Biochemistry and Biophysics Reports 39 (2024) 101783

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

NanoLuciferase technology-based detection of TMPRSS2 as attempt to develop anti-coronavirus agents

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The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has emerged as a global health crisis, affecting millions worldwide and exerting immense pressure on healthcare systems. Despite significant advancements in managing the pandemic, including the rapid development and deployment of vaccines, the cessation of the global pandemic status does not mark the end of challenges. The threat of new diseases, especially those arising from zoonotic spillovers - where pathogens jump from animals to humans - remains high [1]. Environmental factors, such as climate change and habitat encroachment, also contribute to the emergence of new pathogens. The ongoing deforestation and wildlife trade increase human exposure to novel viruses, making the possibility of future pandemics a persistent concern. Moreover, the continuous evolution of viruses through genetic mutations poses additional challenges, necessitating the need for sustained research efforts to monitor and respond to emerging infectious threats [2]. The collective experience with COVID-19 underscores the importance of global collaboration in disease surveillance, research, and the development of therapeutic and preventive strategies.

In the realm of antiviral drug development, small molecule drugs have garnered attention for their potential in combating coronaviruses [3]. These compounds can be designed to interfere with critical stages of the viral life cycle, particularly targeting host cell factors that are exploited by the virus for replication. One of the advantages of small molecule therapeutics is their ability to penetrate cell membranes easily due to their low molecular weight, thereby reaching intracellular targets effectively. They also offer the benefit of oral bioavailability, making them convenient for widespread use. In contrast to biologics like antibodies, small molecules typically have longer shelf lives and lower production costs [4]. Furthermore, the high-throughput screening of small molecule libraries allows for the rapid identification of potential antiviral compounds. However, the design and development of these drugs require a comprehensive understanding of the virus-host interaction mechanisms and the identification of host targets that are crucial for viral replication but have minimal impact on normal cellular functions [5].

TMPRSS2, or Transmembrane Protease, Serine 2, has emerged as a key target in the fight against SARS-CoV-2 [6]. In normal physiology, TMPRSS2 is involved in various cellular processes, including protein processing and activation. It is widely expressed in respiratory, gastrointestinal, and urogenital tracts, playing a role in homeostasis and cellular maintenance. The relevance of TMPRSS2 in COVID-19 stems from its role in priming the spike protein of SARS-CoV-2, facilitating the fusion of the viral and host cell membranes. This proteolytic cleavage by TMPRSS2 is a critical step for viral entry and subsequent infection. Numerous studies have highlighted the importance of TMPRSS2 in the pathogenesis of COVID-19, with evidence showing reduced viral entry and infection in cells where TMPRSS2 activity is inhibited [7]. Several existing drugs, such as camostat mesylate and nafamostat, have been repurposed to target TMPRSS2, demonstrating the potential of TMPRSS2 inhibitors in reducing viral load and severity of infection [8]. These findings underscore the therapeutic promise of TMPRSS2 inhibition in managing SARS-CoV-2 infection and potentially other emerging coronaviruses.

While existing TMPRSS2 inhibitors like nafamostat and camostat have shown potential [9], their efficacy in clinical settings against COVID-19 has been limited. These limitations stem from various factors, including suboptimal pharmacokinetics, where the drug does not reach sufficient concentrations in the relevant tissues, or has a short half-life requiring frequent dosing [10]. Additionally, issues related to drug safety and tolerability have been observed, with some inhibitors

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https://doi.org/10.1016/j.bbrep.2024.101783
Received 27 January 2024; Received in revised form 4 July 2024; Accepted 8 July 2024
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exhibiting off-target effects that may lead to adverse reactions [11]. Moreover, the specificity of these inhibitors towards TMPRSS2 versus other serine proteases remains a concern, as non-specific inhibition could disrupt normal physiological processes [12]. The potential for drug resistance also necessitates the exploration of novel inhibitors with different mechanisms of action [12]. These challenges highlight the need for continued research to develop more effective and selective TMPRSS2 inhibitors. Scientific evidence, including clinical trial data and in-vitro studies, must guide this development process to ensure the safety and efficacy of new therapeutic agents.

The NanoBiT technology represents a significant advancement in the field of molecular biology and drug discovery [13,14]. This technology employs a bioluminescent reporter system for the quantitative analysis of protein-protein interactions, protein abundance, and enzyme activity. The system is based on a split luciferase assay, where the luciferase enzyme is divided into two subunits - Large BiT (LgBiT) and High-affinity BiT (HiBiT). The HiBiT component is particularly noteworthy for its small size and high affinity for LgBiT, facilitating easy tagging of proteins without affecting their native function. When the tagged protein (HiBiT) comes into proximity with LgBiT, a complementation occurs, resulting in a luminescent signal that can be quantitatively measured. This sensitivity and specificity make it an ideal tool for monitoring the expression and interaction of proteins within cells. The application of NanoBiT technology extends beyond basic research to drug screening, where it can be used to identify compounds that modulate specific protein interactions, a crucial aspect in the development of targeted therapeutics.

