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Identification of Aloe-derived natural products as prospective lead scaffolds for SARS-CoV-2 main protease (M\textsuperscript{pro}) inhibitors

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A B S T R A C T

In the past two years, the COVID-19 pandemic has caused over 5 million deaths and 250 million infections worldwide. Despite successful vaccination efforts and emergency approval of small molecule therapies, a diverse range of antivirals is still needed to combat the inevitable resistance that will arise from new SARS-CoV-2 variants. The main protease of SARS-CoV-2 (M\textsuperscript{pro}) is an attractive drug target due to the clinical success of protease inhibitors against other viruses, such as HIV and HCV. However, in order to combat resistance, various chemical scaffolds need to be identified that have the potential to be developed into potent inhibitors. To this end, we screened a high-content protease inhibitor library against M\textsuperscript{pro} in vitro, in order to identify structurally diverse compounds that could be further developed into antiviral leads. Our high-content screening efforts retrieved 27 hits each with > 50% inhibition in our M\textsuperscript{pro} FRET assay. Of these, four of the top inhibitor compounds were chosen for follow-up due to their potency and drugability (Lipinski's rules of five criteria): anacardic acid, aloesin, aloeresin D, and TCID. Further analysis via dose response curves revealed IC\textsubscript{50} values of 6.8 \mu M, 38.9 \mu M, 125.3 \mu M, and 138.0 \mu M for each compound, respectively. Molecular docking studies demonstrated that the four inhibitors bound at the catalytic active site of M\textsuperscript{pro} with varying binding energies (-7.5 to –5.6 kcal/mol). Furthermore, M\textsuperscript{pro} FRET assay kinetic studies demonstrated that M\textsuperscript{pro} catalysis is better represented by a sigmoidal Hill model than the standard Michaelis-Menten hyperbola, indicating substantial cooperativity of the active enzyme dimer. This result suggests that the dimerization interface could be an attractive target for allosteric inhibitors. In conclusion, we identified two closely-related natural product compounds from the Aloe plant (aloesin and aloeresin D) that may serve as novel scaffolds for M\textsuperscript{pro} inhibitor design and additionally confirmed the strongly cooperative kinetics of M\textsuperscript{pro} proteolysis. These results further advance our knowledge of structure-function relationships in M\textsuperscript{pro} and offer new molecular scaffolds for inhibitor design.

The global scourge of the COVID-19 pandemic has enveloped the world for two years, leaving over 5 million dead and 250 million infected in its wake.\textsuperscript{1-3} Despite the emerging success of vaccines, a significant number of people remain unvaccinated, either due to lack of availability or vaccine hesitancy.\textsuperscript{4,5} Furthermore, “breakthrough” infections are becoming more common in the vaccinated with the identification of new variants,\textsuperscript{6-7} leaving few therapeutic options available for life threatening infections.\textsuperscript{8-9} This illustrates the need to develop novel small molecule chemical modalities that can be used both to treat severe SARS-CoV-2 infections and as potential prophylactic agents in high-risk population groups. While the earliest small molecule therapies targeting SARS-CoV-2 directed their activities towards the RNA polymerase target (e.g., remdesivir and molnupiravir), recent attention has turned to the 3C-like viral main protease (3CL\textsuperscript{pro} or M\textsuperscript{pro}) as a possible drug target.\textsuperscript{10-17} Viral protease inhibitors have previously demonstrated high clinical efficacy against other viruses, such as HIV (human immunodeficiency virus)\textsuperscript{18-19} and HCV (hepatitis C virus),\textsuperscript{20-22} making M\textsuperscript{pro} an attractive target for antiviral drug development. Indeed, Pfizer, Inc. has a first-in-class M\textsuperscript{pro} inhibitor currently in Phase III clinical trials,\textsuperscript{23} pointing to M\textsuperscript{pro} inhibition as an effective therapeutic strategy. Despite this, a major liability of viral protease inhibitors is the eventual development of resistance, which necessitates their use as part of combination therapy and also the development of second and third generation compounds. This highlights the need for the identification of new lead structures that may function as M\textsuperscript{pro} inhibitor scaffolds.\textsuperscript{12,14-15} Since the publication of the SARS-CoV-2 M\textsuperscript{pro} crystal structure,\textsuperscript{23} a number of campaigns have been conducted to identify selective M\textsuperscript{pro} inhibitors\textsuperscript{11,12,14,16,17-29} While most of these published accounts have
exclusively involved computational approaches, several have screened existing small molecule libraries for M\(^{\text{pro}}\) inhibition in vitro. However, these approaches have led to a number of redundant hits with similar mechanisms of action. In order to forestall eventual viral resistance, chemical diversity is an important consideration in identification of early protease inhibitor leads. Even small chemical libraries that are highly diverse can lead to the identification of inhibitors with unique mechanisms of action.

