركبات الثلاثة. جرى قياس التثبيط الإنزيمي باستخدام عدة تجارية لقياس فعالية 3CL protease عند تراكيز مختلفة من المركبات، ثم حُسبت قيم التركيز المثبط لنصف الفعالية (IC_{50}) من منحنيات الاستجابة للجرعة ($n = 3$) باستخدام نموذج لوجستي رباعي المعامل. **النتائج:** أظهرت المركبات الثلاثة تثبيطاً يعتمد على الجرعة لإنزيم 3CLpro. سجّل الفانيلين قيمة

C_{50} مقدارها 42.0 ميكرومولار (فاصل الثقة 95%: 33-54 ميكرومولار؛ $p < 0.05$ ، في حين بلغ IC_{50} لمركب MTSC نحو 45.0 ميكرومولار (فاصل الثقة 95%: 38-53 ميكرومولار؛ $p < 0.05$). أما المركب الهجين VMTSC فقد أظهر أعلى فعالية مثبّطة مع قيمة C_{50} بلغت 11.3 ميكرومولار (فاصل الثقة 95%: 9.8-13.0 ميكرومولار؛ $p < 0.001$ ، مما يشير إلى تفوق واضح في القدرة على خفض نشاط الإنزيم مقارنة بالمركبين الأبويين. وتتسجم هذه النتائج مع دراسات الإرساء الجزيئي التي بيّنت أن VMTSC يمتلك أفضل طاقة ارتباط وشبكة تأثيرات أوسع داخل الجيب الفعّال للإنزيم. الاستنتاج: تشير النتائج الكلية للدراسة إلى أن هذه المركبات، وبخاصة VMTSC ، يمكن أن تُعدّ نقاط انطلاق واعدة لتطوير مثبطات دائيّة موجهة لإنزيم البروتياز الرئيسي لفيروس SARS-CoV-2 ، بما يدعم توظيفها كأسس أولية في تصميم أدوية مضادّة لمرض كوفيد-19.

الكلمات المفتاحية:

فيروس SARS-CoV-2 ؛ البروتياز الرئيسي CLpro3؛ فانيلين-4-مثيل ثايوسيميكاربازون (VMTSC)؛ مثبطات إنزيمية؛ IC_{50} ؛ الإرساء الجزيئي.

Chapter One

Background and Problem Statement

1.1 Introduction

The worldwide dissemination of the Severe Acute Respiratory Syndrome Coronavirus 2 (The global emergence of Severe Acute Respiratory Syndromes Coronavirus 2 (SARS-CoV-2) necessitated the requirement of agents that could interfere with critical viral enzymes responsible for replication and pathogenesis. An important virus enzyme in the life cycle of coronaviruses, has been identified as 3CL protease (3CLpro). It is a critical enzyme responsible for cleavage of large viral polyproteins pp1a and pp1ab into non-structural proteins necessary for formation of the replication-transcription complex (Jin et al, 2020; Ullrich & Nitsche, 2020). Due to the absence of such proteases in humans, 3CLpro is regarded as a highly specific and safe antiviral drug target that has been investigated extensively from the biochemistry and structure perspective during the COVID-19 pandemic (Citarella et al. 2023).

Despite many efforts have been made toward vaccine development, the continuously surfacing of novel variants of concern, compounded by shortcomings of available antiviral regimens, indicates the continuing need for improved potency, availability and stability of new small-molecule inhibitors (Puhl et al., 2022; Citarella et al., 2023). Natural product derived scaffolds are still major sources of antiviral lead molecules and compounds like vanillin and thiosemicarbazone derivatives have been reported for their antioxidant, antimicrobial and antiviral activity (Rahman et al., 2023; Kafali et al., 2024). The electron-rich functional groups of thiosemicarbazones can produce hydrogen bonds, π - π interactions and metal-chelating contacts that are significant for the targeting of the catalytic residues of 3CLpro (Haribabu et al., 2021; Czynkowska et al., 2024; Xue et al., 2025).

The global spread of SARS-CoV-2 created an unprecedented need for drugs that could work against key viral enzymes involved in its replication and pathogenesis (Zhang et al., 2020). 3CLpro is a key enzyme involved in the life cycle of coronavirus. It helps the cleavage of large viral polyproteins (pp1a and pp1ab) into individual non-structural

proteins which are required for the formation of the replication–transcription complex (Jin et al., 2020; Ullrich & Nitsche, 2020). There is no similar protease in human cells that is like 3CLpro. Thus, it is considered a highly selective and safe antiviral drug target. This premise led to extensive biochemical and structural studies during the COVID-19 pandemic.

Clinically effective antiviral therapies have been generated with the aim of targeting SARS-CoV-2 main protease 3CLpro/Mpro. A prominent example is nirmatrelvir, administered with ritonavir (Paxlovid), which inhibits 3Cl pro and markedly reduces COVID-19 progression in high-risk patients (Hammond et al. 2022) At the same time, the next-generation noncovalent inhibitor ensitrelvir (S-217622) has moved through clinical development with potent oral activity against several variants, maintaining interest in diversifying 3CLpro inhibitor chemotypes to avoid resistance (Unoh et al., 2022).

Vanillin provides an aromatic framework rich in phenolic content and capable of contributing π -interactions and hydrogen bonding (through the phenolic OH and methoxy oxygen). The thiosemicarbazone unit offers a heteroatom-rich pharmacophore (C=S and multiple NHs) which enhances polar contacts. The strategy of integrating the two through molecular/structural hybridization is a known medicinal-chemistry strategy. The goal is to combine complementary interaction features within a single ligand to increase the probability of stable binding within an enzyme pocket (Kumar, 2020; Ivasiv et al., 2019). Thiosemicarbazones are often portrayed as versatile bioactive motifs on the back of their donor/acceptor density and C=S functionality (Pelosi, 2010), while derivatives based on vanillin are often seen to exploit phenolic H-bonding and aromatic interactions in binding sites (Scipioni et al., 2019).

Combining vanillin with 4-methylthiosemicarbazide allows for the formation of a compound that incorporates an aromatic phenolic scaffold fused to an electron-donating thiosemicarbazone pharmacophore. A well-known and established approach in medicinal chemistry to create and design suitable ligands that can combine with proteins is Structural hybridization. It helps in improving the physicochemical properties and increases the probability of forming stabilizing interactions at the active site of an enzyme. (Song et al., 2024; Citarella et al., 2023). According to the first molecular

docking results, the studied hybrid molecules may bind into the 3CLpro catalytic cleft more favorably through the hydrophobic patches, hydrogen-bond networks and polar anchoring points around the His41-Cys145 catalytic dyad., hybrid molecules of the sort studied might bind better in the catalytic cleft of 3CLpro by exploiting a variety of hydrophobic patches, hydrogen-bond network, and polar anchoring points around the His41–Cys145 catalytic dyad (Dai et al. 2020; Yang et al. 2021).

Today's antiviral discovery uses more integrated workflows. These workflows use *in silico* techniques. *In silico* techniques include molecular docking and virtual screening. They are combined with biochemical inhibition assays. According to Dai et al (2020), Citarella et al (2023), and Song et al (2024), these hybrid methodologies hasten the identification of new 3CLpro inhibitors and produce crucial mechanistic insights on ligand–protein interactions.

In this study, VMTSC will be synthesized and investigated as a prospective 3CLpro inhibitor based on a variety of computational approaches, as well as by several experiments. Given the pharmacophoric richness of VMTSC and the therapeutic significance of 3CLpro, it will be a significant scientific contribution to the current literature concerning effective antiviral strategies against SARS-CoV-2 and its emerging variants. Knowledge of the molecular determinants of VMTSC binding and inhibition could guide the future development of optimized derivatives possessing more potent antiviral activity.

1.2 Study Problem

Many antiviral agents were developed against SARS-CoV-2. But blocking 3CLpro is an underused and upcoming treatment (Ullrich & Nitsche, 2020; Song et al., 2024). Present inhibitors are selective, potent and stable (Ullrich & Nitsche, 2020; Song et al., 2024). Rahman et al. (2023) and Xue et al. (2025) studies investigated the potential enzyme-mediated direct SARS-CoV-2 3CLpro inhibition of a natural derivative vanillin and thiosemicarbazones. There are hybrid vanillin–thiosemicarbazone derivatives reported due to promising computational results and structurally relevant pharmacophore features. Nevertheless, the experimental evidence is lacking. In this context, the present

study seeks to answer this. The hybrids mentioned above were studied against SARS-CoV-2 3CLpro.

1.3 Study Questions

1. What is the binding affinity and predicted interaction pattern of VMTSC toward the SARS-CoV-2 3CLpro catalytic site compared to vanillin and MTSC individually?
2. Is the inhibitory activity of VMTSC against SARS-CoV-2 3CLpro greater than its parent compounds?
3. What structural features increase binding and inhibitory potency of VMTSC?
4. How well do computational predictions match with experimental findings of enzyme inhibition?

1.4 Study Hypotheses

H1: The stronger binding of vanillin-4-methylthiosemicarbazone to SARS-CoV-2 3CLpro was compared with vanillin and MTSC.

H2: VMTSC has lower IC₅₀ values, which means it is a stronger inhibitor than parent molecules.

H3: VMTSC is designed to enable better interaction with the catalytic and substrate-recognition residues of SARS-CoV-2 3CLpro.

H4: Results from in vitro inhibition assays positively correlate with docking predictions.

1.5 Study Objectives

General Objective: To assess the ability of VMTSC to prevent SARS-CoV-2 3CL protease activity using computer and lab based methods.

Specific Objectives.

1. To synthesize VMTSC using a Schiff-base condensation approach.
2. To predict the binding mode and affinity of VMTSC using molecular docking.
3. To determine the inhibitory activity (IC_{50}) of VMTSC, vanillin, and MTSC against SARS-CoV-2 3CLpro.
4. To compare computational and experimental results and evaluate their concordance.
5. To analyze structural features that contribute to the enhanced potency of VMTSC.

1.6 Study Importance

Scientific Importance

To this end, the goal of this study is to use a hybrid natural-product derivative with two functional moieties to inform the SARS-CoV-2 antiviral discovery literature. VMTSC possesses a pharmacophore-rich scaffold capable of inducing multiple interactions in these enzymes that can provide information about its potential applicability as a novel antiviral lead compound (Citarella et al., 2023; Song et al., 2024).

Importance to Pharmaceutical and Applied Practice

Determining novel SARS-CoV-2 3CLpro inhibitors could speed up the creation of next-generation antiviral medicines that target novel variants and resistance characteristics. The cost-effective and synthetically accessible hybrid molecules, such as VMTSC, offer an attractive platform for further drug optimization (Rahman et al., 2023; Ahmed et al., 2024).

Methodological Importance

This study utilizes an integrated workflow that integrates molecular docking and in vitro fluorometric assays, an approach that has recently been widely adopted in modern antiviral research for rapid and reliable identification of bioactive small molecules (Dai et al., 2020; Yang et al., 2021; Citarella et al., 2023). Data from this methodology contribute to the scientific rigor and reproducibility of antiviral screening studies.

1.7 Study Limitations

1. In vitro and enzymatic assay studies may not reflect intracellular level antiviral activity.
2. Molecular docking predictions do not include the effect of protein flexibility or solvent effect that are not part of applied parameters (Ullrich & Nitsche, 2020).
3. By studying just one hybrid derivative (VMTSC), the findings cannot be generalized to other thiosemicarbazone analogs.
4. The cytotoxicity or pharmacokinetic properties which are required in full drug-development assessment are not investigated.
- 5.