Our study employed the NanoBiT technology to create a novel drug screening platform targeting TMPRSS2. We engineered a Beas-2B cell line to stably express a HiBiT-linked TMPRSS2 gene, allowing us to monitor TMPRSS2 expression levels accurately. The establishment of this stable cell line was a critical step, ensuring consistent and reproducible results in our screening assays. We first validated the stability and functionality of the cell line, confirming that the HiBiT-TMPRSS2 fusion protein retained its native activity and did not interfere with normal cellular processes. Subsequently, we conducted a comprehensive screening of three diverse drug libraries to identify compounds that could reduce TMPRSS2 expression levels. The top 1 % of hits from this initial screening were subjected to further analysis to determine their inhibitory concentration (IC50) values. These potential inhibitors were then evaluated using Western blot analysis to confirm their effect on TMPRSS2 expression and high-content screening to assess their impact on cellular functions. This multi-faceted approach not only allowed for the identification of promising TMPRSS2 expression reducers but also provided insights into their mechanisms of action, paving the way for the development of novel anti-coronavirus therapeutics.

1. Methods

1.1. Materials

In this study, Beas-2B cells (CRL-9609) were sourced from ATCC and cultured in DMEM/F-12 medium (11320082) supplemented with Fetal Bovine Serum (FBS) (26140079) obtained from Gibco. Transfection of the cells was carried out using Lipofectamine 3000 Transfection Reagents (L3000015) from Invitrogen to introduce the TMPRSS2-HiBiT construct. TMPRSS2 detection was performed using the TMPRSS2 antibody (PA5-83286) sourced from Thermo Fisher. MG132 (F1100) from UBPBio was utilized as a proteasome inhibitor to prevent ubiquitination degradation of the protein of interest. Various assays, including the CellTiter-Glo 2.0 Cell Viability Assay (G9243), Nano-Glo HiBiT Lytic Detection System (N3040), Nano-Glo HiBiT Blotting System (N2410), and HiBiT CMV-neo Flexi Vectors (N2401, N2391), were supplied by Promega and employed in this study to facilitate the construction of a stable TMPRSS2-HiBiT overexpression cell line and the subsequent validation of TMPRSS2 expression levels. Diversified building block compound library, bioactive compound library and endogenous metabolite compound library were from TargetMol.

1.2. Construct TMPRSS2-HiBiT overexpression Beas-2B monoclonal cells

To establish a stable cell line with TMPRSS2-HiBiT overexpression, a stepwise process was followed. Initially, Beas-2B cells were cultured in DMEM-F12 media supplemented with 1 % Pep/Strep, 1 % 1 M Hepes, 1 % 200 mM L-Glutamine, and 10 % FBS. The full-length TMPRSS2 gene was then cloned into the pFC37K HiBiT CMV-neo Flexi Vector to create an in-frame fusion with the HiBiT tag. Transfection of Beas-2B cells with the TMPRSS2-HiBiT construct was facilitated using Lipo3000 from Invitrogen. Stable cell lines were subsequently generated by introducing a selection marker, geneticin (G418), followed by antibiotic selection to isolate monoclonal cell populations expressing TMPRSS2-HiBiT. The establishment of the stable cell line was confirmed by assessing the TMPRSS2-HiBiT fusion protein's expression and functionality.

1.3. Stable cell line TMPRSS2 expression level detection using HiBiT lytic reagents

The HiBiT technology and the Nano-Glo HiBiT Lytic Detection System were employed to measure bioluminescence as a surrogate index for the TMPRSS2 protein levels. The HiBiT tag, when attached to TMPRSS2, allowed the protein to generate a luminescent signal upon the addition of LgBiT, which is included in the detection kit as a reagent. The luminescent signal directly correlates with the amount of HiBiT-tagged TMPRSS2 protein, providing a sensitive and quantitative measure of TMPRSS2 expression. The NanoBiT technology was chosen over GFP due to its higher sensitivity and ability to quantitatively measure protein expression levels in live cells. The HiBiT system, although requiring the presence of LgBiT for luminescence, provides a dynamic range and sensitivity superior to GFP. During our detection assays, we utilized LgBiT provided in the detection kit as a reagent added to the microplates. When HiBiT-tagged TMPRSS2 in the cells comes into proximity with the added LgBiT reagent, a complementation occurs, resulting in a luminescent signal that can be quantitatively measured. This method ensures precise monitoring of TMPRSS2 expression levels, which is crucial for identifying effective inhibitors. These cells were seeded into 384-well microplates and treated with specific compounds, including MG-132 (a proteasome inhibitor blocking ubiquitination degradation of the protein) and cycloheximide (which blocks protein synthesis). These treatments were aimed at selecting cell strains that exhibited higher and more stable signals in response to MG-132 and cycloheximide. The Z' factor, a reliable statistical metric, was calculated to identify the most robust cell strain for further experiments. This stringent selection process ensured the selection of cells that consistently exhibited the desired TMPRSS2-HiBiT expression and response characteristics.

