



## RESEARCH ARTICLE

### Identification of Antiviral Peptide Ligands Targeting the Capsid Spike Domain of Goose Astrovirus

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#### ABSTRACT

Outbreaks of joint and visceral gout in goslings caused by goose astrovirus (GAstV) continue to affect the goose industry in China. Currently, there is a lack of vaccines and drugs to treat GAstV-associated diseases. Due to the wide spread of GAstV through fecal-oral, vertical, and cross-host transmissions, the need to develop effective antiviral agents is urgent. In this study, the spike domain of the capsid protein of GAstV XX strain was targeted for the development of potential peptide ligands that possessed antiviral effect on GAstV infection. Molecular docking was performed to select the peptides with potential binding to the capsid spike of GAstV. The binding was then validated using an enzyme-linked immunosorbent assay (ELISA). After screening based on immunoassay (IPMA), immunofluorescence assay (IFA), and Western blot, peptide AP21 was demonstrated to inhibit GAstV infection in LMH cells in a dose-dependent manner. The expression of capsid spike reduced significantly in GAstV-infected cells upon pre-incubation of the virus with AP21. In addition, AP21 reduced viral infectivity by more than 10-fold as determined by the 50% tissue culture infectious dose (TCID<sub>50</sub>) assay. Structural analysis showed that the binding sites of AP21 on capsid spike were located on the surface of the protein and these sites were not linear but discontinued. In summary, the identification of antiviral peptides based on virtual screening and experimental validation greatly facilitated the development of antiviral peptides, which is important for the prevention and control of GAstV-associated gout diseases.

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#### INTRODUCTION

Since 2015, a systemic gout disease of goslings has affected many goose farms across China and the causative agent of the disease was identified to be a goose astrovirus (GAstV) (Zhang *et al.*, 2017). GAstV could transmit through fecal-oral and vertical routes and mainly infects goslings aged 5 to 20 days (Yang *et al.*, 2018; Wei *et al.*, 2020; Xu *et al.*, 2024). With an estimated morbidity of 80-90%, GAstV infection leads to swelling of kidney, urate deposition over the heart and liver, and renal tubular necrosis in goslings (Zhang *et al.*, 2018; Xiang *et al.*, 2024). Additionally, GAstV has been isolated in Cherry Valley ducklings and Muscovy ducklings, providing evidence that the virus also has cross-host transmission (Chen *et al.*, 2019; Chen *et al.*, 2021).

GAstV is a positive-sense, single-stranded, and non-

enveloped RNA virus that belongs to the genus Avastrovirus in the Astroviridae family (Liu *et al.*, 2018). Its genome is around 7.2 kb in length consisting of a 5'-untranslated region (UTR), a 3'-UTR, a poly (A) tail, and three open reading frames (ORFs), which are ORF1a, ORF1b, and ORF2 (Niu *et al.*, 2018). ORF1a and ORF1b were known to encode non-structural proteins involved in viral replication, while ORF2 encoded a capsid protein responsible for virion formation (Lewis *et al.*, 1994; Zhang *et al.*, 2018; Zhang *et al.*, 2022). It has been reported that the capsid protein of astroviruses contains a highly basic N-terminal region, a core domain, a spike domain, and a C-terminal acidic region (Krishna, 2005; Bogdanoff *et al.*, 2017). The spike domain existed in homo-dimers and has been shown to function as a receptor-binding domain which mediated viral attachment to host cells (Dong *et al.*, 2011; Bogdanoff *et al.*, 2017). In addition, core antigenic

regions containing immunodominant B-cell epitopes have been demonstrated to be located on the spike domain of capsid protein of GAsV (Ren *et al.*, 2021; Wang *et al.*, 2022). Therefore, the capsid spike is considered as an important target for the development of antiviral biological agents against astroviruses.

Protein and protein interaction constitutes the life cycle of viruses. Blocking the interaction between viral proteins and cellular proteins including cell-surface receptors has been shown to be an effective strategy to inhibit virus infection. For example, antibodies elicited by immunization with recombinant capsid spike have proven to be neutralizing and capable of blocking the attachment of human astrovirus (HAsV) to susceptible cell lines (Bass and Upadhyayula, 1997; Espinosa *et al.*, 2019). However, the production of potent neutralizing antibodies *in vitro* is tricky, because it requires the proper design and display of an immunogen, an appropriate immunization protocol, and a robust immune response. In contrast, peptide ligands that have high binding affinity to viral proteins could avoid these limitations and potentially function to inhibit virus infection during attachment, membrane fusion and entry, and virus assembly (Zhao *et al.*, 2016; Efaz *et al.*, 2021). In addition, peptide ligands are easy to produce chemically and are of low cost compared with the preparation of neutralizing antibodies. Hence, they hold a great promise for being used as prophylactic or therapeutic agents against viral diseases.