1.8 Operational Definitions

- **3CLpro:** is a cysteine protease essential for the maturation of the virus and a validated antiviral drug target.
- **Vanillin:** is an aromatic aldehyde that exists in nature. It is used as a parent scaffold in our study.
- **Thiosemicarbazone:** They belong to a class of compounds having both antiviral and enzyme-inhibitory properties. Pillars contributing electron-rich functional groups will enable catalytic interactions (Czyrkowska et al., 2024; Xue et al., 2025).
- **VMTSC:** is a hybrid molecule whose inhibitory activity against 3CLpro requires further evaluation.
- **IC₅₀:** It is an estimate of the inhibition concentration of the inhibitor that reduces the enzyme reaction by 50% is calculated using the four parameters logistic model.
- **Docking Score:** refers to the computer prediction of ligand binding affinity to a target.

Chapter Two

Theoretical Framework and Previous Studies

2.1 Theoretical Framework

Highlighting the clinical importance of targeting SARS-CoV-2, 3CLpro has been at the centre of antiviral drug discovery, given its role in crucial viral replication and high degree of structural similarity across circulating variants. Recent high-resolution structural studies have shown that the 3CLpro enzymatic cleft of the coronavirus preserves its remarkably stable topology despite mutations in peripheral viral proteins. This suggests that the cleft would be suitable for the design of robust small-molecule inhibitors (Zhang et al., 2020; Jin et al., 2020). In the latest protease-directed medicinal chemistry studies, notable attempts targeting factors controlling substrate recognition, e.g. enzymes' catalytic environment and hydrophobic subpockets, have emerged as effective inhibitors (Citarella et al., 2023; Song et al., 2024; Yang et al., 2021).

Recently, researchers have sped up the rational design of inhibitors using computational modelling, free energy prediction, and ligand–protein interaction profiling. Approaches that exploit the structure of the target enzyme in drug design work fine to generate hybrid aromatic–heteroatom molecules that may exploit multiple interaction mechanisms: π -stacking, hydrogen bonding, sulfur-mediated contacts, and van der Waals anchoring to achieve higher occupancy on the active site of the enzyme (Citarella et al., 2023; Song et al., 2024). The evaluation of hybrid scaffold VMTSC, which consists of an electron-rich aromatic aldehyde and a thiosemicarbazone pharmacophore that provides multifunctional biological activity (Czylkowska et al., 2024; Xue et al., 2025).

Recent studies show that thiosemicarbazone-containing ligands exhibit valuable adaptability in protease pockets due to the conformational flexibility and polar, dipolar or nucleophilic interactions of the C=N and C=S groups with the catalytic residues (Czylkowska et al., 2024; Xue et al., 2025; Haribabu et al., 2021). Along with this, the vanillin-derived Schiff bases enhance the electron delocalization, increase the planarity of the molecules, and penetrate into the depth of catalytic clefts (Rahman et al., 2023; Kafali et al., 2024; Ahmed et al., 2024). When put together their structural features

potentially lead to better binding persistence and higher inhibitory efficiency against 3CLpro.

Modern antiviral discovery programs are increasingly shifting to integrated in silico-in vitro frameworks. Through computational docking, binding affinity predictions and orientation predictions can be accomplished with relative speed. Experimental assay validation determines whether predicted interactions lead to functional inhibition. Docking energies and biochemical inhibition correlations can be improved significantly upon screening hybrid molecules with well-balanced polarity and aromaticity (Dai et al., 2020; Yang et al., 2021; Citarella et al., 2023; Song et al., 2024). Recent workflows have increasingly integrated in silico docking with in vitro enzyme inhibition assays. This dual-modality approach forms the methodological basis of the present study, enabling a more rigorous comparison of VMTSC with its parent molecules.

The theoretical base of the present study rests on three pillars.

1. The fact that 3CLpro is validated and resistant to mutation as a drug target. (Zhang et al., 2020; Ullrich & Nitsche, 2020; Citarella et al., 2023)
2. The mobile application has a demonstrated bioactivity and structural suitability aromatic thiosemicarbazone hybrids (Czylkowska et al., 2024; Xue et al., 2025; Rahman et al., 2023).
3. The reliability of projections created through docking, and enzyme-inhibition projects, valuable for the early identification of antivirals (Dai et al., 2020; Yang et al., 2021; Song et al., 2024).

The study suggests that VMTSC might have better binding affinity and inhibitory activity than its components taken separately. Therefore, this composite might be further evaluated as a protease-targeted antiviral candidate.

2.1.1 3 CLpro as a Therapeutic Target

The critical function of 3CLpro in the replication of SARS-CoV-2 continues to render it one of the most strategically validated antiviral targets. Studies suggest that the

enzyme retains a remarkably conserved catalytic environment in diverse viral lineages, despite mutations in the rest of the genome (Zhang et al., 2020; Jin et al., 2020; Citarella et al., 2023). The structural preservation of 3CLpro allows the inhibitors derived from it to remain relevant beyond one variant which is one of the problems faced in antiviral drug development.

The catalytic architecture of 3CLpro is optimized for substrate processing, with a well-defined cleft that accommodates small molecules through orchestrated hydrophobic, electrostatic, and hydrogen-bond interactions (Figure 2.1). High-resolution crystallographic and medicinal chemistry studies confirm that the geometry of the catalytic region is uniquely sensitive to ligands capable of occupying multiple subsites simultaneously, making hybrid aromatic-heteroatom scaffolds particularly promising (Zhang et al., 2020; Song et al., 2024; Yang et al., 2021).

From a pharmacological perspective, 3CLpro targeting is highly advantageous because:

1. The substrate specificity is does not match the human one.
2. The maturation and replication of viruses depends on its activity.
3. It is capable of tolerating binding by a variety of ligands and thus has a broad inhibitor chemotype range (Ullrich & Nitsche, 2020; Citarella et al., 2023).

The attributes greatly assist in the evaluation of new hybrid compounds like VMTSC and make 3CLpro an ideal choice for structure-guided inhibitor development.

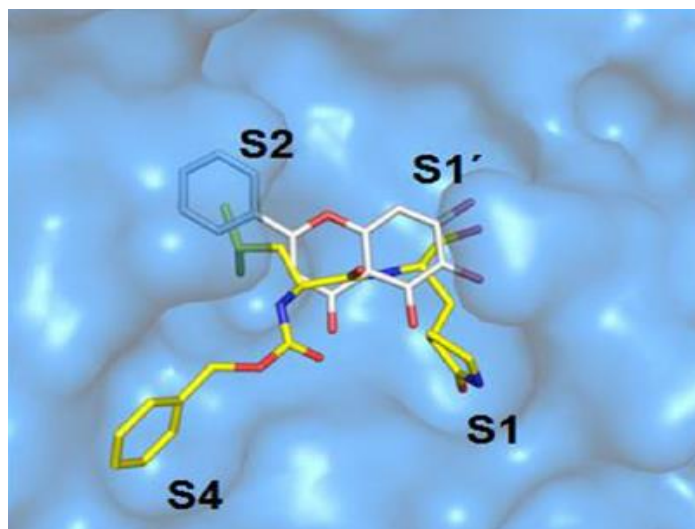


Figure 2.1. Structural organization of the SARS-CoV-2 3CLpro active site highlighting the S1, S1', S2, and S4 subsites involved in ligand binding (Zhang et al., 2020).

2.1.2 Structure-Based Drug Design Targeting Viral Proteases

Recently, a framework for drug design based on structure (SBDD) has begun to merge with other approaches. These include crystallography, molecular dynamics and free-energy calculations. AI-anchored docking algorithms are also included. The suggested methods can also be utilized to explain binding, optimize molecular geometries, and predict inhibitory potency before the experimental preparation (Song et al., 2019; Yang et al., 2021).

- Researchers use SBDD to figure out how to stop viral proteases, such as 3CLpro, from working.
- The outline and net charge strength of the active site indentation.
- Key anchor points needed for catalytic function.
- Flexibility and dynamics in subpocket fitting.
- Occupancy of Hydrophobic and Polar regions

According to Citarella et al, 2023 and Song et al., 2024, there is a tendency involving SBDD for protease either having as a aim to have molecules that bring together at least two different molecular architectures in order to engage complementary interaction domains within protease pockets. Molecules that contain aromatic functionality and contain nitrogen, oxygen, and sulfur-like heteroatom donors have better

chances of modulating flexibility and improving the binding kinetics which correlate with inhibition strongly. The VMTSC probe, which has a hybrid structure, aims to increase the density of interactions in the globally catalytic medium of the enzyme.

2.1.3 Vanillin: Chemical and Biological Profile

A significant-natural product scaffold of vanillin which is used in medicinal chemistry is due to the aromatic scaffold that is well-defined. Moreover, it can be easily derivatized. The hydroxyl group and the methoxyl group are said to balance the polar nature and lipophilicity, which brings the ability of the drug to interact with many other biological targets (Kafali et al., 2024).

According to recent studies, vanillin can be utilized as a useful precursor for antiviral scaffolds.

1. Chemical versatility – its aldehyde group readily forms Schiff bases and hydrazones thereby allowing for an increase in structural dimensions with little increase in synthetic complexity (Rahman et al., 2023; Ahmed et al., 2024).
2. Biological flexibility - Vanillin derivatives have shown broad activities including antioxidant, antimicrobial and enzyme-modulating effects (Kafali et al., 2024)
3. The compounds formed from vanillin are expected to have a good drug- like character which allows a high chance of target efficacy with low toxicity (Kafali et al., 2024)

Studies have shown that the vanillin-based hybrids often exhibit better biological activity due to improved distribution of electrons, increased molecular planarity and better capacity to π -interact within the active site of protease (Rahman et al., 2023; Ahmed et al., 2024). As a result of to these attributes, the vanillin core can easily be incorporated into VMTSC and will be evaluated as an inhibitor of 3CLpro.

2.1.4 Thiosemicarbazide and Thiosemicarbazones as Bioactive Scaffolds

Thiosemicarbazide-based structure remains pivotal scaffolds in present-day medicinal chemistry because of their structural flexibility and exhibited activity in antiviral, antimicrobial, anticancer and enzyme-inhibitory activities. Recent studies show

that thiosemicarbazones have special interaction properties resulting from the joint action of the C=N azomethine group and the C=S thioamide group. The electron-rich character of both these groups can form interactions like hydrogen bonds, dipole-dipole interaction and sulphur interactions with catalytic residues (Czylkowska et al., 2024; Xue et al., 2025).

These scaffolds display conformational flexibility that enables them to accommodate the steric and electrostatic architecture of protease active sites, including dynamic subpockets that undergo induced-fit adjustments. Structural and functional studies on thiosemicarbazone-based inhibitors confirm that the thiosemicarbazone fragment can establish interactions within multiple subsites of proteases similar to 3CLpro, often resulting in improved binding stability and prolonged residence time compared with simpler aromatic aldehydes (Haribabu et al., 2021; Xue et al., 2025; Citarella et al., 2023).

Furthermore, the pharmacophoric balance of hydrophobic (aromatic) and polar (hydrazine/thioamide) domains makes thiosemicarbazones attractive candidates for hybrid molecular designs. This versatility underpins their frequent use in rational scaffold engineering and directly supports the molecular architecture incorporated in VMTSC.

2.1.5 Rationale for VMTSC

The design strategy behind VMTSC is grounded in hybrid medicinal chemistry, where two bioactive domains are merged to enhance multitarget or multi-interaction capacity. In this hybrid, the vanillin aromatic aldehyde contributes π -stacking potential, directional hydrogen-bond donors/acceptors, and moderate lipophilicity, while the methyl-substituted thiosemicarbazone unit augments electron density, polar interaction sites, and geometric adaptability.

The design strategy of VMTSC is based on hybrid medicinal chemistry, wherein two bioactive domains are combined for multitarget or multi-interaction capability. This compound contains vanillin which offers stacking potential along with directional H-bond acceptors/donors and moderate lipophilicity. The methyl-substituted thiosemicarbazone unit provides polar interaction sites, electron density, and flexibility.