In an attempt to expand the chemical diversity of potential scaffolds for SARS-CoV-2 M\(^{\text{pro}}\) inhibitor lead design, our approach focused on a high-content protease inhibitor library to filter out redundant molecules and obtain unique pharmacophores that could be further exploited by chemical optimization. Here, we report the identification of novel Aloe-derived natural product scaffolds that have the potential to be further optimized into effective clinical candidates, given their high drug-like qualities (Lipinski’s rule of five and safety profiles). Additionally, our data confirm that SARS-CoV-2 M\(^{\text{pro}}\) functions as a cooperative dimer, hinting at the possibility of developing allosteric inhibitors that may target and disrupt the enzyme dimer interface.

In order to screen library compounds for inhibitory activity against M\(^{\text{pro}}\) in an in vitro system, we cloned, expressed, and purified the M\(^{\text{pro}}\) viral protein in an E. coli based heterologous expression system. To facilitate production of the native protease, we retained the original viral N-terminal autocatalytic cleavage site (SAVLQ↓SGFRK) and modified the C-terminal sequence with the core amino acids of the HRV (human rhinovirus)-3C protease cleavage site (VTFQ↓GP) to permit removal of the His tag needed for the protein purification (see supporting information, Figure S1). This construct resulted in high yields of the native protein (see supporting information, Figure S2). This construct resulted in high yields of the native protein (see supporting information, Figure S2). This construct resulted in high yields of the native protein (see supporting information, Figure S2). This construct resulted in high yields of the native protein (see supporting information, Figure S2). This construct resulted in high yields of the native protein (see supporting information, Figure S2). This construct resulted in high yields of the native protein (see supporting information, Figure S2).

The resulting kinetic parameters for the Michaelis-Menten and allosteric sigmoidal (Hill) models. B: Eadie-Hofstee transformation of Hill equation at h = 1 (no cooperativity) and h = 1.7 (positive cooperativity). The kinetic assay was performed at 2–128 \(\mu\)M FRET substrate and 100 nM M\(^{\text{pro}}\). Initial velocities in RFU converted to pmol/s with the free Edans calibration curve. Points represent mean of triplicate measurements ± standard deviation.