To accurately quantify the TMPRSS2 expression levels, it is crucial to normalize the bioluminescent signal to the cell number in each well. We utilized the ATP-Glo assay, which measures cellular ATP levels as an indicator of viable cell count. By correlating the HiBiT luminescence signal with ATP levels, we ensured that variations in cell number did not skew the expression data. This normalization process allowed for a more accurate and reliable comparison of TMPRSS2 expression levels across different treatment conditions. Additionally, the ATP-Glo assay was employed to assess the potential cytotoxicity of the identified hits. This assay provided insights into whether the compounds had any adverse effects on cell viability.

1.4. Western blots

Western blot analysis was employed to validate the effects of selected compounds on TMPRSS2 expression in the chosen cell strain. Cells treated with compounds of interest were lysed to extract protein samples, followed by polyacrylamide gel electrophoresis and subsequent

transfer onto membranes for immunoblotting. Specific antibodies targeting TMPRSS2 were utilized for Western blot detection. The resulting protein bands were visualized using chemiluminescence or a suitable detection method.

2. Results

2.1. First round of screening: diversified, bioactive and endogenous metabolite libraries

In our high-throughput screening analysis, the z' factor was employed as a key metric to evaluate the robustness and reliability of the assay across various plates. The average z' factor for the HiBiT(10uM) condition was observed to be 0.554, indicating a moderate level of assay stability, which, while satisfactory, leaves room for improvement in terms of assay quality. In contrast, the HiBiT(1uM) condition demonstrated a lower average z' factor of 0.338, reflecting a diminished assay reliability and increased variability, thus signaling potential issues in assay sensitivity or specificity at this concentration. Remarkably, the ATP-Glo conditions (both at 10uM and 1uM concentrations) exhibited superior assay stability, with average z' factors of approximately 0.695. This suggests a high degree of consistency and a robust distinction between positive and negative controls, thereby underscoring the reliability and precision of these conditions in our screening protocol.

Fig. 1 shows the normalized HiBiT signal representing abundance of TMPRSS2 expression compared to control under treatment of compounds at 10 μM across three different compounds libraries, with adjustment according to the ATP-Glo signal reflecting the cell count of each observed well.

In our high-throughput screening study, we employed a meticulous and data-driven approach to identify potential hits from our comprehensive compound library of diversified building block compound. Utilizing a bespoke algorithm encoded in our screening software, we applied a multi-parametric selection criterion to each compound based

