Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Synthesis and activity of an octapeptide inhibitor designed for SARS coronavirus main proteinase

Yi-Ru Gan a,*, He Huang a, Yong-Dong Huang a, Chun-Ming Rao b, Yang Zhao b, Jin-Sheng Liu a, Lei Wu a, Dong-Qing Wei c

a School of Chemical Engineering & Technology, Tianjin University, Tianjin 300072, China
b National Institute for the Control of Pharmaceutical and Biological Products, Beijing 100050, China
c Tianjin Institute of Bioinformatics and Drug Discovery (TIBDD) and Tianjin Normal University, Tianjin 300074, China

1. Introduction

Severe acute respiratory syndrome (SARS) is a respiratory disease that was originally reported in Asia at the beginning of 2003. Shortly afterwards, it was rapidly spread to North America and Europe. Threatened by such a disease, scientists in all areas are devoted to the research for finding the treatment of SARS. Cumulative evidences indicate that a previously unrecognized coronavirus is the culprit of SARS [4,7,10]. It is also known that the process of cleaving the SARS-CoV polyproteins by a special proteinase, the so-called SARS coronavirus main proteinase (SARS-CoV Mpra, 3CLpr), is a key step for the replication of SARS-CoV. The functional importance of the Mpra in the viral life cycle makes it an attractive target for developing drugs directly against this new disease.

According to the “lock-and-key” mechanism in enzymology, an octapeptide cleavable by the SARS proteinase must have a good fit for binding to its active site [8]. Thus, the cleavable peptide, after some chemical modification, can be converted to a competent inhibitor against the enzyme, as elaborated by Chou [6] in the “distorted key” theory. In view of this, the knowledge about what kind of peptide sequences can be cleaved by 3CLpr is important in designing effective inhibitors against 3CLpr and potential drugs for SARS therapy [17]. The protease-susceptible sites in proteins usually extend to an octapeptide [5,8,15,16], as generally formulated by P1P2P3P4 with the scissile bond located between the subsites P1 and P2 [6]. The SARS coronavirus enzyme and several viral proteinases exhibit Gln (Ser, Ala, and Gly) specificity [4,10]. The octapeptide AVLQSGFR selected in this paper was taken from the paper by Chou et al. [10]. These
authors had done studies of docking the octapeptide to SARS-CoV Mpro based on the three-dimensional structure of SARS coronavirus main proteinase obtained by Anand et al. [4] through a homologous approach. The binding results obtained through docking study [10] and structural bioinformatics [7] show that the octapeptide AFLQSGFR is bound to the SARS proteinase through six hydrogen bonds. The crystal structure of SARS-CoV Mpro has also been determined later by Yang et al. [18], whose results have confirmed the above analysis.

The present study was initiated in an attempt to conduct an in-depth examination of the antiviral activity of the octapeptide AFLQSGFR against SARS-associated coronavirus by biochemical experimental approaches.

2. Materials and methods

Fmoc-Arg (Pbf)-Wang resin, O-benzotriazol-1-yl-N,N,N,N'-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBT) were obtained from GL Biochem (Shanghai, China). Fmoc-Ala-OH, Fmoc-Gln (Trt)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Ser (tBU)-OH, and Fmoc-Val (tBU)-OH were purchased from Advanced Chem-Tech (Louisville, KY, USA). Trifluoroacetic acid (TFA) was from Tedia Company (Fairfield, OH, USA). Acetonitrile, ethanol, and piperidine were purchased from Tianjin Chemical Reagent Company (Tianjin, China).

The desired octapeptide AFLQSGFR was assembled manually by solid-phase synthesis and HBTU coupling of Fmoc-protected amino acids. The N-terminal Fmoc group was removed by treatment with 20% piperidine in NMP. Fmoc amino acids (2 equivalents) were activated by the addition of equimolar amounts of HBTU, HOBt, and DIEA diluted to 0.5 M with DMF. Each coupling efficiency was determined by quantitative ninhydrin assay. Finally, the target peptide was deprotected and cleaved from the resin. We used reversed-phase high-performance liquid chromatography (RP-HPLC) Hewlett-Packard 1100 Series (Agilent, Palo Alto, CA, USA) with gradients of increasing concentrations of acetonitrile in the presence of trifluoroacetic acid to analyze peptide mixtures. The molecular weight of the octapeptide was also tested on an ion trap mass spectrometer LCQ Deca XP (Thermo Finnigan, San Jose, CA, USA).