Fig. 1. Kinetic analysis of FRET substrate proteolysis by M\(^{\text{pro}}\). A: Kinetic data fit to Michaelis-Menten and allosteric sigmoidal (Hill) models. B: Eadie-Hofstee transformation of Hill equation at h = 1 (no cooperativity) and h = 1.7 (positive cooperativity). The kinetic assay was performed at 2–128 \(\mu\)M FRET substrate and 100 nM M\(^{\text{pro}}\). Initial velocities in RFU converted to pmol/s with the free Edans calibration curve. Points represent mean of triplicate measurements ± standard deviation.
| Compound                    | Structure | Inhibition (%) | Compound          | Structure          | Inhibition (%) | Compound                     | Structure | Inhibition (%) |
|-----------------------------|-----------|----------------|-------------------|--------------------|----------------|-------------------------------|-----------|----------------|
| Z-DEVD-FMK                  | ![Z-DEVD-FMK](image1) | 100.0 ± 0.7    | Z-FA-FMK          | ![Z-FA-FMK](image2) | 90.1 ± 0.8      | Leupeptin hemisulfate       | ![Leupeptin hemisulfate](image3) | 59.0 ± 1.8     |
| Calpain Inhibitor II        | ![Calpain Inhibitor II](image4) | 99.8 ± 0.7     | Telaprevir        | ![Telaprevir](image5) | 88.8 ± 0.5      | Celasrol                     | ![Celasrol](image6) | 57.7 ± 1.3     |
| MG-132                      | ![MG-132](image7) | 99.0 ± 1.7     | Aloesin * (aloeresin B) | ![Aloesin B](image8) | 80.1 ± 2.9      | Nelfinavir mesylate         | ![Nelfinavir mesylate](image9) | 56.1 ± 2.2     |
| Calpain Inhibitor XII       | ![Calpain Inhibitor XII](image10) | 98.3 ± 0.3     | Aloeresin D *     | ![Aloeresin D](image11) | 72.6 ± 1.1      | IU1                          | ![IU1](image12) | 54.8 ± 0.1     |
| Boceprevir                  | ![Boceprevir](image13) | 97.4 ± 0.2     | PD 151,746        | ![PD 151,746](image14) | 71.5 ± 1.4      | Tanshinone IIA *             | ![Tanshinone IIA](image15) | 53.9 ± 3.3     |
| Calpeptin                   | ![Calpeptin](image16) | 97.2 ± 0.1     | Mocetinostat *    | ![Mocetinostat](image17) | 66.7 ± 2.1      | Dibenzazepine                | ![Dibenzazepine](image18) | 53.8 ± 2.0     |
| Anacardic acid              | ![Anacardic acid](image19) | 96.1 ± 0.1     | L-685,458         | ![L-685,458](image20) | 65.0 ± 7.1      | Glecaprevir *                | ![Glecaprevir](image21) | 51.7 ± 4.9     |
| Calpain Inhibitor I         | ![Calpain Inhibitor I (MG-101)](image22) | 95.2 ± 0.9     | LDN-57444         | ![LDN-57444](image23) | 61.4 ± 1.0      | MC1568                       | ![MC1568](image24) | 51.4 ± 1.3     |
| TCID *                      | ![TCID](image25) | 94.2 ± 2.5     | 7-O-Methyl-aloeresin A | ![7-O-Methyl-aloeresin A](image26) | 60.5 ± 1.7      | Asunaprevir *                | ![Asunaprevir](image27) | 49.4 ± 4.2     |

Percent inhibition values represent mean of three replicates ± standard deviation. * Compound with background fluorescence.
S/Nmol $M_{\text{PRO}}$, and $S_0 = 24.2 \pm 2.0 \mu M$ and $V_{\text{max}} = 269.6 \pm 11.4 \text{ pmol/}
S/Nmol $M_{\text{PRO}}$ for the allosteric sigmoidal fit (see supporting information, Table S1).

Interestingly, the data best fit to the sigmoidal Hill plot with an $R^2$ of 0.985 and an AICc correct fit probability of 99.98%. This is dramatically different when the data is analyzed by the Eadie-Hofstee transformation (Fig. 1B). The presence of cooperativity in enzyme activity is readily explainable and somewhat expected as the enzyme is reported to be only active as a dimer.$^{48-50}$ However, while allosteric cooperativity has been demonstrated previously for this enzyme, this is the first time that kinetic data have been quantitatively compared to determine the best model for enzyme activity, i.e. either the Michaelis-Menten or the sigmoidal Hill equations (Fig. 1A, Table S1). The clear appearance of allometric kinetics, as represented in the Eadie-Hofstee plot (Fig. 1B), indicates that the allosteric dimer interface may also be a promising inhibitor target, as was found to be the case for the SARS-CoV-1 M$^{\text{PRO}}$ enzyme.$^{50}$