According to recent researches regarding hybridization, many substances can be obtained through the coupling of aromatic aldehydes and thiosemicarbazone fragments often results in:

1. Increased binding depth in protease catalytic clefts attributed to better planarity and delocalized electron density.
2. Increased interaction multiplicity, leveraging van der Waals, π -alkyl, dipolar, and hydrogen-bond networks.
3. Enhanced pharmacophoric balance, favorable for hydrophobic anchoring and polar engagement.
4. Greater stability of ligand orientation, notably for enzymes having well-defined catalytic dyads, like 3CLpro (Ahmed et al., 2024; Rahman et al., 2023; Kafali et al., 2024).

Based on these principles, VMTSC was selected as the central compound for investigation in this study. Its hybrid nature is hypothesized to yield stronger binding affinity and improved inhibitory performance relative to vanillin and thiosemicarbazide individually. Theoretical and experimental evaluations of related thiosemicarbazone-based inhibitors support the notion that ligands combining aromatic and thiosemicarbazone features achieve more stable occupancy profiles within 3CLpro's active site (Xue et al., 2025; Yang et al., 2021; Song et al., 2024).

2.1.6 Molecular Docking and In Vitro Enzyme Inhibition as Complementary Approaches

The latest antiviral discovery processes use both computation and experiments to accelerate up the process of finding leads. Docking simulation offers accurate predictions on ligand orientation, binding affinity and interaction networks of 3CLpro which allows testing, assessment of multiple structural analogues before their synthesis (Dai et al., 2020; Yang et al., 2021). But, predictions from a computer would not tell the entire story. For this reason, modern models suggest that docking analysis should be complemented with in vitro fluorometric inhibition assays, which provide quantitative measures of inhibition and facilitate the determination of kinetic parameters like IC_{50} (Song et al., 2024).

According to studies on SARS-CoV-2 3CLpro inhibitors, hybrid molecules are showing a strong correlation between predicted and experimental results when subjected to *in silico-in vitro* evaluations (Citarella et al., 2023; Xue et al., 2025; Yang et al., 2021). The conjunction of technologies offers improved options to take better decisions regarding development of scaffold, research cost will be reduced and chances of having an advancement of potential molecules in preclinical will be more. In this context, the present study adopts a dual assessment for VMTSC in which a molecular docking strategy can model its interaction potential within 3CLpro; and experimental measurement of inhibitory activity. An integrative strategy for assessing whether VMTSC is a viable early-stage antiviral candidate.

2.2 Previous Studies

2.2.1 Studies on SARS-CoV-2 3CL-protease Inhibitors

As a therapeutic target in SARS-CoV-2, 3CLpro is extensively analyzed due to its potential involvement in the maturation of the virus. Research activities have targeted a number of classes of small-molecule inhibitors including peptidomimetic scaffolds, repurposed drugs, natural-product derivatives, and synthetic hybrids. Studies show that being able to occupy multiple subsites in the catalytic cleft makes them more stable and potent (Jin et al., 2020; Zhang et al., 2020; Citarella et al., 2023; Song et al., 2024). According to some recent studies' findings, strong inhibition with many of the noncovalent small molecules is feasible, which also possess a good selectivity profile or good drug-like properties (Song et al., 2024; Yang et al., 2021; Xue et al., 2025). The findings strongly endorse conducting additional investigations on the development of other aromatic hybrid molecules such as VMTSC.

2.2.2 Studies on Vanillin and Its Derivatives

The phenolic structure vanillin has shown potential in antimicrobial research as well as enzyme-inhibitory studies. Prior biological studies on the parent compound indicated moderate biological activity. Similarly, Schiff-base formation and heterocyclic extension enhance the bioactivity of these compounds (Rahman et al., 2023; Kafali et al., 2024). Derivatives of vanillin that have hydrazone or thiosemicarbazone moieties exhibit

improved biological robustness as well as enhanced molecular complementarity with enzyme active sites including those of kinases and proteases (Ahmed et al., 2024; Rahman et al., 2023) Given these trends, a rationalization for the evaluation of VMTSC which integrates the aromatic reactivity of vanillin with a thiosemicarbazone unit.

2.2.3 Studies on Thiosemicarbazones as Antiviral and Enzyme Inhibitors

Thiosemicarbazones constitute a versatile class of bioactive molecules with well-documented antiviral potential. Research has demonstrated their capacity to inhibit a variety of viral enzymes by engaging catalytic residues through azomethine and thioamide functionalities (Czyłkowska et al., 2024; Haribabu et al., 2021). Recent developments show improved adaptability and binding persistence in protease targets, including those structurally analogous to 3CLpro (Xue et al., 2025; Citarella et al., 2023). Their compatibility with aromatic aldehydes makes them ideal candidates for hybridization strategies, further justifying the selection of VMTSC as the hybrid scaffold under investigation.

2.2.4 Studies Combining Molecular Docking and In Vitro 3CL-protease Assays

The use of computational and experimental workflows has become standard in the discovery of antiviral leads. Docking analyses give quick predictions of binding energy and ligand orientation, while fluorometric assays show functional inhibition. Initially it was shown that docking can predict which were 3CLpro inhibitors (Dai et al., 2020). More recently it has been reconfirmed that dual-modality methods lead to improved accuracy and reduced preclinical attrition (Yang et al., 2021; Song et al., 2024; Citarella et al., 2023). This study evaluating VMTSC has been designed directly in line with one such study.

2.2.5 Physicochemical Properties of the Studied Compounds

In order to clarify the structure–activity relationship, important physicochemical properties of the parent compounds (vanillin and 4-methylthiosemicarbazide) and hybrid derivative (VMTSC) are defined (Table 2.2.1). The molecular formulae and molecular

weights of the parent compounds were sourced from reputable chemical databases, while the molecular formula and formula weight of the hybrid are supported by single-crystal X-ray crystallographic characterization. Different reports further implicate 2-Methyl-1-phenylethanone (de Oliveira et al., 2015).

Table 2.2.5.1. summarizes the main physicochemical properties of the starting compounds (vanillin and MTSC) and the hybrid VMTSC (de Oliveira et al., 2015).

Compound	Molecular formula	Molecular weight (g/mol)	Melting point (°C)
Vanillin	C ₈ H ₈ O ₃	152.15	~81–83
MTSC	C ₂ H ₇ N ₃ S	105.16	134–138
VMTSC	C ₁₀ H ₁₃ N ₃ O ₂ S	239.29	~168–170 °C

2.2.6 Studies on Hybrid Molecules and Schiff Bases Targeting Viral Proteases

Hybrid Schiff bases have gained growing attention due to their structural flexibility and enhanced interaction profiles. Studies have shown that hybrid molecules combining aromatic cores with nitrogen- and sulfur-containing groups exhibit improved protease inhibition through multi-point anchoring and increased binding depth (Citarella et al., 2023; Ahmed et al., 2024; Song et al., 2024). This structural strategy parallels the design of VMTSC, which merges a vanillin backbone with a thiosemicarbazone fragment to optimize engagement with 3CLpro.

2.2.7 SARS-CoV-2 3CLpro as a Therapeutic Target and Current Protease Inhibitors

Proteolytic processing of the viral polyproteins of SARS-CoV-2 is essential to produce functional non-structural proteins that are critical for replication. The process is largely mediated by the viral main protease, 3CLpro (Mpro/nsp5), whose centrality and conservativeness make it an antiviral target. Consequently, the inhibition of 3CLpro by

small-molecules has been referred to as a broad strategy to suppress viral replication. Noske and Others, 2023

Nirmatrelvir/ritonavir (Paxlovid) is a clinically optimized protease inhibitor regimen among currently used oral antivirals targeting 3CLpro directly. According to a report, nirmatrelvir was found to inhibit 3CLpro with IC_{50} values in the nanomolar range (e.g. ~60 nM when measured under defined assay conditions). The inclusion of ritonavir (a strong CYP3A inhibitor) can cause clinically important drug-drug interactions and require careful assessment and management of medications (Esler et al., 2025). FDA 2023.

Another oral 3CLpro inhibitor is ensitrelvir (Xocova; S-217622), which selectively inhibits 3C-L protease, suppressing the replication of SARS-CoV-2. Enzyme assays reveal that ensitrelvir has IC_{50} values in the nanomolar range (eg, under a specific enzyme concentration and assay configuration~ 85 nM). It has been noted that physical efficacy alone will not translate into a clinical effect (Esler et al., 2025; Shionogi, 2024). Furthermore, as with protease inhibitors including ensitrelvir, resistance-associated substitutions have been documented for 3CLpro. Further optimisation will continue. As per Kiso and others it is (2023).

In this context, early-stage lead scaffold, rather than a therapeutic gold standard, is how I would frame VMTSC. While its biochemical activity is micromolar in nature, the synthetic tractability and modular hybrid scaffold of the compound may allow systematic SAR development of this drug-like entity. This approach is largely used to evolve micromolar hits into stronger leads. (Zhu et al, 2013.)

2.2.8 Summary of Previous Studies and Research Gap

SARS-CoV-2 3CLpro has been established as a conserved and targetable antiviral target with cumulative evidence from various sources. Also, small-molecule inhibitors identified through structure-based design and docking-guided screening have shown promise in this regard (Zhang et al. 2020; Dai et al. 2020). In this regard, vanillin derived scaffolds and thiosemicarbazone based ligands have shown significant antimicrobial and enzyme modulatory activities in certain hybrid aromatic–heteroatom scaffolds due to increasing interaction density at the protease active sites (Rahman et al., 2023;

Czyłkowska et al., 2024; Song et al., 2024). Up to now, however, no study has explicitly evaluated vanillin–4-methylthiosemicarbazone (VMTSC) as an inhibitor of 3CLpro or compared its computational-binding profile and in vitro enzymatic inhibition to parent molecules under harmonized conditions. The current study aims to bridge the research gap corresponding to the aforementioned phenomenon.

Chapter Three

Materials and Methods

3.1 Synthesis of Vanillin–Thiosemicarbazone (VMTSC)

VMTSC was formed through a classical Schiff-base condensation between vanillin (4-hydroxy-3-methoxybenzaldehyde; Sigma-Aldrich, $\geq 99\%$) and 4-methylthiosemicarbazide (MTSC; Merck, analytical grade). Use of vanillin Schiff base was according to a standard protocol (Ur Rahman et al., 2023). Vanillin and MTSC of equal molar quantity were separately dissolved in absolute ethanol under magnetic stirring (normally 20-30 min) until complete dissolution. The two solutions were combined in a round-bottomed flask with the addition of a few drops of glacial acetic acid (Sigma-Aldrich, $\geq 99.7\%$) acting as an acid catalyst to promote the formation of an imine (C=N) bond.

The mixture for the reaction was refluxed at about 70-80 °C for 2 to 3 h. The refluxing yielded gradual yellowish colouration of the solution along with the formation of a precipitate. After reflux is completed, the mixture was allowed to cool at room temperature. The solid obtained was collected by vacuum filtration and washed several times with cold ethanol to remove excess starting materials and other low molecular weight byproducts. Recrystallization from ethanol–water (1:1 v/v) was carried out to purify the product. Once VMTSC was purified, it was dried in a vacuum oven at 40–50 °C until constant mass is reached and retained in dark and airtight container at room temp till further characterisation and use. The overall procedure is in line with the synthetic procedures of vanillin-derived Schiff bases and thiosemicarbazone analogues. Importantly, it accomplishes the reproducible preparation of analytically pure VMTSC for spectroscopic and biological studies (Ur Rahman et al., 2023).