Classically, phage display peptide library and overlapping synthetic peptide library are two sources for the identification of peptide candidates which can bind to a specific protein. The phage display technology involves a separating method called affinity selection or biopanning which selects and amplifies phage clones displaying high-affinity ligands to the target from an immensely diverse repertoire (Jaroszewicz *et al.*, 2022). In overlapping peptide scanning, a set of overlapping synthetic peptides covering the entire sequence of a specific protein is created and screened to identify potential target-binding ligands (Hensen *et al.*, 2014). These two techniques contributed greatly to the identification of peptide ligands for diagnostic, prophylactic, and therapeutic purposes (Xu *et al.*, 2017; Vilas Boas *et al.*, 2019). Recently, with the increasing availability of structural information and the advancement of bioinformatics, protein-protein interaction could be simulated *in silico* and antiviral peptides could be designed computationally based on the available structure from Protein Data Bank (PDB) or built by using homology modelling (Wang *et al.*, 2020a). Given the fact that some viruses continue to evolve and viral proteins might mutate to escape antibody binding and neutralization, the demand for production of novel antiviral peptides is ever-increasing. To meet the challenges, the development of appropriate antiviral peptides requires simple, fast, and time-saving procedures, which make computer-aided molecular docking an invaluable choice of method. Computer-aided molecular docking allows the prediction of the binding mode and binding affinity of a ligand to the active site of a protein and may greatly shorten the development cycle of effective antiviral peptides (Xu and Lill, 2011; Yuriev and Ramsland, 2013). It has been extensively used to guide the development of affinity peptides to capsid protein of porcine circovirus type 2 (PCV-2) and antiviral peptides to

classical swine fever virus (CSFV) and fowl adenovirus 4 (FAdV-4) by our teams and others (Yu *et al.*, 2018; Wang *et al.*, 2020b; Chen *et al.*, 2023).

Due to its high transmissibility, albeit a newly emerged astrovirus, GAsV has spread to many provinces of China (Yang *et al.*, 2018; Zhang *et al.*, 2018; Xiang *et al.*, 2024). In 2016, we identified GAsV XX strain (GenBank No. MN337323.1) from diseased goslings in central China's Henan province after blind passage on chicken hepatoma cell line (LMH cells). Whole genome sequencing revealed that the virus had a genome of 7,252 bp and shared more than 98.1% nucleotide sequence similarity with GAsV isolates circulating in other provinces. Currently, there is a lack of vaccines and drugs to treat GAsV-associated diseases and little effort has been made to the development of antiviral peptides against GAsV. Hence, in this study, computer-based virtual screening technology was initially applied to design peptide ligands that possibly possess high binding affinity to the capsid spike of GAsV XX strain. Then, the potential antiviral ability of the peptides was evaluated and confirmed by immunoperoxidase monolayer assay (IPMA), immunofluorescence assay (IFA), Western blot, and virus titration. The structural information on the binding sites of the antiviral peptides on capsid spike was analyzed using PyMOL software. These results were the first to demonstrate the antiviral ability of peptide ligands to GAsV and provided evidence for using small peptides to effectively control GAsV-associated diseases.

## MATERIALS AND METHODS

**Reagents:** All the peptide ligands and biotinylated peptides were synthesized by GL Biochem Ltd. (Shanghai, China). Cell counting kit-8 (CCK-8) and commercial radioimmunoprecipitation assay (RIPA) buffer were bought from Jiangsu Beyotime Biotechnology, Inc. (Jiangsu, China). Streptavidin-horseradish peroxidase conjugate (SA-HRP) was purchased from Beijing Bioss Inc. (Beijing, China). Anti-beta-actin monoclonal antibody was purchased from Shanghai Universal Biotech Company (Shanghai, China). Alexa Fluor 488-conjugated goat anti-mouse IgG, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and enhanced chemiluminescence (ECL) detection kit were bought from Proteintech Wuhan Sanying (Wuhan, China). 4',6-diamidino-2-phenylindole (DAPI) was bought from Solarbio Life Sciences (Beijing, China). Recombinant capsid spike of GAsV XX strain, GAsV-specific monoclonal antibody 2B4, and rabbit polyclonal antibodies to GAsV XX strain were previously produced in our laboratory.