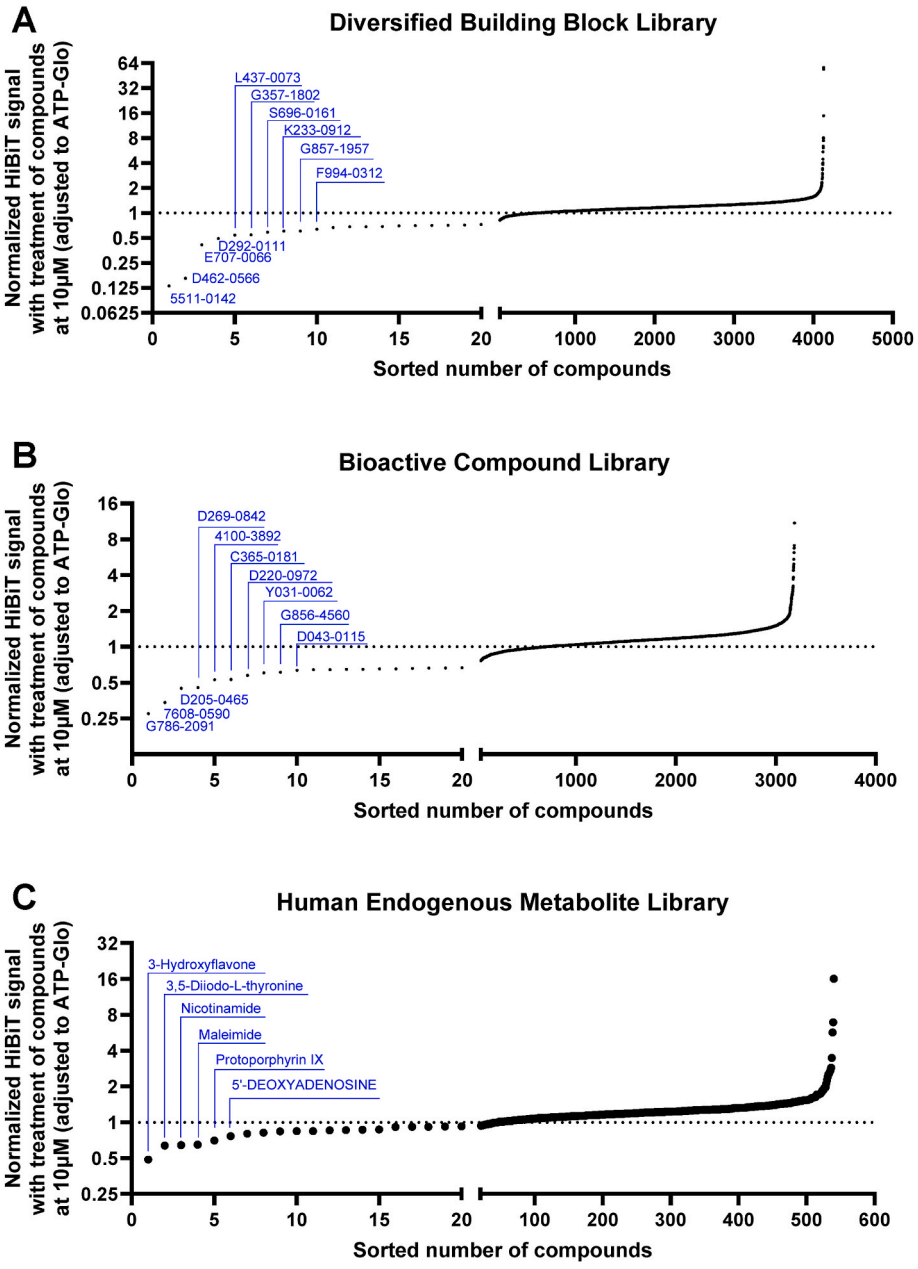

Fig. 1. Normalized TMPRSS2-HiBiT signal under treatment of compounds from (A) diversified building block library, (B) bioactive compound library, or (C) human endogenous metabolite library, at concentration of 10 μM and adjusted to corresponding ATP-Glo signal.

on its performance across various assays. Specifically, our criteria were centered on normalized and adjusted HiBiT signals at different drug concentrations, which were corrected for cell viability using ATP-Glo signals. The algorithm was configured to flag a compound as a 'hit' if it satisfied any of the following conditions: a normalized HiBiT signal at a particular concentration falling below 0.76, a combined threshold where either of the adjusted HiBiT signals was less than 0.76 and the normalized HiBiT signal was less than 0.86, or a scenario where another signal metric was below 0.76 and the normalized HiBiT signal was under 1. These stringent yet comprehensive criteria ensured that only compounds showing significant modulation of the HiBiT signal, indicative of potential biological activity, were selected. Through this rigorous screening process, we successfully identified 37 distinct hits from our extensive library, each representing a promising candidate for further pharmacological investigation and drug development.

In our high-throughput screening of a bioactive compound library for TMPRSS2 modulators, we identified a varied set of potential targets, each embodying unique biological roles. Key targets included Inositol Monophosphatase 1, involved in the phosphatidylinositol signaling pathway; Beta-lactamase AmpC, linked to antibiotic resistance; Thyroid Hormone Receptor Beta-1, a nuclear receptor influencing gene expression; and Prelamin-A/C, associated with nuclear structure and function. These targets, spanning a range of classes from enzymes to transcription factors, suggest a multifaceted approach in the modulation of TMPRSS2.

In the TMPRSS2 reducer screening from the human endogenous metabolite library, key pathways were identified that potentially influence TMPRSS2 modulation. Notably, the thyroid hormone regulation pathway emerged as a significant player, with Liothyronine acting on TRα and TRβ receptors, suggesting its role in TMPRSS2 activity. The metabolism of bile acids also showed a possible connection to TMPRSS2, highlighted by Lithocholic Acid's targeting of FXR and PXR receptors. Additionally, purine metabolism pathways were implicated in the modulation of TMPRSS2, as evidenced by 8-Azaguanine's targeting of purine nucleoside phosphorylase.

2.2. IC50 determination of hits from screening results

The secondary phase of drug screening involved determining the IC50 values for promising compounds identified in the initial screening. This phase commenced with the preparation of a concentration gradient for each drug through serial dilutions in a 1:3 ratio, yielding concentrations ranging from 30 to 0 μM. Human cell lines expressing TMPRSS2 were uniformly seeded in 384-well plates and treated overnight with these drug dilutions, ensuring consistent exposure while maintaining cellular viability. The assessment of TMPRSS2 expression level was conducted using HiBiT lytic reagents, followed by measuring bioluminescence intensity, leveraging the sensitivity of NanoLuciferase technology. This bioluminescence served as a direct indicator of TMPRSS2 expression level under various drug concentrations, facilitating the precise calculation of IC50 values for each compound.

The IC50 determination for TMPRSS2 reducers across three distinct libraries has elucidated a spectrum of inhibitory potentials, with certain compounds emerging as notably potent. Within the diversified skeleton library, the compound D462-0566 stood out with an IC50 of 0.2301 μM, followed closely by 5511–0142 with an IC50 of 0.3696 μM, and E707-0066 exhibiting an IC50 of 7.393 μM, indicating significant inhibitory activity. The known bioactive library presented G786-2091 with an IC50 of 1.982 μM, 7608–0590 with an IC50 of 2.504 μM, and 4903–2167 with an IC50 of 3.461 μM, reflecting a collection of pharmacologically active compounds with targeted TMPRSS2 inhibition. Lastly, the human endogenous metabolite library revealed Nicotinamide as a prominent inhibitor with an IC50 of 0.9926 μM, 3-Hydroxyflavone with 2.333 μM, and 5′-Deoxyadenosine with 20.12 μM, underscoring the therapeutic potential inherent in endogenous substances.