For our high-content library screening, a total of 236 compounds were obtained from different vendors: Protease Inhibitor Library (catalog no. L2500) and PF-00835231 from Selleck Chemicals (Houston, TX); ebselen from Sigma-Aldrich (St. Louis, MO); aloin B, alo-emodin, calpain inhibitor II, calpain inhibitor III, calpain inhibitor VI, calpain inhibitor XII, 2-cyano-pyrimidine, E-64d, oeselamivir (phosphate), PD 150606, PSI-7977, tosyllysine chloromethyl ketone HCl, and pimodivir from Cayman Chemical (Ann Arbor, MI); aloeresin D from eNovation Chemicals (Green Brook, NJ); and 7-O-methylaloeresin A from Muse Chem (Fairfield, NJ). The inhibition screening assays were performed at 50 nM $M_{\text{PRO}}$, 10 μM FRET substrate, and 100 μM inhibitor (1.5% final volume DMSO). 100 μM inhibitor concentration was selected as our goal was not to identify highly potent compounds, but rather chemically diverse leads. The Pfizer $M_{\text{PRO}}$ inhibitor, PF-00835231, was used as a positive control for inhibition at a concentration of 1 μM. The reactions were initialized at room temperature by the addition of 50 μL FRET substrate and inhibitor mixture, dissolved in reaction buffer, to 50 μL of $M_{\text{PRO}}$ also prepared in reaction buffer. The reaction buffer consisted of 20 mM Tris HCl, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT, pH 7.3 at room temperature. Fluorescence was monitored for 1 h, with readings taken every 2.5 min. The first 20 min were used to calculate the initial velocity, and percent inhibition was calculated from the slope compared to the DMSO control. Background from the FRET substrate was subtracted from all samples. All experiments were performed in triplicate and the presented values represent the mean ± standard deviation. Positive “hits” were defined as any compound that inhibited $M_{\text{PRO}}$ activity by 50% or more. As can be seen in Fig. 2, 27 compounds fell into this category, indicating an assay hit rate of approximately 11.4%. The structures and percent inhibition are shown in Table 1, demonstrating the chemical diversity present in the hits obtained from the initial library screening. To assess assay robustness, the $Z'_{-factors}$ were between 0.727 and 0.969, well within the guidelines of 0.5 to 1 for a high-throughput assay.$^{24}$

Inherent limitations of the FRET-based assay led to 19 of the initial library compounds (8.1%) being eliminated from the assay screen due to either intrinsic fluorescence from the compounds themselves or issues stemming from inner filter effects. Some of the compounds we identified have been previously reported as SARS-CoV-2 $M_{\text{PRO}}$ inhibitors (see supporting information, Figure S4). The resulting $Z'_{-factors}$ were between 0.727 and 0.969, well within the guidelines of 0.5 to 1 for a high-throughput assay.$^{24}$

Aloesin and aloeresin D come from the Aloe plant, including Aloe perryi and A. barbadensis (aka, A. vera) species.$^{55-56}$ These compounds have a long history of medicinal use as stimulant-laxatives to treat constipation,$^{57}$ anti-inflammatory compounds to promote wound healing,$^{58}$ and bittering agents in food supplements, thereby demonstrating their positive safety profile and bioavailability in humans. Furthermore, aloesin is a known tyrosinase inhibitor with an $IC_{50}$ of 0.9 mM against mushroom tyrosinase.$^{59-60}$ Anacardic acid is a natural product from the cashew nut that exhibits anti-inflammatory and antinociceptive properties.$^{61}$

Due to the chemical novelty of the natural products identified and the availability of readily accessible functional groups on these compounds for further modification, we decided to...
focus on the top three natural products with high percent inhibition in the 100 μM screen: anacardic acid (96.1% ± 0.1), aloesin (80.1% ± 2.9), and aloeresin D (72.6% ± 1.1).