**Cell and virus:** The chicken hepatoma cell line, LMH, were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F12) media (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) supplemented with penicillin (100U/mL) and streptomycin (100µg/mL) at 37°C in 5% CO<sub>2</sub>. GAsV XX strain (GenBank No. MN337323.1) was isolated from a diseased field sample collected in Xinxiang region, Henan province of China, in July of 2018 and propagated in LMH cells.

**In silico prediction of GAsV capsid spike-targeted peptide ligands:** The three-dimensional structure of the capsid spike of GAsV XX strain was built using SWISS-MODEL (<http://swissmodel.expasy.org>) with the crystal structure of the capsid spike of turkey astrovirus 2 (TastV-2) as the template for homology modelling (PDB ID: 3TS3). Potential receptor binding pockets on the surface of the capsid spike of GAsV XX strain were determined based on findings from that of TastV-2 (DuBois *et al.*, 2013). Potential peptides for binding to the capsid spike of GAsV XX strain were selected using a virtual docking method based on SYBYL-X 2.1.1 software.

Prior to molecular docking, the capsid spike of GAsV XX strain was analyzed using the option of Prepare Protein Structure in the SYBYL-X 2.1.1 software. The backbone, side-chain, termini treatment, and protonation type of the capsid spike of GAsV XX strain were fixed according to the software's default recommendations. Biopolymer Hydrogens, Waters, and Side-chains were minimized 100 times per step. In the Surflex-Dock-Define SFXC File option, the Threshold value was set to 0.5, the Bloat value to 10, while the remaining parameters were left at their default settings. A Surflex-Dock Screen (SFXC) file was then generated for the subsequent docking process.

Virtual peptide library design was performed according to previously described (Hao *et al.*, 2019; Chen *et al.*, 2023). The initial conformation of the docking peptide was obtained using the Biopolymer/Build/Build Protein module embedded in SYBYL-X 2.1.1 software. A peptide library consisting of a series of random peptides of varying lengths (2–9 amino acids) was designed following hydrogenation, MMFF94 charge addition, and energy gradient optimization. Molecular docking between the peptide and capsid spike was carried out using the Surflex-Dock Program in the SYBYL-X 2.1.1 software. The docking results were evaluated and analyzed using a software-based scoring system. Consensus Score (Cscore) was used as the criterion to evaluate the binding affinity between peptide ligands and the capsid spike.

**Immunoperoxidase monolayer assay:** Immunoperoxidase monolayer assay (IPMA) was performed to visualize viral infection under a light microscope. Briefly, LMH cells were grown in 96-well cell culture plates to a confluency of 80% before infection with GAsV XX strain at a multiplicity of infection (MOI) of 0.1 for 36h. After being fixed with precooled methanol containing 1% H<sub>2</sub>O<sub>2</sub> for 20min at room temperature, the cells were incubated with 5% skimmed milk at 37°C for 1h. Rabbit polyclonal antibodies to GAsV XX strain were used as primary antibodies to incubate with the cells at 37°C for 1h. HRP-conjugated goat anti-rabbit IgG was then added and incubated with the plate as described above. During each step, the plates were washed six times with phosphate-buffered saline containing 0.05% Tween-20 (PBST). Finally, 3-amino-9-ethylcarbazole (AEC) was added for color development and the presence of red-brown precipitates in the wells represents a positive reaction. The average number of GAsV-infected cells per microscopic field was determined according to a protocol for a systematic randomization procedure (Jaspers *et al.*, 2005).

**Screening and selection of potential antiviral peptides:** A blocking IPMA was used to determine the ability of synthetic peptides to inhibit GAsV infection. Briefly,

GAsV XX strain (MOI=0.1) was pre-incubated with a final concentration of 100µM peptide at 37°C for 1h. Then, the mixture was added to monolayers of LMH cells grown in 96-well cell culture plate and incubated with the cells at 37°C for 2h. After washing the cells with sterile PBS, DMEM/F12 media containing 2% FBS was added to the wells and the plates were cultured at 37°C in 5% CO<sub>2</sub> for 36h. An IPMA read-out was obtained as described above.