The results indicated that the compounds reducing TMPRSS2 expression levels did not significantly impact cell viability, as reflected by the ATP-Glo data. This suggests that the identified hits are effective in reducing TMPRSS2 expression without causing substantial cell death.

See Table 1 for the top hits from the three compound libraries we performed the TMPRSS2 reducer screening.

2.3. Western blot validation of hits

During the validation phase of our screening process for TMPRSS2 expression inhibitors, we encountered challenges with the specificity and sensitivity of available TMPRSS2 antibodies, resulting in a prevalence of non-specific bands in Western blot assays and suboptimal performance in high-content cell imaging via immunofluorescence. These issues led to inconsistencies in the Western blot results, which lacked the desired level of replicability. Despite these technical hurdles, the reproducibility of the screening results for certain hits suggests their genuine potential as TMPRSS2 expression modulators. Given the promising nature of these findings and the time-sensitive objectives of the project, we have proceeded to publish our data, advocating further investigation into the mechanisms of action of these putative hits.

In a parallel screening endeavor, we utilized a second cell line stably expressing a different protein of interest, tagged with HiBiT for bioluminescence-based detection, and treated it with the same diversified skeleton drug library. A comparative analysis between this dataset and our TMPRSS2-focused study allowed us to identify and eliminate non-selective agents that likely act as general modulators of bioluminescence rather than specific inhibitors of protein expression. This stringent cross-validation excluded compounds with non-specific activity, thereby refining our selection of candidate molecules. Subsequent Western blot analyses corroborated the specificity of two compounds, F994–0312 and V011-3681 (Fig. 2), which exhibited a more pronounced dose-dependent diminution of TMPRSS2 protein levels. These findings warrant a deeper mechanistic investigation into their mode of action, potentially contributing to the development of selective TMPRSS2 modulatory therapies.

3. Discussion

The high-throughput screening data we've gathered points to several common targets among the identified bioactive compounds, offering new perspectives in the search for anti-coronavirus agents. A key focus is on enzymes like Inositol Monophosphatase 1, Beta-lactamase AmpC,

Table 1
Top hits of diversified building block compound library, bioactive library and endogenous metabolite library.

Diversified building block compound Library		Bioactive Compound Library		Endogenous Metabolite Compound Library	
	IC50		**IC50**		**IC50**
D462-0566	0.2301	G786-2091	1.982	Nicotinamide	0.993
5511–0142	0.3696	7608–0590	2.504	3-Hydroxyflavone	2.333
E707-0066	7.393	4903–2167	3.461	5′-DEOXYADENOSINE	20.12
D292-0111	10.66	Y031-0062	4.87	8-Azaguanine	30.67
C053-0444	11.68	G856-4560	5.557	Protoporphyrin IX	31.24
L437-0073	11.69	D205-0465	6.425	Methylcobalamin	101.1
F994-0312	12.96	D220-0972	7.539	Maleimide	404.9
K233-0912	13.53	C365-0181	8.018	4-Hydroxy-3-methoxymandelic acid	415.2
L537-1077	15.36	K405-3422	9.613		
3329–3776	17.75	6284–0045	10.31		
V011-3681	17.91	D269-0842	11.15		
S607-0198	22.34	Y040-6798	11.54		
G857-1957	26.28	C660-0630	12.01		
4088–0109	27.71	G751-2945	13.67		
V015-7168	28.28	6466–1109	14.43		
G868-0980	28.54	D043-0183	16.57		
G747-0308	29.34	7039–5403	17.36		
V024-9580	34.62	G020-0030	17.82		
D430-1152	35.57	D341-1144	18.55		
G006-0026	36.68	D301-0252	20.77		

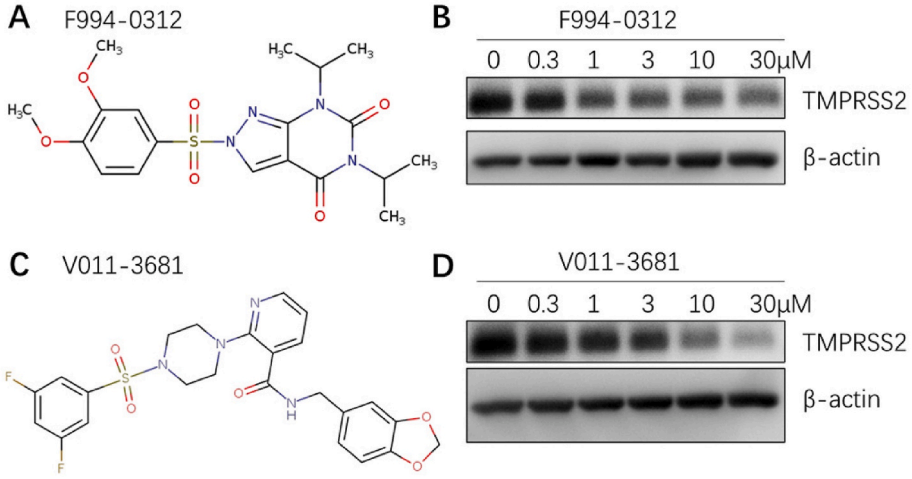

Fig. 2. Structure and Western blot analysis of hit compounds F994–0312 and V011–3681. (A) Structure of F994-0312. (B) Dose course effect of F994-0312 on the endogenous TMPRSS2 expression level. (C) Structure of V011-3681. (D) Dose course effect of V011-3681 on the endogenous TMPRSS2 expression level.

