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Synthesis and activity of an octapeptide inhibitor designed for SARS coronavirus main proteinase

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ABSTRACT

The outbreak of SARS, a life-threatening disease, has spread over many countries around the world. So far there is no effective drug for the treatment of SARS. Stimulated by the binding mechanism of SARS-CoV M^{pro} with the octapeptide AVLQSGFR reported recently as well as the "Chou's distorted key" theory, we synthesized the octapeptide AVLQSGFR for conducting various biochemical experiments to investigate the antiviral potential of the octapeptide against SARS coronavirus (BJ-01). The results demonstrate that, compared with other compounds reported so far, AVLQSGFR is the most active in inhibiting replication of the SARS coronavirus, and that no detectable toxicity is observed on Vero cells under the condition of experimental concentration.

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 M^{pro}, $3CL^{pro}$), is a key step for the replication of SARS-CoV. The functional importance of the M^{pro} 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 $3CL^{pro}$ is important in designing effective inhibitors against $3CL^{pro}$ 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 $P_{-4}P_{-3}P_{-2}P_{-1}\downarrow P_1P_2P_3P_4$ with the scissile bond located between the subsites P_{-1} and P_1 [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

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authors had done studies of docking the octapeptide to SARS-CoV M^{pro} 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 AVLQSGFR is bound to the SARS proteinase through six hydrogen bonds. The crystal structure of SARS-CoV M^{pro} 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 AVLQSGFR 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 ChemTech (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 AVLQSGFR 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% (CC_{50}) to the concentration of the octapeptide needed to inhibit the cytopathic effect to 50% of the control value (EC_{50}). The cytotoxicity of the peptide was determined with an MTS Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA).

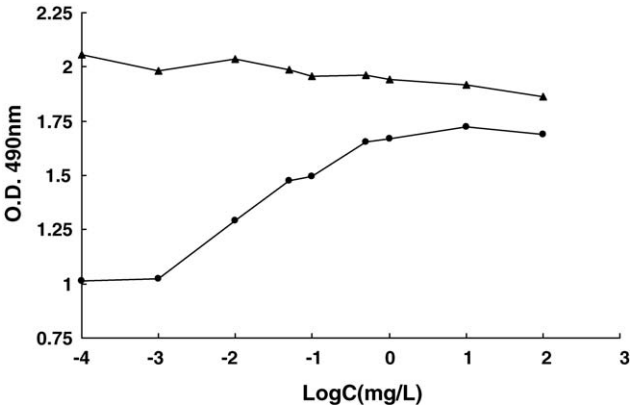
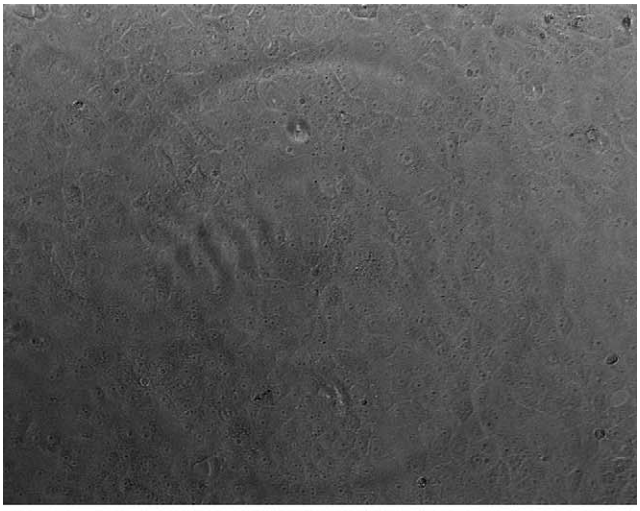


Fig. 1 – The relation of OD_{490} vs. log *C* showing the octapeptide's toxicity on Vero cells and the inhibitory effect of the octapeptide against SARS coronavirus in Vero cells. Here (▲) represents the normal Vero cells without infection and treated with the octapeptide (cell control OD_{490} = 2.014), whereas (●) represents the infected cells treated with the octapeptide (cell control OD_{490} = 2.1067; virus control OD_{490} = 1.0192). Note that the two curves approach with each other when the concentration of the octapeptide gets larger, indicating that the octapeptide AVLQSGFR is an effective inhibitor against SARS-CoV.