#### Measurement of the cytotoxicity of the peptides:

Cytotoxicity of the potential antiviral peptides selected by IPMA was measured using the cell counting kit-8. In brief, LMH cells grown to a confluency of 80% in 96-well cell culture plates were incubated respectively with 0, 50, 100, or 150µM of the synthetic peptides diluted in DMEM/F12 media containing 2% FBS at 5% CO<sub>2</sub> for 48h before the addition of 10µL CCK-8 reagent. The absorbance (A) was measured at a wavelength of 450nm using a microplate reader and expressed as an optical density at 450nm (OD<sub>450</sub>). Cell viability (%) was expressed as  $[A (\text{Peptide}+) - A (\text{Blank})] / [A (\text{Peptide}-) - A (\text{Blank})] \times 100\%$ , where A (Peptide+) represents OD<sub>450</sub> value of wells with cells, CCK-8, and peptides, A (Peptide-) represents OD<sub>450</sub> value of wells with cells, CCK-8, but without peptides, A (Blank) represents OD<sub>450</sub> value of wells with culture medium and CCK-8, but without cells.

**Enzyme-linked immunosorbent assay:** Enzyme-linked immunosorbent assay (ELISA) was performed to determine the binding capacity of the peptide with recombinant capsid spike of GAsV XX strain expressed using the *Escherichia coli* system. Briefly, 96-well microtiter plates were coated with 100µL purified capsid spike (2.0µg/mL) in carbonate buffered saline (pH 9.6) overnight. After three washes with PBST, the plates were blocked with 5% skimmed milk at 37°C for 1h and then incubated with 100µL biotinylated peptides (1.0µg/mL) at 37°C for 30min. After washing six times with PBST, the plates were incubated with 100µL SA-HRP at 37°C for 30min. Then, the plates were washed with PBST and finally incubated with 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich, Saint Louis, MO, USA) for color development for 10min. The enzymatic reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and OD<sub>450</sub> values were obtained using an automatic plate reader (Bio-Tek Instruments, USA). All data were measured in triplicate and the mean OD<sub>450</sub> value (X) and standard deviation (SD) were calculated.

**Immunofluorescence assay:** Immunofluorescence assay (IFA) was carried out to confirm the antiviral activity of the peptides. GAsV XX strain (MOI=0.1) was pre-incubated with a final concentration of 0, 5, 20, or 80µM peptide at 37°C for 1h, respectively. Then, the mixture was added to monolayers of LMH cells grown in 96-well cell culture plate and incubated with the cells at 37°C for 2h. After washing the cells with PBS, DMEM/F12 media containing 2% FBS was added to the wells and the plates were cultured at 5% CO<sub>2</sub> for 48h. After being fixed with precooled methanol containing 1% H<sub>2</sub>O<sub>2</sub> for 20min at room temperature, the cells were incubated with 5% skimmed milk at 37°C for 1h. Monoclonal antibody 2B4 specific to the capsid spike of GAsV XX strain was used as primary antibody to incubate with the cells at 37°C for 1h. Alexa

Fluor 488-conjugated goat anti-mouse IgG was used as the secondary antibody and incubated with the plate as described above. During each step, the plates were washed six times with PBST. A blue-fluorescent DNA stain, 4',6-diamidino-2-phenylindole (DAPI), was applied to stain nuclear DNA. The results were observed under a fluorescence microscope and images were merged to localize positive reactions.

**Western blot:** Western blot was performed to further confirm the antiviral activity of the peptides. In brief, GAsV XX strain (MOI=0.1) was pre-incubated with a final concentration of 0, 20, or 80µM peptide diluted in DMEM/F12 media at 37°C for 1h. Then, the mixture was added to monolayers of LMH cells grown in 24-well cell culture plate and incubated with the cells at 37°C for 2h. After washing the cells with PBS, DMEM/F12 media containing 2% FBS was added to the wells and the plates were cultured at 5% CO<sub>2</sub> for 48h. Then, the cells were washed with PBS and mixed with 100 µL RIPA lysis buffer. After centrifugation at 10,000g for 3min, the supernatant was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After protein transfer, the polyvinylidene fluoride (PVDF) membrane was sealed with 5% skimmed milk at 37°C for 1h and incubated with GAsV-specific monoclonal antibody 2B4 or anti-beta-actin monoclonal antibody at 37°C for 1h followed by incubation with HRP-conjugated goat anti-mouse IgG at 37°C for 1h. During each step, the membrane was washed with PBST for six times. The signal was detected using ECL detection reagents and the image was captured using the Chemiluminescent Imaging System (Tanon Science & Technology Co., Shanghai, China).