Preparative RP-HPLC purification was carried out here using a Waters Prep LC 4000 System (Waters Associates, Milford, MA, USA) linked to a Delta-Pak™ semipreparative C18 column (25 mm × 100 mm, 15 μm, 100 Å). We used a linear gradient (20–50% over 40 min) of acetonitrile/trifluoroacetic acid (99.9:0.1, v/v) at a flow rate of 5 mL min⁻¹ to separate the target peptide. UV Absorbance was monitored at 214 nm. The desired product was finally pooled and freeze-dried.

The toxicity of the octapeptide in Vero cells was assessed and its antiviral activity was measured with SARS coronavirus BJ-01 strain, which was isolated from a SARS patient in Beijing, China. The cytopathogenicity induced by the virus 24–96 h after infection in 96-well microplates on confluent layers of Vero cells was visually scored. The selectivity index was determined as the ratio of the concentration of the octapeptide that reduced cell viability to 50% (CC₅₀) to the concentration of the octapeptide needed to inhibit the cytopathic effect to 50% of the control value (EC₅₀). The cytotoxicity of the peptide was determined with an MTS Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA).

| Compound       | EC₅₀ (mg L⁻¹)a | CC₅₀b | Selectivity index |
|----------------|---------------|-------|------------------|
| AFLQSGFR       | 2.7 × 10⁻²    | >100  | >3704            |
| 6-Azauridine   | 16.8 (2.9)    | 104 (18) | 6                |
| Pyrazofurin    | 4.2           | 52 (9.6) | 12              |
| Mycophenolic acid | >50       | >50   | NCd              |
| Ribavirin      | >1000         | >1000 | NCd              |
| Glycyrrhizin   | 300 (51)      | >20000 | >67             |

a EC₅₀, effective concentration of compound needed to inhibit the cytopathic effect to 50% of control value.
b CC₅₀, cytotoxic concentration of the compound that reduced cell viability to 50%.
c See Anand et al. [4].
d NC: not calculable.
3. Results

Excellent results were obtained by solid-phase methodology using Fmoc/HBTU chemistry. The crude AVLQSGFR showed 87.6% yield and greater than 72.8% purity with molecular weight of 877.3, which exactly matches the theoretical value. The yield capacity of this purification process reached 10 mg per circle, and the purity of the desired octapeptide was 98%.

The octapeptide AVLQSGFR we synthesized in this study has no toxicity on Vero cells within the experimental concentration, as shown in Fig. 1. It also has no recognizable effect of Vero cells observed with the help of a microscope. Also, as shown in Fig. 1, the inhibitory effect of the octapeptide against SARS coronavirus in Vero cells is dose-dependent. The cultures’ absorbance at 490 nm was measured using a 96-well plate ELISA reader. CC50 and EC50 were then determined by the computer program Prizm 2.0. The octapeptide’s EC50 is 2.7 × 10^-2 mg L^-1, and its selectivity index is more than 3704 (Table 1).

We also detected the effect of the octapeptide AVLQSGFR on replication of SARS-associated coronavirus in Vero cells (Fig. 2). It was observed that, for the infected cells treated with 1 mg L^-1, the octapeptide AVLQSGFR obviously blocked replication of the virus.

4. Discussion

The molecular mechanism of the octapeptide inhibiting the activity of SARS-CoV may be illuminated by the binding mechanism of the enzyme SARS-CoV M-Pro with its ligands. It has been pointed by Chou et al. [10] that there are six hydrogen bonds formed between the octapeptide AVLQSGFR and the SARS-CoV M-Pro. Those residues involved in forming the hydrogen bonds from the enzyme are: Arg-40, His-41, Phe-185, Asp-187, and Gln-189. The interaction is so strong that the octapeptide naturally becomes an ideal competitive inhibitor for the SARS proteinase [1–3,9]. Accordingly, our results obtained through biochemical experiments are fully in consistent with those of the computational docking studies performed by Chou et al. [10,12–14,17].

Furthermore, our results are also compatible with those of Cinatl et al. [11], who assessed the antiviral potential of ribavirin, 6-azauridine, pyrazofurin, mycophenolic acid, and glycyrrhizin against two SARS coronavirus strains (FFM-1 and FFM-2) isolated from Germany patients. The EC50 for AVLQSGFR we recorded shows the lowest concentration among those existing compounds and a higher selective antiviral activity (SI > 3704) against SARS-CoV. This suggests that AVLQSGFR can serve as a starting entity in the course of discovering effective drug candidates for the treatment of SARS.