We also chose to include the non-natural product TCID (a selective ubiquitin C-terminal hydrolase-L3 inhibitor), due to its performance in the high-content screen (94.2% ± 2.5).

In order to quantitatively assess the extent of M<sub>pro</sub> inhibition, we performed IC<sub>50</sub> experiments using the screening assay that we had already developed. The dose response curves in Fig. 3 demonstrate IC<sub>50</sub>’s in the micromolar range. It should be noted that 0% inhibition (100% activity) was not reached in all cases due to assay background fluorescence.

TCID (IC<sub>50</sub> = 138.0 μM ± 12.9), was ruled out as a possible scaffold due to its higher IC<sub>50</sub> value and potential toxicity. While the most potent inhibitory compound was anacardic acid (IC<sub>50</sub> = 6.8 μM ± 1.0), its toxic liabilities may also prevent it from further development as an antiviral agent, whereas the two Aloe compounds (aloesin IC<sub>50</sub> = 38.9 μM ± 8.6, aloeresin D IC<sub>50</sub> = 125.3 μM ± 24.5), have a more favorable safety and efficacy profile in humans. The four hits were also pre-incubated with and without the enzyme in order to assess time-dependent inhibition (TDI) (see supporting information, Figure S5).

While a single inhibitor concentration is not conclusive, the data suggest that the compounds exhibit a moderate amount of TDI. Experiments involving multiple concentrations points will need to be carried out to further delineate the TDI potential of these compounds.

Table S3 (see supporting information) illustrates Lipinski’s rule of five for the four inhibitors: aloesin and TCID meet all four criteria, while anacardic acid and aloeresin D have one and two violations respectively.化合物 meeting at least three of the four of Lipinski’s criteria generally make excellent orally active drugs.

In an effort to expand our knowledge as to the specific mode of action of these compounds, a molecular docking study was undertaken using the AutoDock Vina algorithm, v. 1.1.2. Briefly, the SARS-CoV-2 M<sup>pro</sup> crystal structure (PDB entry 6Y2E) was prepared for docking by removing ions and water molecules and adding polar hydrogens using MGL AutoDock Tools v. 1.5.7 (UCSD Molecular Graphics Lab and The Scripps Research Institute). Inhibitor (PF-00835231, anacardic acid, aloesin, aloeresin D, and TCID) structural coordinates were obtained from the Protein Data Bank (PDB: https://www.rcsb.org/) and parameterized by adding polar hydrogens and identifying rotatable bonds. The receptor docking grid was defined by the following parameters - grid box center: x-center = -16.022, y-center = -32.73, z-center = 4.648, and the total number of grid points in each dimension being: x-dimension = 80, y-dimension = 48, and z-dimension = 62. To facilitate an efficient docking routine, a configuration file docking script was prepared using MS Notepad in simple text format with the energy range set to 4 and the exhaustiveness search parameter set to 24. AutoDock Vina was executed using the configuration file with both PDBQT.out and log.out file options selected. The PDBQT.out file contains all of the ligand binding poses for any particular docking simulation. Output files were analyzed using the ViewDock function of UCSF Chimera v.1.15, and ranked according to binding energy (ΔG). To test the validity of AutoDock Vina to predict the correct binding pose for our selected inhibitors, we first attempted to dock PF-00835231 using the parameters described above. The reported IC<sub>50</sub> of Pfizer’s reference inhibitor is in the low nanomolar...
range (6.9 nM). Here, we found that the lower IC50 value correlated with the lower docking score, both indicating higher affinity for the enzyme. The most energetically stable pose found the compound bound in the active site within close proximity of the catalytic dyad (Cys 145 and His 41) (see supporting information, Figure S6). This docking pose is strikingly similar, although not quite superimposable, with the location of the compound found in PDB entry 6HXM, where it is covalently adducted to Cys 145. The difference in position is likely due to the covalent adduct formed between PF00835231 and Cys 145 during catalysis.