**Determination of 50% tissue culture infectious dose (TCID<sub>50</sub>):** The GAsV XX strain was diluted serially at 1:10 from 10<sup>-1</sup> to 10<sup>-10</sup> using DMEM/F12 media. LMH cells grown in 96-well cell culture plates to a confluency of 80% were infected with 100µL of the diluted GAsV XX strain for 2h. After removal of viral sample, the cells were washed with sterile PBS and then cultured in 100µL DMEM/F12 media containing 2% FBS at 5% CO<sub>2</sub> for 5 days. The endpoint result was determined using the IPMA read-out and the viral titer was expressed as TCID<sub>50</sub>/0.1mL as calculated with the Reed-Muench method.

**Confirmation of the antiviral activity of the peptides using virus titration:** The antiviral activity of the peptides was further confirmed by measuring changes in viral titers after pre-treatment of GAsV XX strain with the peptides. Specifically, GAsV XX strain (MOI=0.1) was pre-incubated with a final concentration of 0 or 80µM peptide diluted in DMEM/F12 media at 37°C for 1h. Then, the mixture was added to monolayers of LMH cells grown in 24-well cell culture plate and incubated with the cells at 37°C for 2h. After washing the cells with sterile PBS, DMEM/F12 media containing 2% FBS was added to the wells and the wells were cultured at 37°C in 5% CO<sub>2</sub> for 12, 24, 36, and 48h, respectively. After three repeated freeze-thawing and centrifugation at 2,000g for 15min, the supernatants from each infection group were harvested and viral titer was obtained by determination of TCID<sub>50</sub> using the method described above.

**Analysis of the binding sites of the antiviral peptides on capsid spike:** Molecular docking between the identified peptide and capsid spike was performed using SYBYL software. A complex of the two molecules was obtained by projecting peptide onto the structure of capsid spike. PyMOL software was used to visualize and analyze potential binding sites of the antiviral peptide and their structural information on capsid spike.

## RESULTS

**Virtual screening of peptide ligands against the capsid spike of GAsV:** The sequences of capsid spike of GAsV XX strain and TAsV-2 were found to share 44.85% homology using BLAST. Based on the crystal structure of TAsV-2 capsid spike, structural information on the capsid spike of GAsV XX strain was obtained through homology modelling with SWISS-MODEL. Interestingly, it was shown that the structure of the capsid spike of GAsV XX strain resembled that of TAsV-2. Both existed in the form of a homodimer and shaped like a tightly packed heart (DuBois *et al.*, 2013). According to the location of the receptor binding pockets found on the surface of TAsV-2 capsid spike, potential receptor binding pockets on the capsid spike of GAsV XX strain were determined to be within residues 455-493aa and 524-598aa. Then, these areas were specifically targeted for molecular docking with peptide ligands under the multi-channel surface mode using SYBYL software. Through screening with the SurFlex-Dock protocol, thirty peptides targeting the pocket area of capsid spike protein of GAsV XX strain were selected and synthesized chemically. The CScore values of the docking between each peptide and the capsid spike were listed in Table 1.