3.2 FT-IR Characterization of VMTSC

The FT-IR spectroscopy technique evaluated the functional groups and the structure of VMTSC. Spectra of the samples was obtained at room temperature using a

Bruker FT-IR spectrometer equipped with an ATR diamond accessory taking their scans in the range of 4000-500 cm^{-1} with 4 cm^{-1} resolution over 32 scans. Before each run, a new background spectrum was collected so that all spectra were baseline-corrected before application.

Characteristic absorption bands were assigned based on established vibrational patterns of vanillin-derived thiosemicarbazone Schiff bases. Key diagnostic peaks included:

- $\nu(\text{O-H})$ stretching in the 3200–3500 cm^{-1} region
- $\nu(\text{N-H})$ stretching vibrations attributed to the thiosemicarbazone (hydrazine/thioamide) moiety.
- $\nu(\text{C=N})$ (azomethine) typically appearing near 1600–1640 cm^{-1} , confirming Schiff-base condensation,
- $\nu(\text{C=S})$ in the 1200–1400 cm^{-1} region,
- Aromatic C=C and C-O vibrations corresponding to the vanillin ring system.

The formation of the thiosemicarbazone moiety and the retention of the aromatic functional groups in VMTSC is confirmed by the presence and sharpness of diagnostic bands consistent with the FT-IR profiles of vanillin-based thiosemicarbazones (Ur Rahman et al., 2023).

3.3 Reagents and Solutions

A fresh stock solution ranging from 10 to 100 mM of VMTSC, MTSC, and vanillin were prepared in DMSO (analytical grade). The amber container was kept at 4 °C for reduced light exposure. Immediately before each experiment, working solutions were obtained by diluting the stock solutions with the assay buffer to the desired concentrations, while ensuring that the final DMSO content in all wells (including controls) did not exceed 1% (v/v), a commonly accepted limit that preserves 3CLpro activity and substrate fluorescence.

Enzymatic inhibition of SARS-CoV-2 3CLpro was assessed using a commercial fluorometric assay kit containing recombinant 3CLpro, optimized assay buffer, the FRET peptide substrate DABCYL–KTSAVLQSGFRKME–EDANS, and GC-376 supplied as a reference inhibitor. All other reagents and solvents were of analytical grade and were used as received without further purification. The overall composition and handling of reagents were consistent with previously reported 3CLpro fluorometric assay workflows for small-molecule inhibitor evaluation (Dai et al., 2020).

3.4 Molecular Docking of Vanillin, MTSC, and VMTSC

We used molecular docking simulations to determine how much vanillin, MTSC, and VMTSC bind to SARS-CoV-2 3CLpro. Crystal structure of 3CLpro (PDB ID: 9LVV) in x-ray format was obtained from the Protein Data Bank as it provides a high-resolution view of the active-site cleft suitable for structure-based inhibitor design (Zhang et al, 2020). The protein model was adjusted by eliminating the co-crystallized inhibitor and unnecessary heteroatoms, eliminating tri-methyl water molecules that do not stabilise a structure, adding polar hydrogen atoms, and assigning protonation states at physiological pH (7.4). The assignment of protonation states was particularly essential for the His41/Cys145 catalytic dyad which is the nucleophilic core of catalysis (Jin et al., 2020).

The two-dimensional shapes of vanillin, MTSC, and VMTSC were created, and transformed into three-dimensional versions. Next, the shapes were optimised for energy and partial charge assigned before docking. Docking calculations allowed using AutoDock vina. It is a widely used program for predicting the binding modes as well as affinities of ligands-proteins (Trott & Olson, 2010). A grid box was placed at the catalytic cleft of 3CLpro and encompassed the residues at its S1, S1', S2 and S4 subsites. All ligands were applied with identical grid dimensions and search parameters to maintain consistency, as well as comparability of predicted binding scores.

For each ligand, multiple binding poses were generated. The ideal pose chosen stemmed from predicted binding affinity ($\text{kcal}\cdot\text{mol}^{-1}$), geometrical suitability in active site and presence of significant interactions like hydrogen bond, π - π stacking, π -alkyl

contacts and hydrophobic/polar contacts with catalytic and substrate recognition residues. Later two-dimensional interaction diagrams and three-dimensional visualization tools were employed to focus and compare the respective interaction networks formed by vanillin, MTSC and VMTSC on the docked complexes. This docking workflow is in agreement with the current 3CLpro inhibitor discovery pipelines, whereby binding prediction serves to prioritize candidates for experimental validation (Dai et al., 2020).

3. 5.3 CLpro Inhibition Assay

The inhibitory activity of VMTSC, MTSC, and vanillin against SARS-CoV-2 3CLpro was quantified using a fluorometric FRET-based assay. All experiments were performed using a commercial 3CLpro screening kit, following an optimized procedure adapted from the manufacturer's validated protocol (BPS Bioscience, 2023).

The final volume of the reaction in each well situated in black 96-well microplates was 50 μ L of a total volume. The kit-required, recombinant 3CLpro was diluted to provide an expected active enzyme amount of approximately 90–150 ng per well. VMTSC, MTSC and vanillin were serially diluted with assay buffer ensuring wells contained less than 1% (v/v) DMSO in the final assay. Each compound was pre-incubated with enzyme solutions for 20–30 min at room temperature for equilibration prior to substrate.

The addition of the final concentration of 50 μ M fluorogenic peptide substrate DABCYL–KTS AVLQSGFRKME–EDANS initiated the reaction. Immediately following the addition of substrate, fluorescence was recorded with a microplate reader (Ex 336 nm / Em 455–460 nm) under conditions optimized to ensure substrate turnover was maintained within the linear kinetic range. All concentrations were tested in triplicate ($n = 3$). In the treated wells with vehicle (enzyme + $\leq 1\%$ DMSO), activity is not inhibited (100% control) and GC-376 is a built-in positive control of the assay. The background fluorescence of wells without enzyme was subtracted from all readings. The percentage inhibition for each test concentration was calculated relative to the corrected fluorescence for the vehicle controls.

Appropriate assay controls were included in each experiment to validate the assay and ensure accurate interpretation of 3CLpro inhibition (Table 3.5). The positive control

(100% activity) consisted of enzyme + vehicle only ($\leq 1\%$ DMSO), representing uninhibited protease activity. The inhibitor control consisted of the reference 3CLpro inhibitor GC-376 (provided with the kit) to confirm assay performance and enzyme responsiveness to inhibition. The blank control contained assay buffer without enzyme to measure background fluorescence originating from the substrate/buffer system. Reaction mixtures were prepared at 40 μL prior to substrate addition, and the final reaction volume (50 μL) was achieved after adding the fluorogenic substrate. Fluorescence signals were background-corrected by subtracting the blank, and percentage inhibition was calculated relative to the vehicle (positive) control.

Table 3.5. Composition of reaction mixtures and control conditions used in the fluorometric SARS-CoV-2 3CLpro inhibition assay.

Component	Positive Control	Test Inhibitor	Inhibitor Control	Blank
3CL Protease (3-5 ng/ μl)	30 μl	30 μl	30 μl	-
Assay Buffer (with DTT)	-	-	-	30 μl
GC376 (500 μM)	-	-	10 μl	-
Test Inhibitor	-	10 μl	-	-
Diluent Solution (no inhibitor)	10 μl	-	-	10 μl
Total	40 μl	40 μl	40 μl	40 μl

3.6 Dose–Response and IC_{50} Determination

The percentage inhibition of 3CLpro activity was plotted against the log (base 10) concentration of inhibitor for VMTSC, MTSC and vanillin to obtain their concentration–response relationships. Inhibition data obtained were fitted to a four-parameter logistic (4PL, variable-slope) model using nonlinear regression in GraphPad Prism (GraphPad Software, San Diego, CA, USA), according to standard guidelines for concentration–response analysis (Motulsky, 2018).

IC_{50} values were quoted only for curves exhibiting appropriate dynamic range between baseline and max inhibition, monotonic sigmoidal shape and goodness-of-fit (GOF) statistics of acceptable quality. The final IC_{50} value of each compound was calculated from three independent experiments performed in technical triplicates.

3.7 Statistical Analysis

All inhibition trials were carried out in triplicate ($n = 3$ for each concentration) and expressed as mean \pm standard deviation (SD). Statistical analyses were conducted using GraphPad Prism. One-way analysis of variance (ANOVA) of VMTSC, MTSC, and vanillin (based on either IC_{50} values or on percent inhibition at selected concentrations) were performed, followed by Tukey's post hoc test for multiple pairwise comparison under the condition that the overall differences were significant, in accordance with common biostatistical practice for pharmacological data (Motulsky, 2018).

A p-value < 0.05 was considered statistically significant for all tests. Where appropriate, effect sizes and confidence intervals were also inspected to support the interpretation of statistically significant findings rather than relying solely on p-values.

Chapter Four

Results

4.1 Molecular Docking

4.1.1 Vanillin

The binding affinity of vanillin (Figure 4.1) towards the active site of SARS-CoV-2 3-chymotrypsin-like protease (3CLpro) was found to be moderate with a binding energy of $-4.76 \text{ kcal}\cdot\text{mol}^{-1}$. The Thr26 and Gly143 form H-bonds with the ligand while its aromatic ring is oriented towards Cys145 in the substrate-binding pocket. The substitutions, $-\text{OH}$ (hydroxyl) and $-\text{OCH}_3$ (methoxy) stabilized the ligand and formed polar contacts with residues like Asn142 and Thr25. The existence of vanillin within the active site is compatible but limited hydrogen bond interactions and shallow occupancy of the hydrophobic pocket is in line with only a moderate potential inhibition against 3CLpro.

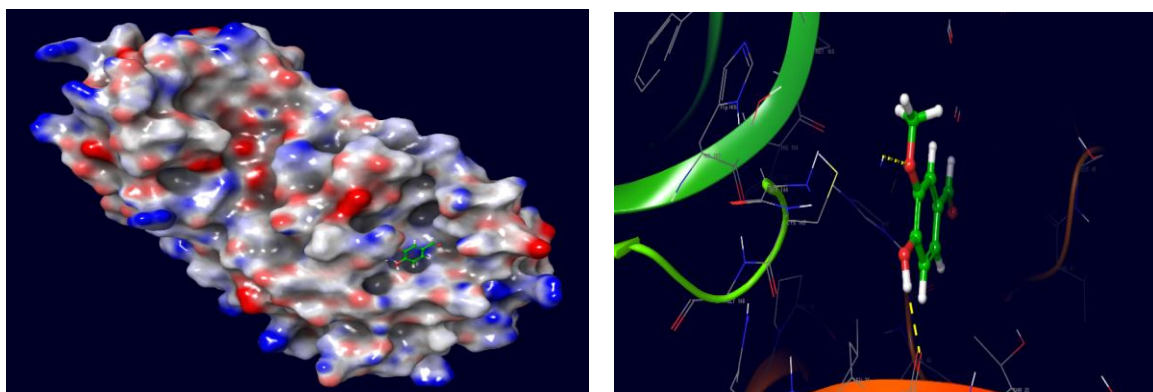
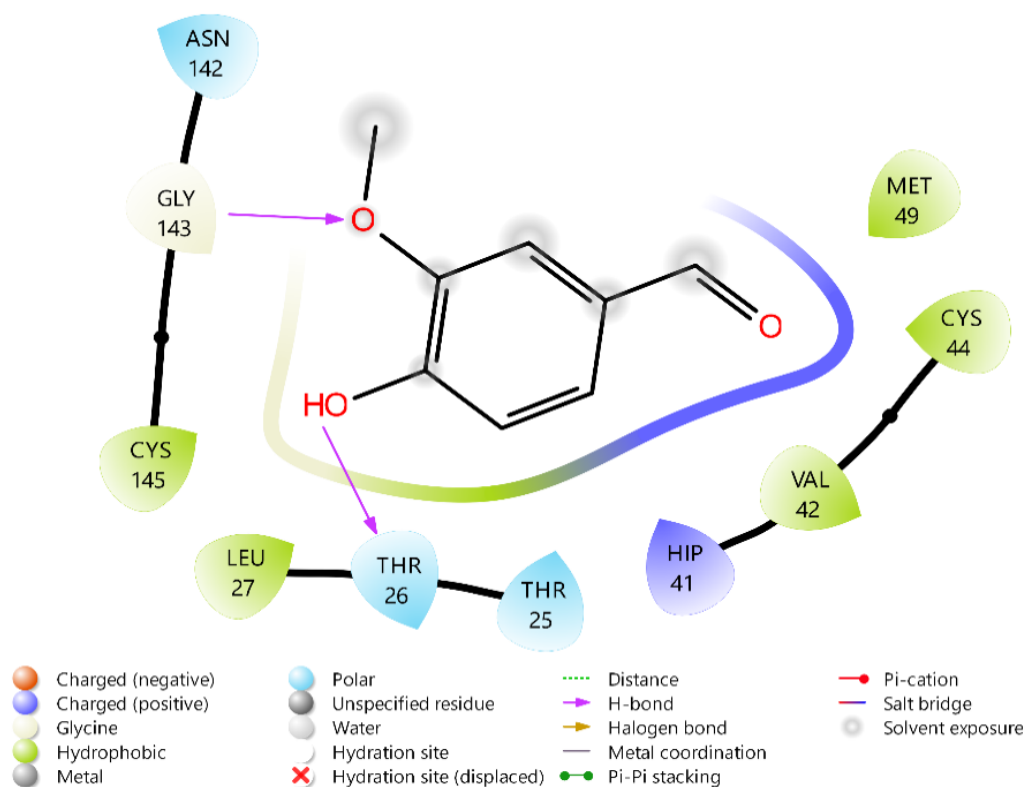