Acknowledgement

D.Q. Wei is supported by the grants from the Tianjin Commission of Sciences and Technology under the contract number 033801911 and the special fund for intensive computation.
REFERENCES

[1] Althaus IW, Chou JJ, Gonzales AJ, Diebel MR, Chou KC, Kezdy FJ, et al. Kinetic studies with the nonnucleoside HIV-1 reverse transcriptase inhibitor U-88204E. Biochemistry 1993;32:6548–54.
[2] Althaus IW, Chou JJ, Gonzales AJ, Diebel MR, Chou KC, Kezdy FJ, et al. Steady-state kinetic studies with the non-nucleoside HIV-1 reverse transcriptase inhibitor U-87201E. J Biol Chem 1993;268:6119–24.
[3] Althaus IW, Gonzales AJ, Chou JJ, Diebel MR, Chou KC, Kezdy FJ, et al. The quinoline U-78036 is a potent inhibitor of HIV-1 reverse transcriptase. J Biol Chem 1993;268:14875–80.
[4] Anand K, Ziebuhr J, Wadhwani P, Mesters JR, Hilgenfeld R. Coronavirus main proteinase (3CLpro) structure: basis for design of anti-SARS drugs. Science 2003;300:1763–7.
[5] Chou JJ. Predicting cleavability of peptide sequences by HIV protease via correlation-angle approach. J Protein Chem 1993;12:291–302.
[6] Chou KC. Review: prediction of HIV protease cleavage sites in proteins. Anal Biochem 1996;233:1–14.
[7] Chou KC. Review: structural bioinformatics and its impact to biomedical science. Curr Med Chem 2004;11:2105–34.
[8] Chou KC. A vectorized sequence-coupling model for predicting HIV protease cleavage sites in proteins. J Biol Chem 1993;268:16938–48.
[9] Chou KC, Kezdy FJ, Reusser F. Review: steady-state inhibition kinetics of processive nucleic acid polymerases and nucleases. Anal Biochem 1994;221:217–30.
[10] Chou KC, Wei DQ, Zhong WZ. Binding mechanism of coronavirus main proteinase with ligands and its implication to drug design against SARS. Biochem Biophys Res Commun 2003;308:148–151 [Erratum: ibid., 2003. vol. 310, 675].
[11] Cinatl J, Morgenstern B, Bauer G, Chandra P, Rabenau H, Doerr HW. Glycyrrhizin: an active component of liquorice roots, and replication of SARS-associated coronavirus. Lancet 2003;361:2045–6.
[12] Du QS, Wang S, Wei DQ, Sirois S, Chou KC. Molecular modelling and chemical modification for finding peptide inhibitor against SARS CoV Mpro. Anal Biochem 2005;337:262–70.
[13] Du QS, Wang SQ, Jiang ZQ, Gao WN, Li YD, Wei DQ, et al. Application of bioinformatics in search for cleavable peptides of SARS-CoV Mpro and chemical modification of octapeptides. Med Chem 2005;1:209–13.
[14] Du QS, Wang SQ, Wei DQ, Zhu Y, Guo H, Sirois S, et al. Polyprotein cleavage mechanism of SARS CoV Mpro and chemical modification of octapeptide. Peptides 2004;25:1857–64.
[15] Miller M, Schneider J, Sathyanarayana BK, Toth MV, Marshall GR, Clawson L, et al. Structure of complex of synthetic HIV-1 protease with a substrate-based inhibitor at 2.3 A resolution. Science 1989;246:1149–52.
[16] Schechter I, Berger A. On the size of the active site in protease. I. Papain. Biochem Biophys Res Commun 1967;27:157–62.
[17] Sirois S, Wei DQ, Du QS, Chou KC. Virtual screening for SARS-CoV protease based on KZ7088 pharmacophore points. J Chem Inf Comput Sci 2004;44:1111–22.
[18] Yang H, Yang M, Ding Y, Liu Y, Lou Z, Zhou Z, et al. The crystal structures of severe acute respiratory syndrome virus main protease and its complex with an inhibitor. Proc Natl Acad Sci USA 2003;100:13190–5.