and 6-phospho-1-fructokinase, as well as on unclassified proteins like Prelamin-A/C and Nuclear factor erythroid 2-related factor 2 (Nrf2). These targets are implicated in diverse cellular processes, from metabolic regulation to stress response, highlighting their potential roles in viral pathogenesis or host-virus interactions. Several of the identified hits are known ligands or inhibitors of cellular proteins, which provides insights into their potential mechanisms of action regarding TMPRSS2 modulation. For instance, Inositol Monophosphatase 1, targeted by compounds like in ID number 4903–2167, plays a role in phosphoinositide signaling, which is crucial for various cellular functions including membrane trafficking. This pathway could be significant in the context of viral entry and replication. Beta-lactamase AmpC, a recurring target in our dataset, is traditionally linked with antimicrobial resistance, but its consistent emergence in our screening suggests a potential, yet unexplored, relevance in viral infection mechanisms. Furthermore, 6-phospho-1-fructokinase, identified by compound C365-0181, is a key enzyme in fructose metabolism, indicating a possible link between carbohydrate metabolism and virus-host dynamics. The targeting of Prelamin-A/C and Nrf2, as seen in compounds G751-2945 and Y031-0062 respectively, opens avenues into understanding how alterations in nuclear architecture and oxidative stress responses may influence viral infection processes. These common targets, highlighted by our screening, suggest novel pathways and mechanisms that could be exploited in the development of antiviral strategies. Future research will aim to validate these targets experimentally, delve into their specific roles in coronavirus pathogenesis, and explore how modulation of these pathways could lead to effective therapeutic interventions.

NRF2, known for its role in regulating cellular redox balance and immune responses, emerges as a critical factor in mitigating the severity of viral infections. In the case of COVID-19, characterized by intense inflammatory responses and oxidative stress, NRF2's role becomes particularly significant [15,16]. It has been observed that activation of NRF2 can lead to the downregulation of ACE2 and TMPRSS2 mRNA expression, the latter being crucial for SARS-CoV-2 entry into host cells. This suggests that NRF2 activation could potentially reduce the susceptibility of cells to the virus by diminishing the expression of TMPRSS2, thereby hindering the virus's entry mechanism. Furthermore, NRF2's anti-inflammatory actions, including the suppression of cytokines involved in the COVID-19 "cytokine storm", align with the need for interventions that can moderate the severe immune reactions seen in advanced stages of the disease. DCAF1, an E3 ubiquitin ligase, is recognized as a regulator of protein stability and has been linked to both NRF2 and TMPRSS2 [17,18]. It plays a role in the proteasomal degradation of NRF2, thus impacting the cellular antioxidative and anti-inflammatory response. Intriguingly, DCAF1 is also implicated in the regulation of TMPRSS2 stability. Compounds that target DCAF1, such as halofuginone, have been shown to reduce TMPRSS2 protein levels, potentially through a pathway that involves proteasomal-mediated degradation. This dual influence of DCAF1 on both NRF2 and TMPRSS2 positions it as a critical nexus in the interplay between these two proteins. By stabilizing NRF2, which in turn could downregulate TMPRSS2, and directly influencing TMPRSS2 stability via its regulatory role, DCAF1 emerges as a promising therapeutic target. Targeting DCAF1 might not only enhance the NRF2-mediated antioxidant and anti-inflammatory responses, crucial in mitigating the severe symptoms of COVID-19, but also reduce TMPRSS2 levels, thus potentially blocking the entry of the virus into host cells. This complex relationship between NRF2, TMPRSS2, and DCAF1 underscores a multifaceted approach to COVID-19 therapy, where modulation of these interconnected pathways could provide a more comprehensive strategy in combating the disease.

The compounds 3-Hydroxyflavone, 5′-Deoxyadenosine, 8-Azaguanine, Protoporphyrin IX, and Nicotinamide each play roles in biological processes that might intersect with TMPRSS2. 3-Hydroxyflavone, as a flavonol, could influence TMPRSS2's functionality through its impact on cellular membranes or proteins. According to Istifli et al., 3-Hydroxyflavone, a fundamental flavonol, demonstrates a significant interaction with TMPRSS2 [19]. This interaction is characterized by van der Waals contacts and classical hydrogen bonds, indicating a strong affinity between 3-Hydroxyflavone and key active amino acid residues of TMPRSS2, particularly His296, Ser441, and Asp345. The study highlights 3-Hydroxyflavone's notable binding free energy with TMPRSS2, suggesting its potential as an inhibitory agent against the protease. These findings open avenues for considering 3-Hydroxyflavone and its derivatives as possible therapeutic agents in managing COVID-19 by targeting and potentially reducing TMPRSS2 expression levels.