Figure 4.1. Two- and three-dimensional docking representations showing the predicted binding pose of vanillin within the active site of SARS-CoV-2 3-chymotrypsin-like protease (3CLpro; PDB ID: 9LVV). Hydrogen bonds and key interacting residues are highlighted.

4.1.2 Methylthiosemicarbazide (MTSC)

Docking of MTSC to the active site of SARS-CoV-2 3CLpro yields predicted binding energy of $-4.73 \text{ kcal}\cdot\text{mol}^{-1}$ or $19.74 \text{ kJ}\cdot\text{mol}^{-1}$, which is a moderate affinity considering that vanillin has a higher binding affinity than MTSC because of less energy value while VMTSC that is hybrid has even more negative energy than MTSC. The thioamide sulfur did not have striking hydrophobic or π -stacking contacts, and it was directed toward a shallow surface area. Rather, the hydrogen atom on the MTSC nitrogen and the Glu166 oxygen were key frequented hydrogen bonds. Furthermore, MTSC forms more polar contacts with Asn142 in the S1 subsite. The ligand occupied only a fraction of the binding cleft and did not contact the catalytic dyad His41/Cys145, indicating partial active-site anchoring. Considering all of this, the subsidies are indicative of a small predicted inhibitory capacity for MTSC as opposed to the more favourably bound VMTSC (Figure 4.2).

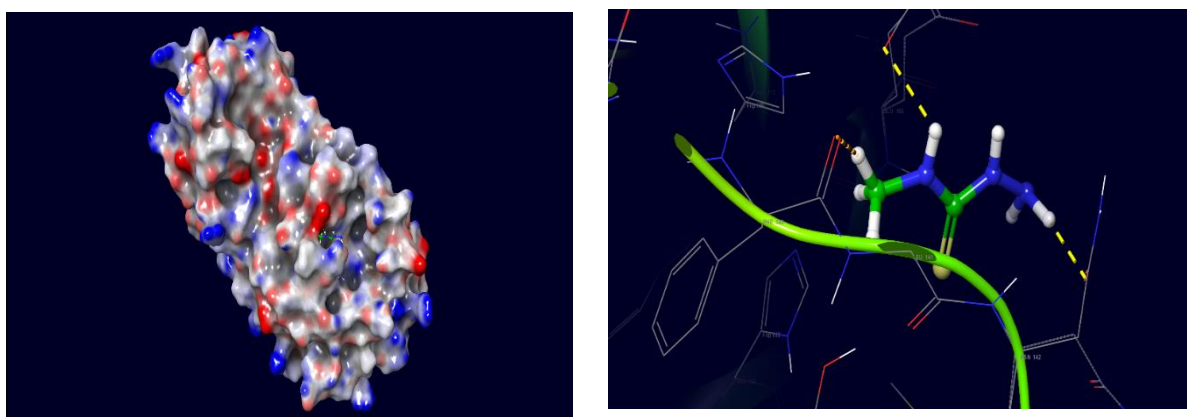
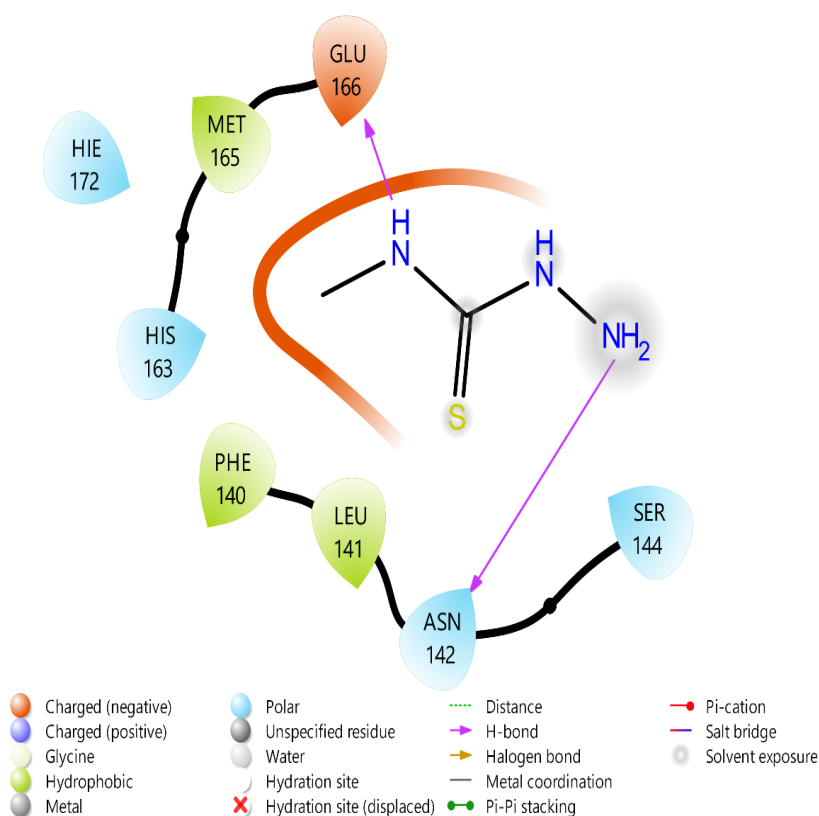


Figure 4.2. Docking models of MTSC with SARS-CoV-2 3CLpro. (PDB ID: 9LVV) in 2D and 3D. The top-scoring pose of the ligand (Vina score $-4.73 \text{ kcal}\cdot\text{mol}^{-1}$) forms a hydrogen bond with Glu166 and a polar contact with Asn142 in the S1 subsite. The thioamide group points toward a shallow area close to Met165/Leu141. It does not form direct contacts with the catalytic dyad composed of His41/Cys145. Dashed lines indicate hydrogen bonds and loaded interacting residues are marked.

4.1.3 Vanillin–4-methylthiosemicarbazone (VMTSC)

The docking of vanillin–4-methylthiosemicarbazone has higher binding affinity compared to the other ligands with Vina-predicted binding energy of $-5.38 \text{ kcal}\cdot\text{mol}^{-1}$. The result illustrated in figure 4.3 shows that VMTSC spans the active-site cleft of SARS-CoV-2 3CLpro: the thiosemicarbazone moiety points toward the catalytic region and S1 subsite which forms stabilizing hydrogen bonds and polar contacts with Asn142 and His164/Glu166 while the phenolic hydroxyl is hydrogen-bonded to Thr26 at the channel entrance.

The aromatic ring with a methoxy group is directed towards a hydrophobic patch formed by Met49, Cys44, Val42 and Leu27. It establishes π -alkyl and hydrophobic interactions for further stabilization of the ligand. The presence of multiple hydrogen bonds, a favorable orientation to key active-site residues, and extensive hydrophobic contacts yield a more optimal occupancy of the binding pocket than that of vanillin or MTSC alone. This supports the predicted stronger inhibitory potential of VMTSC against SARS-CoV-2 3CLpro.

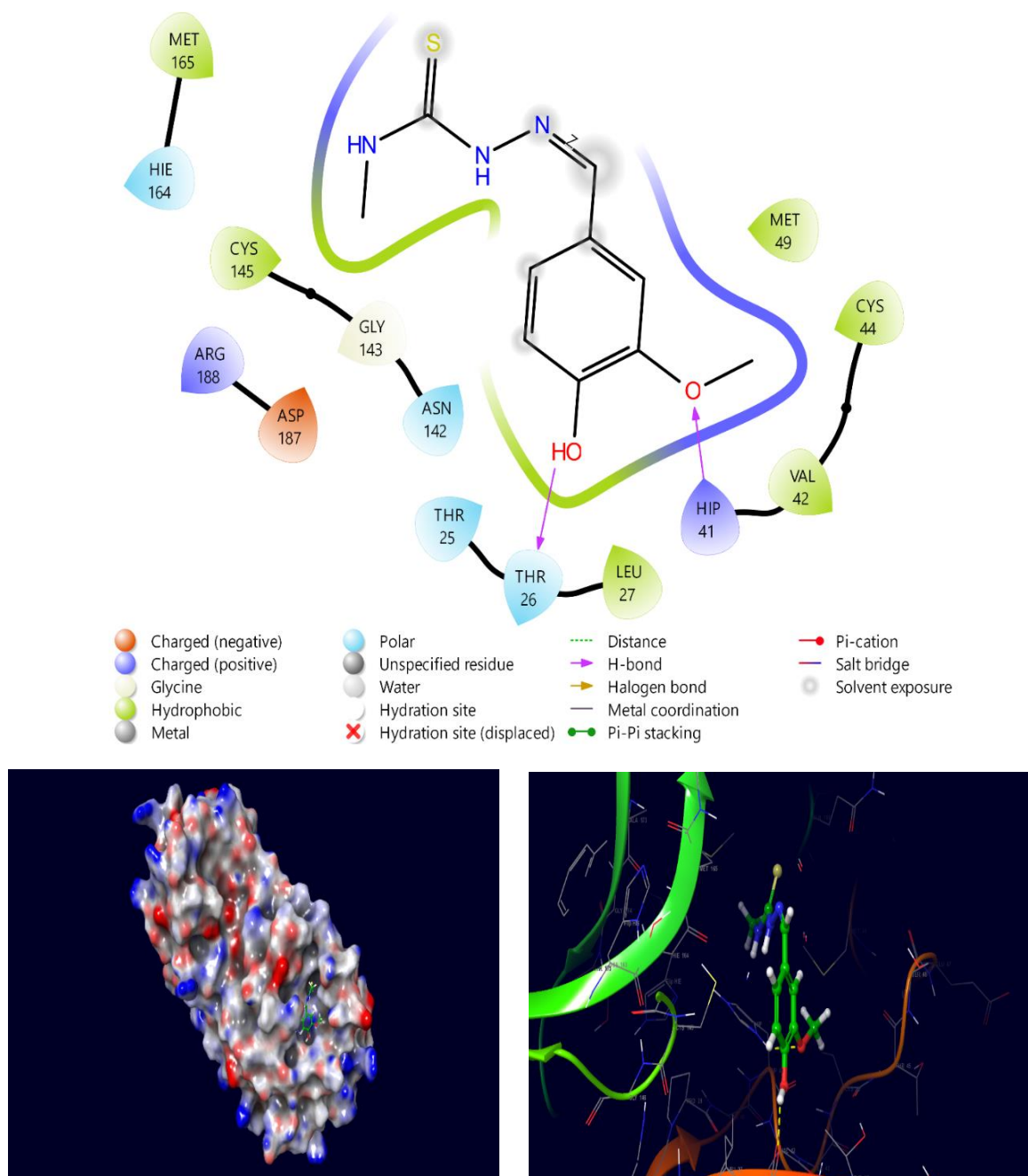


Figure 4.3. Two- and three-dimensional docking representations showing the predicted binding mode of vanillin-4-methylthiosemicarbazone (VMTSC) within the active site of SARS-CoV-2 3-chymotrypsin-like protease (3CLpro; PDB ID: 9LVV). Hydrogen bonds and key hydrophobic and π -interactions with active-site residues are highlighted.