**Table 1:** CScore obtained from molecular docking.

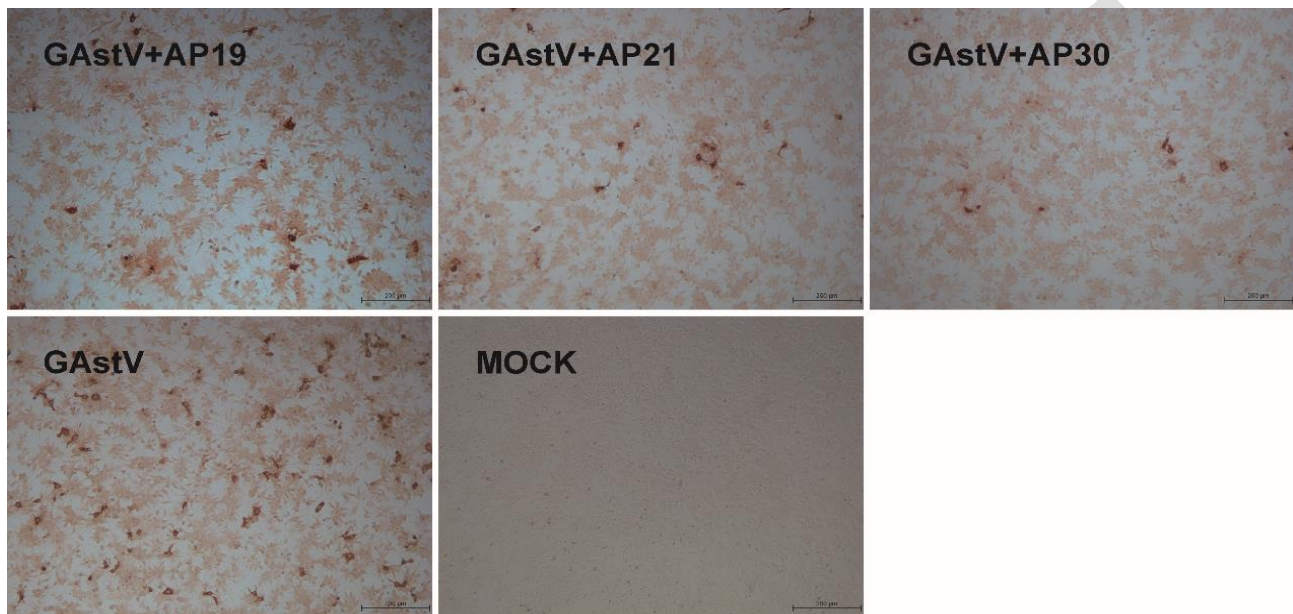
Peptide No.	Peptide sequence	CScore
AP01	RQRNAY	14.0852
AP02	RQRMPR	14.0447
AP03	RQRMWS	14.0180
AP04	RQRDWW	13.9700
AP05	RQRHNW	13.8278
AP06	RQRNHR	13.6760
AP07	RQRYQR	13.6757
AP08	RQRWAH	13.6415
AP09	RQRLRA	13.4479
AP10	RQRWWW	13.1821
AP11	WRCCGW	16.3134
AP12	WRCGWW	16.2390
AP13	WRCKYS	15.0803
AP14	WRCTYR	14.8152
AP15	WRCALR	14.4080
AP16	WRCFMR	14.3275
AP17	WRCSRH	14.1984
AP18	WRCFQK	13.9556
AP19	WRCKVR	13.9425
AP20	WRCHIH	13.9349
AP21	WKHKRR	15.0526
AP22	WKHGHR	14.5379
AP23	WKHRRK	14.4059
AP24	WKHLRQ	14.2738
AP25	WKHWKE	14.0893
AP26	WKHWRQ	13.9211
AP27	WKHIGK	13.6912
AP28	WKHKGR	13.6437
AP29	WKHDPK	13.5709
AP30	WKHWYK	13.4408

Peptide ligands that gave a CScore greater than 5.0 were considered to possess high binding affinity with capsid spike.

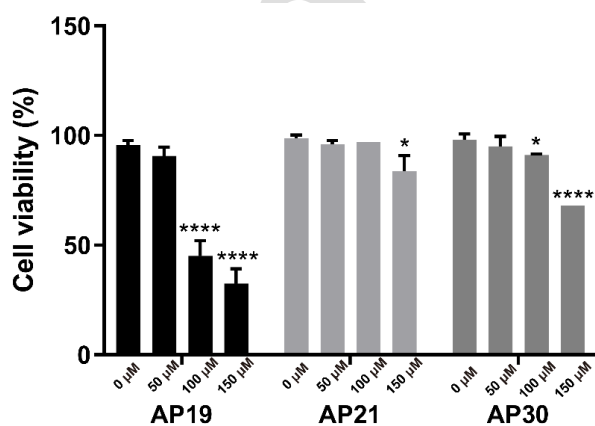
**Screening of potent antiviral peptides using IPMA:** A set of 30 peptide ligands with 6 amino acids each was prepared and tested using a blocking IPMA to evaluate their ability to inhibit GAsV infection. GAsV XX strain was pre-incubated with 100 $\mu$ M of each synthetic peptide at 37°C for 1h before infection and the number of virus-infected LMH cells was counted. After determination of the average number of immunoenzymatically stained cells in different treatments, it was found that the peptide ligands AP19, AP21, and AP30 exhibited obvious degrees of inhibitory effect on GAsV infection. Fig. 1 showed that treatment with AP19, AP21, or AP30 significantly decreased the number of GAsV-infected LMH cells, indicating that these peptide ligands possessed potent antiviral activity against GAsV infection in cell culture.