As seen in Fig. 4, all four inhibitors docked within the active site of MPB, near the catalytic dyad of Cys 145 and His 41, although with differing binding energies. Aloesin bound with the lowest predicted free energy, −7.5 kcal/mol, followed by aloeresin D at −6.8 kcal/mol, TCID at −5.8 kcal/mol, and anacardic acid at −5.6 kcal/mol. TCID, the smallest molecule, docked near the Cys 145 at subsite S1 (Fig. 4D). Both aloesin and anacardic acid are bound in extended conformations, occupying the entirety of the active site, including subsites S1, S1’, S2, and S4 (Fig. 4A, B). In contrast, aloeresin D (Fig. 4C) is bound in the active site in a more sterically constrained conformation, with the vinylphenol ring hovering just above the glycosyl moiety, only occupying subsites S1 and S4. This correlates well with the IC50 potencies, where aloesin D was approximately 3-fold less potent than aloesin (Fig. 3), and may indicate that the vinylphenyl group of aloeresin D sterically hinders binding in the active site and in fact decreases the affinity of the ligand for MPB. Removal and/or substitution of this group will not result in producing significant toxic liabilities. Given the limited number of validated chemical scaffolds currently available for MPB inhibitors, identification of these compounds further adds to the medicinal chemist’s toolkit for SARS-CoV-2 antiviral drug design.

In conclusion, here we have identified two natural product compounds, aloesin and aloeresin D, as novel SARS-CoV-2 MPB inhibitors through screening of a high-content protease inhibitor library. Both of these compounds are safe in humans and have functional groups that are readily accessible for modification allowing them to serve as leads for MPB inhibitors. Anacardic acid and TCID performed well in our study but were both ruled out due to potential toxicity. Additionally, we have confirmed that SARS-CoV-2 MPB exhibits cooperative kinetics during catalysis, suggesting that the protein dimer interface may be an attractive target for allosteric inhibitors. Combined, these results advance our knowledge of structure–function relationships in MPB and offer new molecular scaffolds for inhibitor design.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2022.128732.