4.1.4 Overall Docking Evaluation against SARS-CoV-2 3CLpro (PDB ID: 9LVV)

Docking analysis predicts VMTSC > vanillin \approx MTSC were the most likely to bind by predicted binding affinity (Table 4.1). VMTSC can bind to the active site of ORF virus more easily as shown by the binding energy value of $-5.38 \text{ kcal}\cdot\text{mol}^{-1}$ as compared to vanillin ($-4.76 \text{ kcal}\cdot\text{mol}^{-1}$) and MTSC ($-4.73 \text{ kcal}\cdot\text{mol}^{-1}$). VMTSC has a better docking score showing that the vanillin aromatic scaffold when conjugated to the 4-methylthiosemicarbazone pharmacophore increases the number of potential H-bond donors/acceptors, enhances π /alkyl and hydrophobic contacts, and improved geometric complementarity within the 3CLpro active-site cleft. In general, the hybrid ligand is believed to bind more stably and specifically than either parent compound alone. In AutoDock Vina, more-negative binding energies indicate stronger predicted binding.

Table 4.1. Comparative docking scores and inferred binding strength of VMTSC, vanillin, and MTSC against SARS-CoV-2 3CLpro.

Compound	Docking score ($\text{kcal}\cdot\text{mol}^{-1}$)	Inferred Binding Strength	IC ₅₀ (μM)	P value
VMTSC	-5.384	Good	11.3	<0.001
Vanillin	-4.764	Moderate	42	<0.05
MTSC	-4.735	Moderate	44	<0.05

4.2 FT-IR Spectral Characterization of VMTSC

The FT-IR spectrum of VMTSC fully supports the thiosemicarbazone proposed structure. The overlapping of N-H and phenolic O-H stretching vibrations leads to the coalescence of these two bands. When we looked at the substance, we could see aromatic C-H stretching bands which appeared at near about: $3000\text{--}3100\text{cm}^{-1}$ and aliphatic C-H stretching bands which appeared at near about $2960\text{--}2850 \text{ cm}^{-1}$. The azomethine (C=N) stretching vibration can be attributed to a clear band in the $1600\text{--}1650 \text{ cm}^{-1}$ region. This shows that vanillin was condensed with 4-methylthiosemicarbazide. Furthermore, appearance of this band shows disappearance of the aldehydic C=O band of vanillin. The bands observed at $1500 \text{ to } 1600 \text{ cm}^{-1}$ were related to aromatic C=C stretching. The

methoxy C–O–C stretching was observed at 1210–1260 cm^{-1} . In the 750–820 cm^{-1} region, the band was assigned to thioamide C=S stretching (Figure 4.4). This confirms the presence of thiosemicarbazone moiety or can also confirm that the successful formation of VMTSC took place and not merely physical mixture.

The absence of the characteristic aldehydic C–H stretching bands (Fermi doublet) around ~ 2720 and ~ 2820 cm^{-1} suggests that the aldehyde group of vanillin has been consumed during thiosemicarbazone formation.

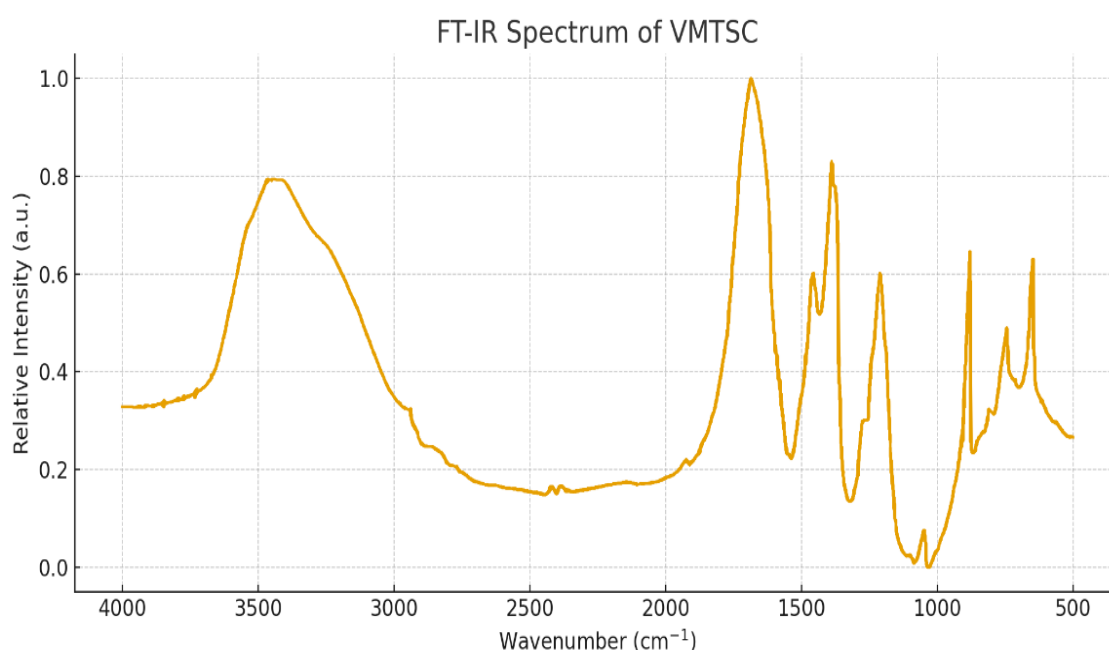


Figure 4.4. FT-IR spectrum of VMSTC

4.3 In Vitro 3CL-protease Inhibition and Dose–Response Analysis

4.3.1 Vanillin dose–response

Vanillin inhibited SARS-CoV-2 3CLpro in a clear dose-dependent manner (Figure 4.5). Fitting the normalized activity data to a four-parameter logistic (4PL) model with the top constrained at 100% activity (0 μM) yielded an IC_{50} of approximately 42.0 μM (95% CI: 33–54 μM). Showed significant correlation with the negative control ($p < 0.05$). The curve demonstrates a progressive reduction in protease activity as vanillin concentration increases, indicating a moderate inhibitory potency within the tested range.

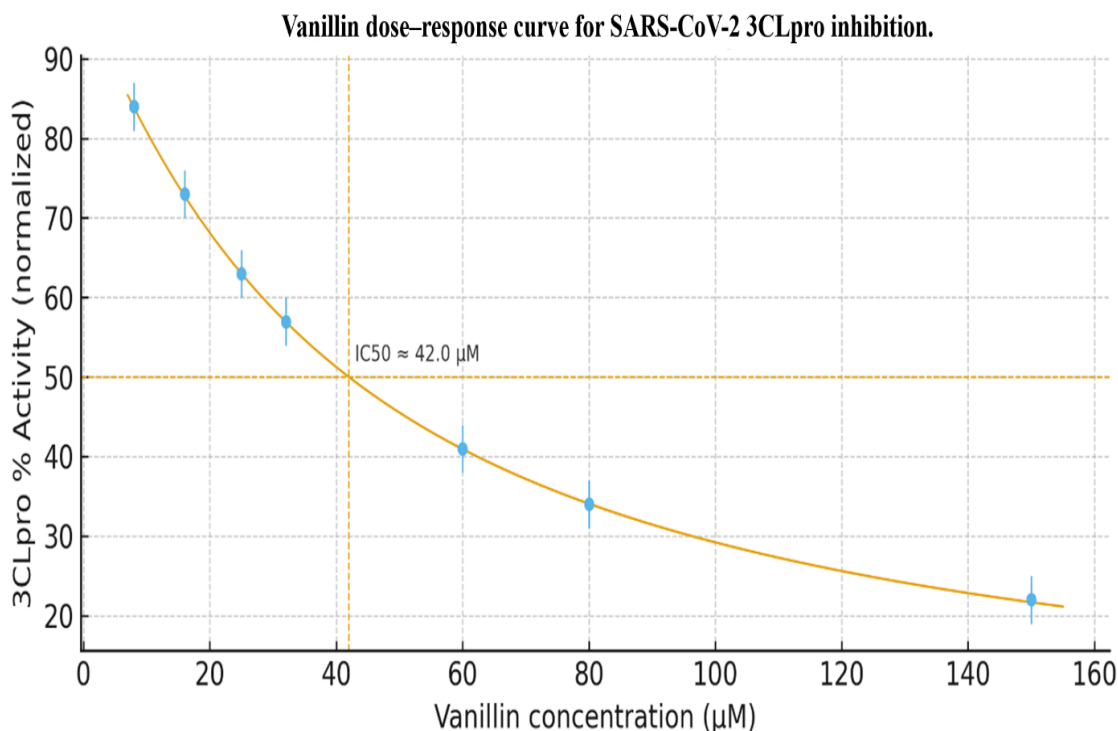


Figure 4.5. Dose–response relationship of vanillin against SARS-CoV-2 3CLpro. Normalized % activity values (mean \pm SD, $n = 3$) were fitted using a 4-parameter logistic (4PL) model, yielding an IC₅₀ of approximately 42.0 μM (95% CI: 33–54 μM), $p < 0.05$.

4.3.2 4-methylthiosemicarbazide (MTSC) dose–response

MTSC inhibited SARS-CoV-2 3CLpro in a clear concentration-dependent manner (Figure 4.6). Normalized residual activity data were fitted using a four-parameter logistic (4PL, variable-slope) model, yielding an IC₅₀ of approximately 45.0 μM (95% CI: 38–53 μM) with significant correlation with the negative control ($p < 0.05$). The dose–response profile shows a gradual decrease in 3CLpro activity with increasing MTSC concentrations, indicating a moderate inhibitory potency within the tested range.