5′-Deoxyadenosine and 8-Azaguanine, by affecting nucleoside metabolism and DNA repair, might indirectly modulate cellular pathways relevant to TMPRSS2 expression or activity. Protoporphyrin IX, involved in heme biosynthesis, and Nicotinamide, a component of NAD, a coenzyme in redox reactions, could influence TMPRSS2 either through changes in redox balance or by affecting cellular energy and health states. These interactions, although not direct, highlight the complexity of metabolic and biochemical networks in which TMPRSS2 is embedded, potentially offering novel insights for therapeutic strategies against COVID-19.

The screening of a diverse scaffold library has yielded promising results, with compounds such as F994–0312 and V011-3681 displaying

notable potency. However, it is important to note that Western blot validation did not yield satisfactory outcomes for some other compounds. It is our belief that the shortcomings in these validations are primarily attributed to issues related to the antibodies utilized for detection. To address these challenges comprehensively, we intend to undertake a meticulous optimization process encompassing various sample preparation conditions. This optimization will encompass factors such as sonication, denaturation, buffer composition, and other critical parameters. Additionally, the selection of specific detection antibodies will be a pivotal aspect of this optimization process. Once these hit compounds are confirmed to effectively reduce endogenous TMPRSS2 expression, we will proceed to the next phase of validation. This subsequent validation will employ a range of assays designed from diverse perspectives, including pseudovirus entry assays, high content screening, and others. The goal is to comprehensively assess the functional attributes of these compounds. Subsequently, refined compounds will be generated based on the outcomes of these assays. These refined compounds will then undergo further evaluation through in vivo validation, exploration of structure-activity relationships, and, ultimately, assessment of their antiviral activity. It is important to acknowledge that due to constraints related to time and funding within the scope of this project, we were unable to conduct all the proposed assays. Nevertheless, we maintain the conviction that the high throughput screening strategy, employing the TMPRSS-HiBiT stable cell line, holds significant promise in identifying novel reagents with the potential to exhibit robust anti-coronavirus activity. This approach represents a valuable contribution to the ongoing efforts to develop effective anti-coronavirus agents.

CRediT authorship contribution statement

Yanwen Chen: Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Yunqi Li:** Validation, Methodology, Investigation. **Ye Zhao:** Validation, Investigation. **Lei Pei:** Validation. **Ling Zhang:** Supervision, Project administration. **Duowu Zou:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have already uploaded full data as supplementary files.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (grant numbers 82061138003 and 82100543).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2024.101783.

References

[1] J.H. Ellwanger, J.A.B. Chies, Zoonotic spillover: understanding basic aspects for better prevention, Genet. Mol. Biol. 44 (2021) e20200355, https://doi.org/10.1590/1678-4685-GMB-2020-0355.

[2] D. Singh, S.V. Yi, On the origin and evolution of SARS-CoV-2, Exp. Mol. Med. 53 (2021) 537–547, https://doi.org/10.1038/s12276-021-00604-z.

[3] S. Lei, X. Chen, J. Wu, X. Duan, K. Men, Small molecules in the treatment of COVID-19, Signal Transduct. Targeted Ther. 7 (2022) 387, https://doi.org/10.1038/s41392-022-01249-8.

[4] F.D. Makurvet, Biologics vs. small molecules: drug costs and patient access, Medicine in Drug Discovery 9 (2021) 100075, https://doi.org/10.1016/j.medidd.2020.100075.

[5] Y.-W. Zhou, Y. Xie, L.-S. Tang, D. Pu, Y.-J. Zhu, J.-Y. Liu, X.-L. Ma, Therapeutic targets and interventional strategies in COVID-19: mechanisms and clinical studies, Signal Transduct. Targeted Ther. 6 (2021) 317, https://doi.org/10.1038/s41392-021-00733-x.

[6] M. Hoffmann, H. Kleine-Weber, S. Schroeder, N. Krüger, T. Herrler, S. Erichsen, T. S. Schiergens, G. Herrler, N.-H. Wu, A. Nitsche, M.A. Müller, C. Drosten, S. Pöhlmann, SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor, Cell 181 (2020) 271–280.e8, https://doi.org/10.1016/j.cell.2020.02.052.

[7] B.J. Fraser, S. Beldar, A. Seitova, A. Hutchinson, D. Mannar, Y. Li, D. Kwon, R. Tan, R.P. Wilson, K. Leopold, S. Subramaniam, L. Halabelian, C.H. Arrowsmith, F. Bénard, Structure and activity of human TMPRSS2 protease implicated in SARS-CoV-2 activation, Nat. Chem. Biol. 18 (2022) 963–971, https://doi.org/10.1038/s41589-022-01059-7.

[8] K.D. Sonawane, S.S. Barale, M.J. Dhanavade, S.R. Waghmare, N.H. Nadaf, S. A. Kamble, A.A. Mohammed, A.M. Makandar, P.M. Fandilolu, A.S. Dound, N. M. Naik, V.B. More, Structural insights and inhibition mechanism of TMPRSS2 by experimentally known inhibitors Camostat mesylate, Nafamostat and Bromhexine hydrochloride to control SARS-coronavirus-2: a molecular modeling approach, Inform. Med. Unlocked 24 (2021) 100597, https://doi.org/10.1016/j.imu.2021.100597.