References

1. (CDC) US CDC. Provisional Counts for Coronavirus Disease 2019 (COVID-19). 2021; https://www.cdc.gov/nchs/nsvr/covid19/index.htm. Accessed 12/05/2021.
2. University JH. Johns Hopkins Coronavirus Resource Center; 2021. https://coronavirus.jhu.edu/. Accessed 12/04/21 2021.
3. Burkert FR, Lansen L, Bellmann-Weiler R, Weiss G. Coronavirus disease 2019: clinical, treatment, and prevention. Front Microbiol. 2021;12:761887.
4. Anjorin AA, Odetokun IA, Abiyeo AI, et al. Will Africans take COVID-19 vaccination? PLoS ONE. 2021;16:e0250575.
5. Sarwar A, Nazar N, Nazar N, Qadir A. Measuring vaccination willingness in response to COVID-19 using a multi-criteria decision-making method. Hum Vaccin Immunother. 2021;1–6.
6. Bergwerk M, Gonen T, Lustig Y, et al. COVID-19 Breakthrough Infections in Vaccinated Health Care Workers. N Engl J Med. 2021;385:1474–1484.
7. Hsu I, Gröne B, Bues M, et al. COVID-19 breakthrough infections and transmission risk: real-world data analyses from Germany’s largest public health department (Cologne). Vaccines (Basel). 2021;9.
8. Islam A, Ahmed A, Naqvi IH, Parveen S. Emergence of severe acute respiratory coronavirus-2 during 2019–2020. Virusdisease. 2020;31:1–9.
9. Gazendam A, Nucci N, Ekhtiari S, et al. Trials and tribulations: so many potential treatments, so few answers. Int Orthop. 2020;44:1467–1471.
10. Gao K, Wang R, Chen J, Tepe JJ, Huang F, Wei GW. Perspectives on SARS-CoV-2 Main Protease Inhibitors. J Med Chem. 2021.
11. Boras B, Jones RM, Anson BJ, et al. Preclinical characterization of an intravenous coronavirus 3CL protease inhibitor for the potential treatment of COVID-19. Nat Commun. 2021;12:6055.
12. Kundra D, Selvaraj C, Singh SK, Dubey VK. Identification of anti-nCoV drug chemical compounds from Indian spices exploiting SARS-CoV-2 main protease as target. J Biomed Struct Dyn. 2021;39:3428–3434.
13. Ma C, Tan H, Choza J, Wang Y, Wang J. Validation and invalidation of SARS-CoV-2 main protease inhibitors using the Flip-GFP and Protease-Glo luciferase assays. Acta Pharm Sin B. 2021.
14. Al-Karmalawy AA, Farid MM, Mostafa A, et al. Naturally available flavonoid aglycones as potential antiviral drug candidates against SARS-CoV-2. Molecules. 2021;26.
15. Ma C, Xia Z, Sacco MD, et al. Discovery of Di- and trihaloacetamides as covalent SARS-CoV-2 main protease inhibitors with high target specificity. J Am Chem Soc. 2021.
16. Toygi M. Potential anti-SARS-CoV-2 drug candidates identified through virtual screening of the CHEMBL database for compounds that target the main coronavirus protease. FEBS Open Bio. 2020;10:995–1004.
17. Ulrich S, Nitsche C. The SARS-CoV-2 main protease as drug target. Bioorg Med Chem Lett. 2020;30, 127377.
18. Weber IT, Wang VF, Harrison RW. HIV Protease: Historical Perspective and Current Research. Viruses. 2021;13.
19. Weichselderflor M, Reitz M, Latiniotic O. Past HIV-1 medications and the current status of combined antiretroviral therapy options for HIV-1 patients. Pharmacutics. 2021;13.
20. Pecoraro V, Banzi R, Cariani E, et al. New direct-acting antivirals for the treatment of patients with hepatitis C virus infection: a systematic review of randomized controlled trials. J Clin Exp Hepatol. 2019;9(2):52, 538.
21. Zappolo E, Scorto R, Buononato AR, Marzolo AE, Pinchera B, Gentile I. Efficacy and safety of a fixed dose combination tablet of asunaprevir + beclabuvir + daclatasvir for the treatment of Hepatitis C. Expert Opin Pharmacother. 2020;21:261–273.
22. Xiao M, Jing X, de Clercq E, Li G. Danoprevir for the treatment of hepatitis C virus infection: design, development, and place in therapy. Int J Antimicrob Agents. 2021;1004.
23. Zhang L, Lin D, Sun X, et al. Crystal structure of SARS-CoV-2 main protease provides insights for design of improved a-ketoamide inhibitors. Science. 2020;368:409–412.
24. Zhang W, Li X, Yu Z, Wu S, Ding L, Liu J. Identification of lactoferrin-derived peptides as potential inhibitors against the main protease of SARS-CoV-2. Lebenm Wiss Technol. 2022;154, 112684.
25. Bernardi M, Ghamsi MR, Bayardet O. Phenylethanol glycosides as a possible COVID-19 protease inhibitor: a virtual screening approach. J Mol Model. 2021;27:341.
26. Arshia AH, Shadravan S, Solhiho A, Shaktteman A, Sam A. De novo design of novel protease inhibitor candidates in the treatment of SARS-Cov-2 using deep learning, docking, and molecular dynamic simulations. Comput Biol Med. 2021;139, 104967.
Costanzi E, Kuzikov M, Esposito F, et al. Structural and biochemical analysis of the dual inhibition of MG-132 against SARS-CoV-2 main protease (Mpro/3CLpro) and human carpehinin-L. Int J Mol Sci. 2021;22.

28 Amin MR, Yasin H, Hosen MA, et al. Synthesis, antimicrobial, anticancer, PASS, molecular docking, molecular dynamic simulations & pharmacokinetic predictions of some methyl β-D-galactopyranoside analogs. Molecules. 2021;26.