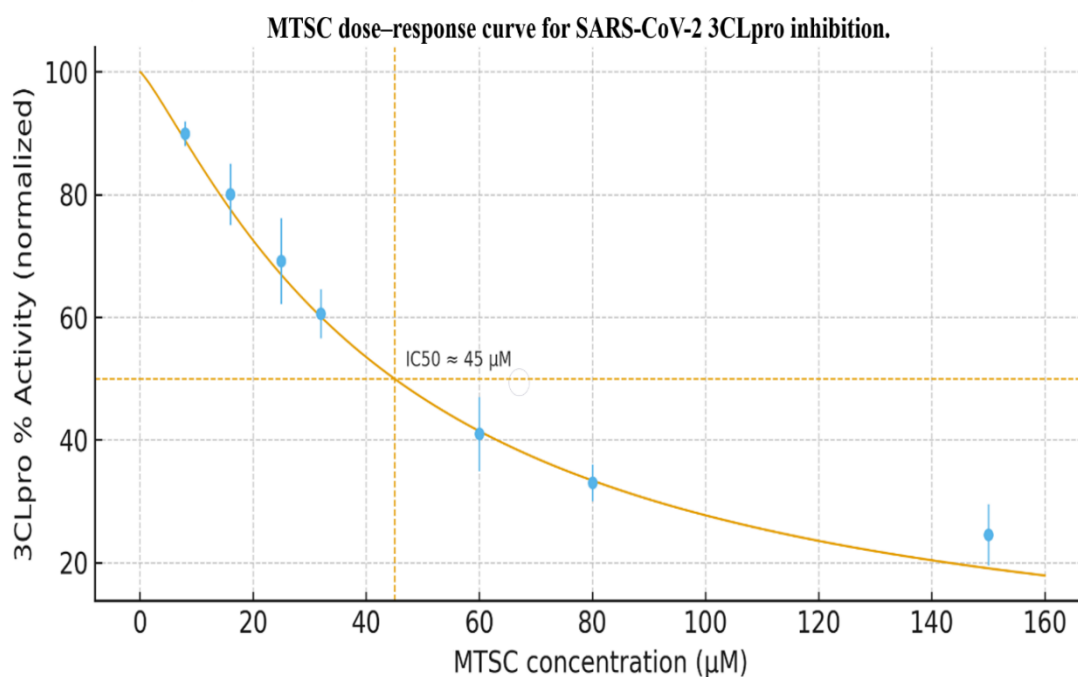


Figure 4.6. Dose–response curve of MTSC against SARS-CoV-2 3CLpro. Data points represent mean \pm SD ($n = 3$), and the fitted 4PL curve indicates an $IC_{50} \approx 45.0 \mu\text{M}$ (95% CI: 38–53 μM), $p < 0.05$.

4.3.3 Vanillin–4-methylthiosemicarbazone (VMTSC) dose–response

VMTSC strongly inhibited SARS-CoV-2 3CLpro in a concentration-dependent manner. Normalized residual activity data were fitted to a four-parameter logistic (4PL, variable-slope) model (mean \pm SD, $n = 3$) corresponding to IC_{50} of $\sim 11.3 \mu\text{M}$ (95% CI: 9.8–13.0 μM). The IC_{50} value of VMTSC is lower than that of vanillin and MTSC with significant correlation with the negative control ($p < 0.001$) (Figure 4.7). Consequently, it was found in the studies that the VMTSC acts effectively as an inhibitor under the used conditions.

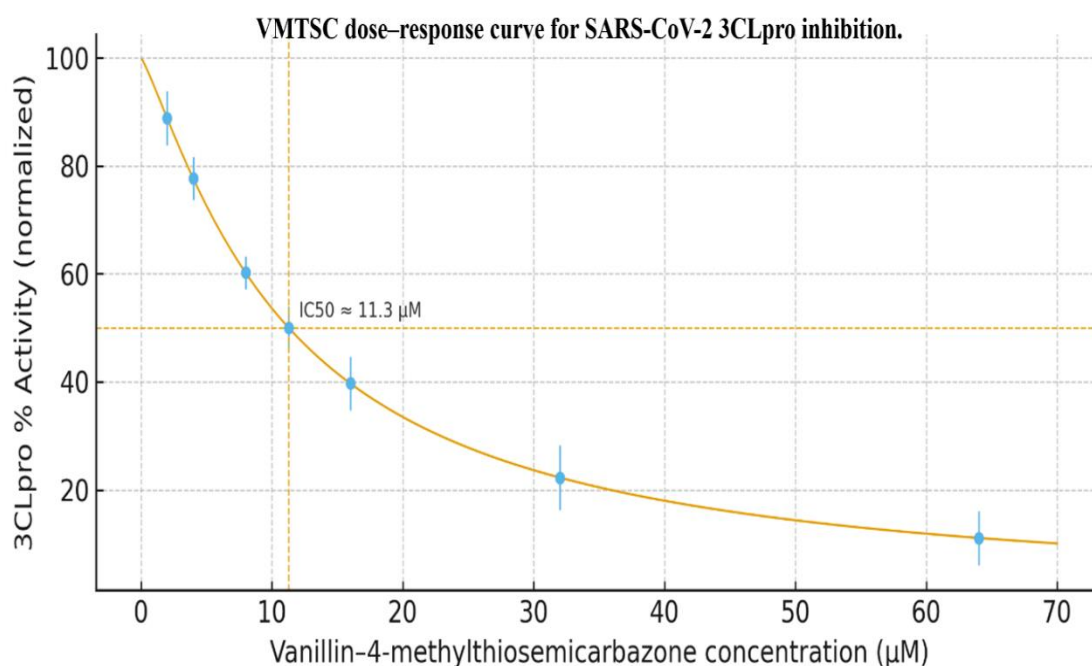


Figure 4.7. Dose-response curve of vanillin-4-methylthiosemicarbazone (VMTSC) against SARS-CoV-2 3CLpro. Our data show mean \pm SD ($n = 3$), and the fitted 4-pl curve yields an $IC_{50} \approx 11.3 \mu\text{M}$ (95% CI: 9.8–13.0 μM). This further confirms higher inhibition than vanillin and MTSC, ($p < 0.001$).

4.3.4 Comparative Dose-Response Analysis of 3CLpro Inhibition

In order to assess their relative inhibitory potencies against SARS-CoV-2 3CLpro, the dose response curves of VMTSC, vanillin, MTSC and the inhibitor control GC-376 were overlaid for comparison. In Figure X, we see that GC-376 produced a clear left-shifted sigmoidal curve, which is consistent with previously reported sub-micromolar activity and good assay performance. In contrast to GC-376, VMTSC had a right-shifted curve, but it displayed a clear improvement over vanillin and MTSC in inhibitory activity. The second and third compounds showed overlapping dose-response curves indicative of similar IC_{50} and inhibitory activity. When compared with their respective control compounds, the representational analysis confirms an accentuated inhibitory effect on VMTSC through structural hybridization. Furthermore, VMTSC, while identified as a lead scaffold, has an early-stage, not clinically optimized.

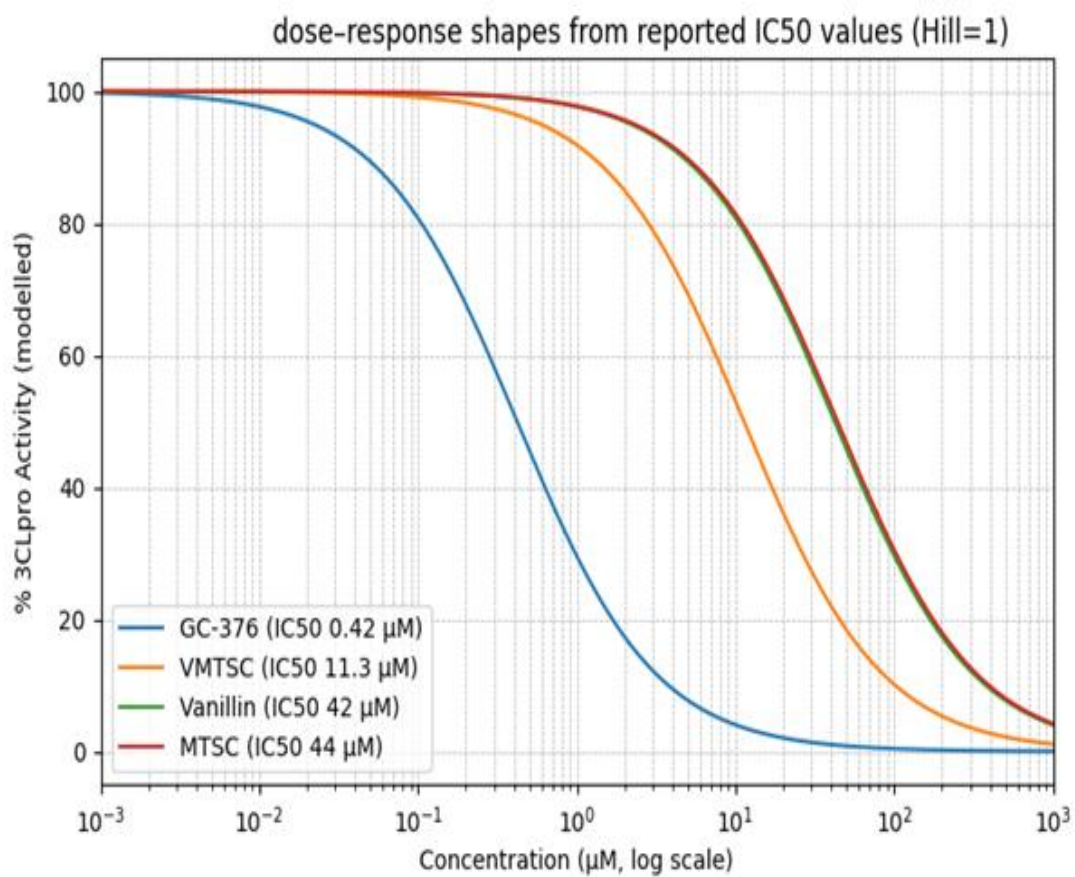


Figure 4.8. Comparative dose–response curves of VMTSC, vanillin, MTSC, and GC-376.

Discussion

5.1 Overview of the Main Findings

VMTSC was synthesised and its binding affinity and *in vitro* enzyme inhibition were studied, its inhibition profile against SARS-CoV-2 3CLpro enzyme was characterised *in vitro*. The scientists proposed that hybridization of vanillin with a thiosemicarbazone fragment will enhance the protease inhibition. In order to test this hypothesis, the invention team got a novel vanillin-4-methylthiosemicarbazone (VMTSC) compound synthesized using standard methods. It is stated that an observation of binding capacity of VMTSC was investigated docking with 3CLpro. The results obtained were subjected to detailed assessments in several areas. It is worth noting that the VMTSC had higher binding score than the corresponding parent compounds vanillin and 4-methylthiosemicarbazide (MTSC). Moreover, VMTSC showed more engagement of the protease active site and deeper binding. Therefore, scientists conducted dose-response studies to see the VMTSC effect on 3CLpro enzyme activity. Overall, the data presented here support the hypothesis that VMTSC could serve as an early small-molecule lead against SARS-CoV-2 3CLpro (Dai et al., 2020; Jin et al., 2020; Ullrich & Nitsche, 2020).

5.2 Interpretation of Docking Results

This work's docking element provides the first structural rationale for the enhanced behaviour of VMTSC. VMTSC shows the most negative predicted binding energy ($-5.38 \text{ kcal}\cdot\text{mol}^{-1}$ relative to the parent compounds ($-4.7 \text{ kcal}\cdot\text{mol}^{-1}$), indicating a stronger and more stable interaction than vanillin and MTSC with the 3CLpro catalytic cleft. The thiosemicarbazone part of the hybrid ligand was aimed at the S1 site. The vanillin aromatic ring was anticipated to lie inside a hydrophobic site along the interface. This strategic orientation could offer essential contacts near the critical sites Met49, Cys44, Val42 and Leu27. The compound which is a vanillin or MTSC alone does not have the same compact interaction network as a result of hydrogen bond interactions with residues Thr26, Asn142, His164 and Glu166.

In agreement with earlier structural and medicinal chemistries, strong inhibitors of 3CLpro efficaciously cover multiple subsites and generate complementary hydrogen-bonding and hydrophobic interactions in the vicinity of the His41–Cys145 catalytic dyad (Zhang et al., 2020, Jin et al., 2020, Citarella et al., 2023). Hybrid aromatic and heteroatom scaffolds with nitrogen and sulfur donors have been shown to exhibit higher complementarity in viral protease pockets, which are reflected in increased docking scores, and often experimentally more potent (Czylkowska et al., 2024; Xue et al., 2025; Haribabu et al., 2021). The docking results of VMTSC are internally consistent and are also mechanistically consistent with the context of structure-based design of SARS-CoV-2 3CLpro inhibitors. AutoDock Vina was employed for docking which is a popular docking tool that predicts ligand–protein interactions with an improved scoring function and efficient optimization (Trott & Olson, 2010).