**Cytotoxicity of the antiviral peptides against GAsV:** To test the influence of each peptide ligand on cell viability, LMH cells were incubated with 0, 50, 100, or 150 $\mu$ M of AP19, AP21, or AP30 for 48h, respectively. As shown in Fig. 2, cell viability was significantly affected with the treatment of 100 or 150 $\mu$ M AP19, while AP21 and AP30 possessed no obvious influence on cell viability within the range of 0-100 $\mu$ M.

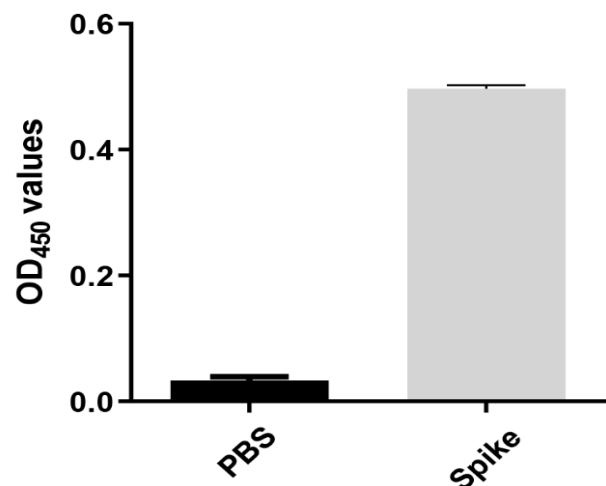
**Confirmation of the antiviral activity of the peptides:** Initially, ELISA was performed to test if the peptide could bind to recombinant capsid spike. The binding between AP21 and recombinant capsid spike was confirmed and amplified by the Biotin-Streptavidin System (BAS). It was found that there existed a clear binding reaction between peptide AP21 and recombinant capsid spike (Fig. 3).



**Fig. 1:** The blockade of GAsV-infection by different peptide ligands. GAsV XX strain (MOI=0.1) was pre-incubated with 100 $\mu$ M of AP19, AP21, or AP30 at 37°C for 1h before incubation with LMH cells grown in 96-well cell culture plate at 37°C for 2h. After washing with PBS, the cells were cultured in DMEM/F12 media containing 2% FBS at 37°C and 5% CO<sub>2</sub> for 36h. In IPMA, rabbit polyclonal antibodies to GAsV XX strain were used as primary antibodies and HRP-conjugated goat anti-rabbit IgG was used as secondary antibodies. An IPMA read-out was obtained with the addition of AEC substrate and observed under a light microscope. The presence of red-brown precipitates represented a positive infection. GAsV-infected LMH cells and mock-infected LMH cells were used as controls.



**Fig. 2:** Cytotoxicity of peptide ligands on LMH cells. After LMH cells were incubated with 0, 50, 100, or 150 $\mu$ M of the peptides for 48h, cytotoxicity of the peptides was measured using CCK-8 assay. \*denotes the significant change in cell viability compared with the blank control ( $P < 0.05$ ).