[9] M. Hoffmann, S. Schroeder, H. Kleine-Weber, M.A. Müller, C. Drosten, S. Pöhlmann, Nafamostat mesylate blocks activation of SARS-CoV-2: new treatment option for COVID-19, Antimicrob. Agents Chemother. 64 (2020), https://doi.org/10.1128/AAC.00754-20.

[10] M.P. Hernández-Mitre, S.Y.C. Tong, J.T. Denholm, G.J. Dore, A.C. Bowen, S. R. Lewin, B. Venkatesh, T.E. Hills, Z. McQuilten, D.L. Paterson, S.C. Morpeth, J. A. Roberts, Nafamostat mesylate for treatment of COVID-19 in hospitalised patients: a structured, narrative review, Clin. Pharmacokinet. 61 (2022) 1331–1343, https://doi.org/10.1007/s40262-022-01170-x.

[11] A. Sauvat, F. Ciccosanti, F. Colavita, M. Di Rienzo, C. Castilletti, M.R. Capobianchi, O. Kepp, L. Zitvogel, G.M. Fimia, M. Piacentini, G. Kroemer, On-target versus off-target effects of drugs inhibiting the replication of SARS-CoV-2, Cell Death Dis. 11 (2020) 656, https://doi.org/10.1038/s41419-020-02842-x.

[12] T. Shapira, I.A. Monreal, S.P. Dion, D.W. Buchholz, B. Imbiakha, A.D. Olmstead, M. Jager, A. Désilets, G. Gao, M. Martins, T. Vandal, C.A.H. Thompson, A. Chin, W. D. Rees, T. Steiner, I.R. Nabi, E. Marsault, J. Sahler, D.G. Diel, G.R. Van de Walle, A. August, G.R. Whittaker, P.-L. Boudreault, R. Leduc, H.C. Aguilar, F. Jean, A TMPRSS2 inhibitor acts as a pan-SARS-CoV-2 prophylactic and therapeutic, Nature 605 (2022) 340–348, https://doi.org/10.1038/s41586-022-04661-w.

[13] A.S. Dixon, M.K. Schwinn, M.P. Hall, K. Zimmerman, P. Otto, T.H. Lubben, B. L. Butler, B.F. Binkowski, T. Machleidt, T.A. Kirkland, M.G. Wood, C.T. Eggers, L. P. Encell, K.V. Wood, Nanoluc complementation reporter optimized for accurate measurement of protein interactions in cells, ACS Chem. Biol. 11 (2016) 400–408, https://doi.org/10.1021/acschembio.5b00753.

[14] R. Cooley, N. Kara, N.S. Hui, J. Tart, C. Roustan, R. George, D.C. Hancock, B. F. Binkowski, K.V. Wood, M. Ismail, J. Downward, Development of a cell-free split-luciferase biochemical assay as a tool for screening for inhibitors of challenging protein-protein interaction targets, Wellcome Open Res 5 (2020) 20, https://doi.org/10.12688/wellcomeopenres.15675.1 [version 1; peer review: 1 approved with reservations].

[15] J.M. McCord, B.M. Hybertson, A. Cota-Gomez, K.P. Geraci, B. Gao, Nrf2 activator PB125® as a potential therapeutic agent against COVID-19, Antioxidants 9 (2020), https://doi.org/10.3390/antiox9060518.

[16] H. Khan, S. Patel, A. Majumdar, Role of NRF2 and sirtuin activators in COVID-19, Clin. Immunol. 233 (2021) 108879, https://doi.org/10.1016/j.clim.2021.108879.

[17] Y. Chen, T.B. Lear, J.W. Evankovich, M.B. Larsen, B. Lin, I. Alfaras, J.R. Kennerdell, L. Salminen, D.P. Camarco, K.C. Lockwood, F. Tuncer, J. Liu, M.M. Myerburg, J. F. McDyer, Y. Liu, T. Finkel, B.B. Chen, A high-throughput screen for TMPRSS2 expression identifies FDA-approved compounds that can limit SARS-CoV-2 entry, Nat. Commun. 12 (2021) 3907, https://doi.org/10.1038/s41467-021-24156-y.

[18] Y. Chen, J.W. Evankovich, T.B. Lear, F. Tuncer, J.R. Kennerdell, D.P. Camarco, M. S. Shishido, Y. Liu, B.B. Chen, A small molecule NRF2 activator BC-1901S ameliorates inflammation through DCAF1/NRF2 axis, Redox Biol. 32 (2020) 101485, https://doi.org/10.1016/j.redox.2020.101485.

[19] E.S. Istifli, A. Şihoğlu Tepe, P.A. Netz, C. Sarikürkcü, İ.H. Kiliç, B. Tepe, Determination of the interaction between the receptor binding domain of 2019-nCoV spike protein, TMPRSS2, cathepsin B and cathepsin L, and glycosidic and aglycon forms of some flavonols, Turk. J. Biol. 45 (2021) 484–502, https://doi.org/10.3906/biy-2104-51.