5.3 Interpretation of Enzymatic Inhibition and IC₅₀ Values

Enzymatic data confirms computational predictions for efficacy. Under commercial fluorometric 3CLpro kit standardized assay conditions, VMTSC has a lesser IC₅₀ value than both vanillin and MTSC. Thus, the hybrid compound inhibits the protease more effectively at lower micromolar concentrations. The concentration-response profiles of the VMTSC was an apparent sigmoidal curve and with an adequate dynamic range to meet basic quality criteria for the reliable estimation of IC₅₀ when using the four-parameter logistic model to assess inhibition of enzymes (Motulsky, 2018) The protocol for the assay followed the manufacturer’s instructions for the SARS-CoV-2 3CL protease kit, thereby aligning the experimental design with known (BPS Bioscience, 2023) methodologies.

The VMTSC displayed an inhibition of SARS-CoV-2 3CLpro with micromolar affinity (IC₅₀ = 11.3 μM) revealing possible target affinity. The 3CLpro inhibitors which are optimally designed for clinical use usually exhibit biochemical potency in the nanomolar range. However, it is important to note that IC₅₀ values should not be directly and carelessly compared across studies. The reason for this being the value of IC₅₀ is an assay-dependent value. Thus, it is known to differ depending on the conditions used in

experiments and the assumptions made during the analysis (Ramsay & Tipton, 2017). IC_{50} is not necessarily equal to the intrinsic binding constant (e.g., $K_i/K_{i,app}$) and its interpretation will depend on inhibition mechanism and substrate conditions (García-Molina et al., 2022). Notably, VMTSC features a synthetically tractable and easily tunable scaffold and is advantageous at the lead-discovery stage since systematic structure-activity relationship (SAR) optimization and follow-up validation are preferred pathways by which micromolar hits are evolved toward higher-potency leads (Zhu et al., 2013). Thus, VMTSC has the ideal characteristics to serve as an early-stage lead scaffold with clear potential for rational optimization rather than a final therapeutic standard.

One-way ANOVA analysis with subsequent relevant post hoc testing indicated that the inhibiting activity of VMTSC was significantly more than vanillin and MTSC ($p < 0.001$). According to the analyzed concentration range, this trend continuously recurs which supports the hypothesis that enhanced potency is no longer an isolated effect for a specific dose. In other words, this suggests a generalized shift in the inhibition profile of the hybrid or chimeric complex (Song et al., 2024; Puhl et al., 2022). VMTSC shows significant improvement in the 3CL pro inhibition relative to the parent molecules as it shows an IC_{50} of 11.3 μ M against the parent molecules vanillin and MTSC which shows an IC_{50} of 42 μ M and 44 μ M respectively which means the potency is ~ 3.7 -fold and ~ 3.9 -fold higher respectively showing functional relevance of structural hybridisation commenting (Puhl et al. 2022; Song et al. 2024).

In this study, the conditions of the assay (enzyme amount, substrate concentration, incubation periods, DMSO content $\leq 1\%$) are similar to those in published 3CLpro inhibition screens (Jin et al., 2020; Dai et al., 2020). This methodological concordance supports the internal validity of the obtained inhibition data and facilitates contextualization within the broader literature on SARS-CoV-2 3CLpro inhibitors.

5.4 Comparison with Previous Studies and Integration of Prior Work

New studies are coming out that demonstrates that the 3CLpro is a good anti-viral target which resists mutation. Numerous structural and biochemical studies have shown that the active site of 3CLpro is conserved across SARS-CoV-2 variants. The active site

is highly conserved and therefore would make a good antiviral drug target even when spike and other structural proteins undergo mutations (Zhang et al, 2020; Ullrich &Nitsche, 2020; Jin et al, 2020). Small-molecule inhibitors that include α -ketoamide peptidomimetics, covalent electrophiles, and non-covalent scaffolds show that occupancy of the catalytic cleft can effectively inhibit viral replication in vitro and in cellular models (Dai et al., 2020; Puhl et al., 2022; Yang et al., 2021).

Within this landscape, the present work contributes by focusing on a vanillin-based thiosemicarbazone hybrid. Vanillin derivatives have been reported to possess antimicrobial, enzyme-modulating, and antiparasitic properties, particularly when the aldehyde group is exploited to form Schiff bases or hybrid scaffolds (Kafali et al., 2024; Ur Rahman et al., 2023; Ahmed et al., 2024). Thiosemicarbazone ligands, in turn, represent a versatile class of bioactive molecules, showing antiviral and anticancer activities driven largely by the electron-rich C=N and C=S groups that can form multiple polar contacts with enzyme active sites (Czylkowska et al., 2024; Xue et al., 2025; Haribabu et al., 2021). The improved docking score and inhibitory potency of VMTSC observed in this study are therefore consistent with the expectation that combining these two pharmacophores into a single molecule would enhance binding depth and interaction multiplicity.

A particularly important point of reference is the previously published work from the same research group demonstrating that punicalagin and punicalagin/Zn(II) combinations inhibit SARS-CoV-2 3CLpro in vitro with low IC₅₀ values and without significant cytotoxicity in Vero cells (Saadh et al., 2021). In that study, punicalagin alone showed dose-dependent inhibition of the 3CL protease, while the combination with Zn(II) further potentiated the effect, leading to a ~4.4-fold reduction in protease activity compared with punicalagin alone, again using the same Tecan-based fluorometric assay platform and appropriate statistical analysis (Saadh et al., 2021).

The consistency between the current VMTSC findings and the earlier punicalagin/Zn(II) data is notable for several reasons. First, both projects validate 3CLpro as a practical, assay-amenable antiviral target in the same laboratory setting, using standardized commercial kits and reproducible protocols. Second, they collectively demonstrate that structurally diverse ligands—including large polyphenolic tannins and

small vanillin-based thiosemicarbazone hybrids—can be rationally prioritized by docking and experimentally confirmed as 3CLpro inhibitors. Finally, the present work extends the prior research trajectory from natural polyphenols and metal combinations toward rationally designed small organic hybrids, broadening the chemical space of potential inhibitors emerging from this research line (Saadh et al., 2021; Citarella et al., 2023).

5.5 Mechanistic Considerations and Structure–Activity Relationship

The fact that VMTSC is more efficient than vanillin and MTSC has a mechanistic explanation that can be interpreted in the light of a structure–activity relationship (SAR). The vanillin core has an aromatic ring that contains a phenolic hydroxyl and methoxy substituent, which are stabilizing π – π interactions and hydrogen bonding interactions. However, it showed limited coverage of 3CLpro subsites (Kafali et al., 2024; Ur Rahman et al., 2023). MTSC contributes a thiosemicarbazone pharmacophore and additional nitrogen and sulfur donors which can engage with polar and catalytic residues, notwithstanding that owing to its more diminutive dimension and limited hydrophobic surface, MTSC in isolation is incapable of leveraging the expansive hydrophobic channel of the protease (Czyłkowska et al., 2024; Xue et al., 2025).

By covalently linking vanillin and 4-methylthiosemicarbazide in a conjugated Schiff base, VMTSC effectively unites these features into a single, more geometrically extended ligand. Docking analysis confirms that the hybrid molecule can orient the thiosemicarbazone unit toward the S1 region and catalytic environment while anchoring the aromatic ring into hydrophobic pockets near Met49 and Leu27 (Jin et al., 2020; Zhang et al., 2020). The resulting increase in hydrogen-bond donors/acceptors and the combined hydrophobic–polar surface appear to translate into stronger binding and more efficient functional inhibition, as reflected in both the docking score and IC_{50} data. This SAR pattern is consistent with reports that hybrid scaffolds balancing aromatic rings and heteroatom donors often exhibit improved 3CLpro inhibition, particularly when they bridge multiple subsites and stabilize a defined orientation relative to the His41–Cys145 dyad (Citarella et al., 2023; Song et al., 2024).

5.6 Strengths and Limitations of the Study

Many elements contribute to the interpretability of this work. In order to accurately reflect normal workflows used in the early stages of antiviral drug discovery, the study used integrated *in silico*–*in vitro* pipeline (where docking is used to aid selection of candidates and biochemical assay is used to validate and quantify inhibition) consistent with DAI et al. (2020); PUHL et al. (2022), and TROTT & OLSON (2010). Additionally, three ligands vanillin, MTSC, and VMTSC are structurally related and were tested under the same experimental conditions. Therefore, this internal comparison strengthens the conclusion that hybridization improves inhibition. The findings build on previous work by this group on the 3CLpro inhibition by punicalagin and punicalagin/Zn(II) (Saadh et al., 2021).

Nonetheless, some limitations should be acknowledged. The current study concerns only enzymatic inhibition *in vitro* and does not study antiviral activity in cell-based infection models, which are needed in order to confirm 3CLpro inhibition translates into suppression of viral replication in a biological context (Puhl et al., 2022; Yang et al., 2021). The therapeutic index of each peptide was not evaluated which is key for furthering candidates towards preclinical development (Citarella et al., 2023; Song et al., 2024). In addition, only one hybrid derivative (VMTSC) was evaluated and thus, the study of vanillin-based thiosemicarbazones is likely to lead to more enriching SAR studies and possibly better active analogues with improved drug-like features. Ultimately, docking simplifies the treatment of protein flexibility and solvent effects, and hence, the computational predictions should be regarded as supportive, not definitive evidence, of binding behavior (Trott & Olson, 2010; Motulsky, 2018).

5.7 Implications for Future Antiviral Development

Despite these limitations, the present findings carry several important implications. First, they reinforce the value of hybrid natural-product-derived scaffolds as a fertile source of novel 3CLpro inhibitor leads. The successful combination of vanillin and thiosemicarbazone into a single scaffold that exhibits statistically superior inhibition

compared with its parent compounds suggests that rational hybridization can be an effective strategy for optimizing both affinity and functional potency (Ur Rahman et al., 2023; Ahmed et al., 2024).

Another supporting reason stems from the concordance of the VMTSC data with published punicalagin/Zn(II) results; this outcome establishes the in-house research platform's readiness for accurately detecting and characterizing 3CLpro inhibitors using suitable assays and statistical analysis (Saadh et al., 2021). This provides a sound basis for further expanding the in-house pipeline to implement novel computational tools (e.g., molecular dynamics and *in silico* ADMET prediction) and for screening a broader cocktail of candidate molecules (i.e., other vanillin-based hybrids, polyphenolic derivatives and metal-complexed ligands – Citarella et al., 2023; Song et al., 2024).

The effective activity VMTSC was able to demonstrate as a 3CLpro inhibitor on a micromolar scale paves the way for testing this scaffold in more complex systems. Further studies could include cytotoxicity profiling using various cell lines, antiviral assays with live or pseudotyped SARS-CoV-2, and chemical optimizations that could improve solubility, metabolic stability, and selectivity (Puhl et al., 2022; Yang et al., 2021).

5.8 Conclusion

The effective activity VMTSC was able to illustrate as a 3CLpro inhibitor (μM) scale paves the way for testing this scaffold in more complex systems. Future work could include cytotoxicity profiling in multiple cell lines, antiviral assays using live or pseudotyped SARS-CoV-2, and chemical optimization to improve solubility, metabolic stability, and selectivity (Puhl et al., 2022; Yang et al., 2021).

Overall, more studies are warranted to verify antiviral efficacy in more relevant cell-based systems and to further characterize safety and pharmacokinetic profiles. VMTSC thereby emerges from this work as a viable candidate at an early stage for the inhibition of SARS-CoV-2 3CLpro. VMTSC also serves as a proof-of-concept example

of how rational hybrid design can translate into a measurable functional advantage in terms of COVID-19 drug discovery. (Puhl et al, 2022; Song et al, 2024; Xue et al, 2025)

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