29 Bahadur Gurung A, Ajmal Ali M, Al-Hemaid F, El-Zaidy M, Lee J. In silico analyses of major active constituents of Fingerroot (Boesenbergia rotunda) unveils inhibitory activities against SARS-CoV-2 main protease enzyme. Saudi. J Biol Sci. 2021.

30 Bastys T, Gapsys V, Walter H, et al. Non-active site mutants of HIV-1 protease. Antiviral Res. 2021;180:104934.

31 Costa E, Kuzikov M, Esposito F, et al. Structural and biochemical analysis of the dual inhibition of MG-132 against SARS-CoV-2 main protease (Mpro/3CLpro) and human carpehinin-L. Int J Mol Sci. 2021;22.

32 Baker JD, Uhrich RL, Kraemer GC, Love JE, Kraemer BC. A drug repurposing screen identifies hepatitis C antivirals as inhibitors of the SARS-CoV-2 main protease. PLoS ONE. 2021;16, e0245962.

33 Chowdhury KH, Chowdhury MR, Mahmud S, et al. Drug repurposing approach against novel coronavirus disease (COVID-19) through virtual screening targeting SARS-CoV-2 main protease. Biomedicines. 2021;9, 254.

34 de Vera IM, Smith AN, Dancel MC, Huang X, Dunn BM, Fanucci EL. Elucidating a relationship between conformational sampling and drug resistance in HIV-1 protease. Biochemistry. 2013;52:3278–3288.

35 Bastys T, Gapsys V, Walter H, et al. Non-active site mutants of HIV-1 protease influence resistance and sensitisation towards protease inhibitors. Retrovirology. 2020;17:13.

36 Bahadur Gurung A, Ajmal Ali M, Al-Hemaid F, El-Zaidy M, Lee J. In silico analyses of major active constituents of Fingerroot (Boesenbergia rotunda) unveils inhibitory activities against SARS-CoV-2 main protease enzyme. Saudi. J Biol Sci. 2021.

37 King NM, Prabu-Jeyabalan M, Nalivaika EA, Schiffer CA. Combating susceptibility to drug resistance: lessons from HIV-1 protease. J Virol. 2020;84:4620–4629.

38 Iketani S, Forouhar F, Liu H, et al. Lead compounds for the development of SARS-CoV-2 main protease inhibitors. Cell Biosci. 2021;11:199.

39 Huff S, Kummetha IR, Tiwari SK, et al. Discovery and mechanism of SARS-CoV-2 main protease inhibitors. Cell Biosci. 2021;11:199.

40 Ibbeson BM, Laraia L, Alza E, et al. Diversity-oriented synthesis as a tool for planning and methodology. Nat Rev Drug Discov. 2019;18;17:333–352.

41 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.

42 Cai X, Huang Y, Zhang Y, et al. Antiviral activity of SARS-CoV-2 main protease inhibitor through reversible glutathionylation of Cysteine 300. J Biomol Screen. 2021;26.

43 Bahadur Gurung A, Ajmal Ali M, Al-Hemaid F, El-Zaidy M, Lee J. In silico analyses of major active constituents of Fingerroot (Boesenbergia rotunda) unveils inhibitory activities against SARS-CoV-2 main protease enzyme. Saudi. J Biol Sci. 2021.

44 Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational planning and methodology. Nat Rev Drug Discov. 2018;17:333–352.

45 Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational planning and methodology. Nat Rev Drug Discov. 2018;17:333–352.

46 Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational planning and methodology. Nat Rev Drug Discov. 2018;17:333–352.

47 Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational planning and methodology. Nat Rev Drug Discov. 2018;17:333–352.

48 Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational planning and methodology. Nat Rev Drug Discov. 2018;17:333–352.

49 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.

50 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.

51 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.

52 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.

53 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.

54 Davis DA, Bulut H, Shrestha P, et al. Regulation of the Dimerization and Activity of Aloe vera. J Am Podiatr Med Assoc. 1989;79:559–562.