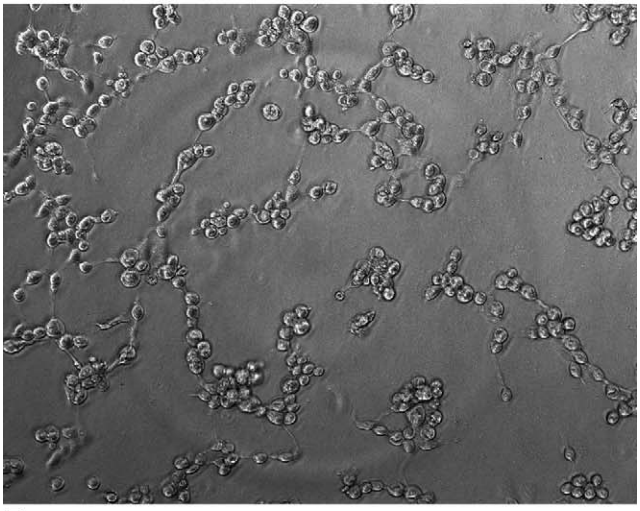
Table 1 – Activity of compounds against SARS-related coronavirus in Vero cells

Compound	EC_{50} (mg L^{-1})[a]	CC_{50}[b]	Selectivity index
AVLQSGFR	2.7×10^{-2}	>100	>3704
6-Azauridine[c]	16.8 (2.9)	104 (18)	6
Pyrazofurin[c]	4.2	52 (9.6)	12
Mycophenolic acid[c]	>50	>50	NC[d]
Ribavirin[c]	>1000	>1000	NC[d]
Glycyrrhizin[c]	300 (51)	>20000	>67

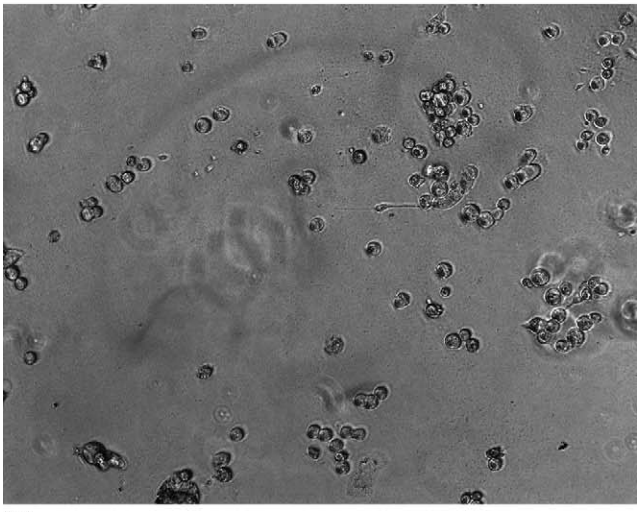
[a] EC_{50}, effective concentration of compound needed to inhibit the cytopathic effect to 50% of control value.
[b] CC_{50}, cytotoxic concentration of the compound that reduced cell viability to 50%.
[c] See Anand et al. [4].
[d] NC: not calculable.



(A)



(B)



(C)

Fig. 2 – Effect of the octapeptide AVLQSGFR on replication of SARS-associated coronavirus in Vero cells: (A) showing mock infected cells, (B) showing infected cells treated with 1 mg L^{-1} the octapeptide AVLQSGFR, and (C) showing infected cells without treatment.

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. CC_{50} and EC_{50} were then determined by the computer program Prizm 2.0. The octapeptide's EC_{50} 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 EC_{50} 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.

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