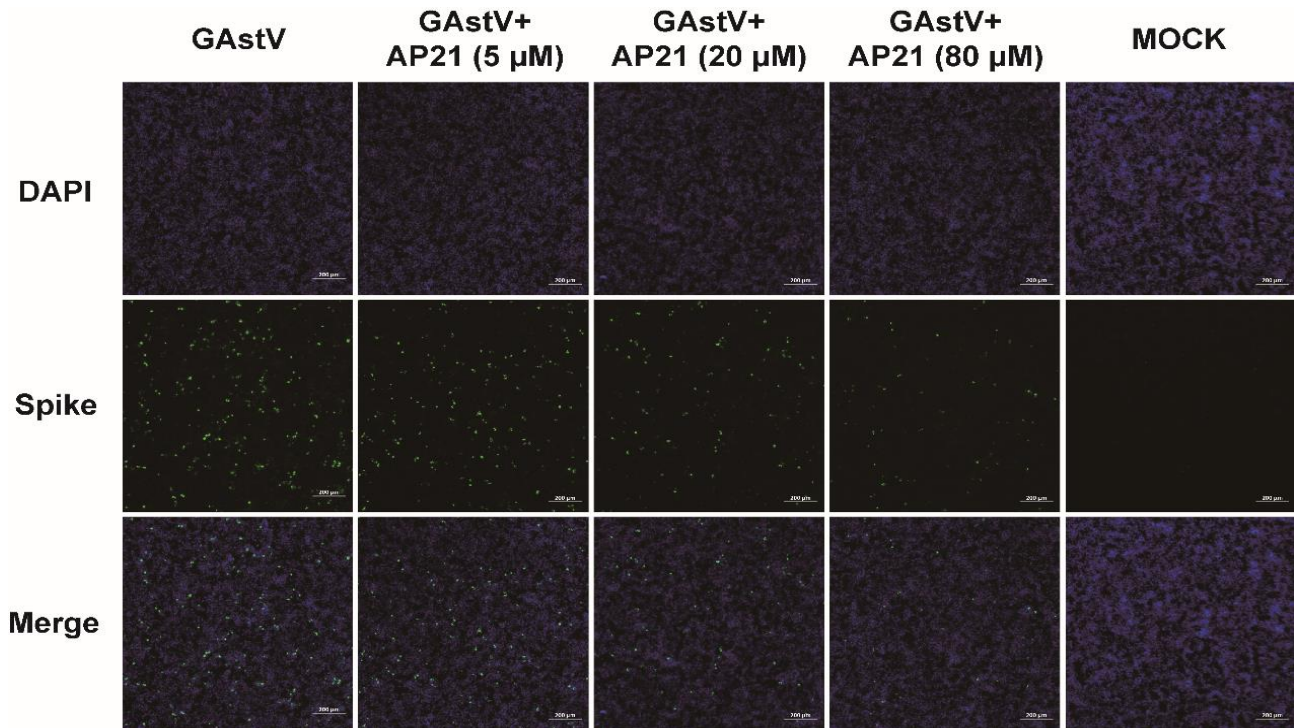
**Fig. 3:** Reaction between recombinant capsid spike and peptide ligand AP21 in ELISA. Biotinylated AP21 was allowed to incubate with recombinant capsid spike before the addition of SA-HRP conjugate. \* denotes the significant change in OD<sub>450</sub> values ( $P < 0.05$ ).



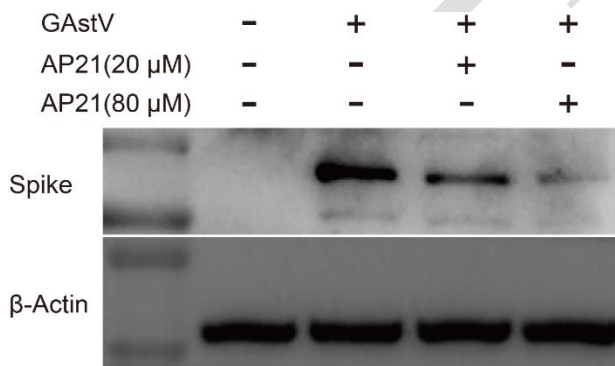
To confirm the antiviral activity of peptide AP21 against GAsV infection, IFA and Western blot were performed after pre-incubation of GAsV XX strain with different concentrations of AP21. As shown in Fig. 4, the number of GAsV-infected cells decreased obviously with the increase of AP21 concentration from 5 to 20 and 80  $\mu$ M, suggesting that there existed a dose-dependent antiviral effect of AP21 on GAsV infection. In addition, the result was further confirmed by Western blot in which the amount of capsid spike from infected cells reduced significantly after pre-incubation of GAsV XX strain with 20  $\mu$ M or

80  $\mu$ M of AP21 (Fig. 5).

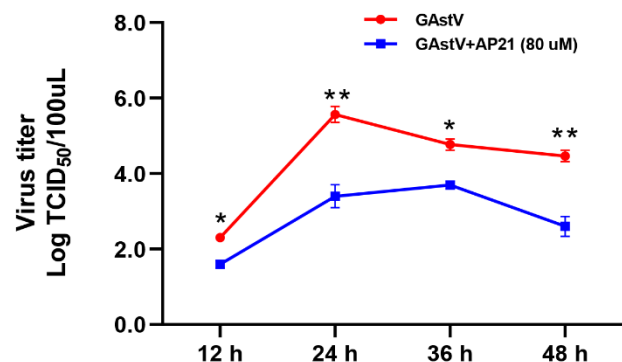
To test if pre-incubation of GAsV XX strain with AP21 could lead to reduced viral infectivity, viral titers at 12, 24, 36, and 48 h post-infection were measured after harvesting viral samples with or without AP21 pre-treatment. It was shown that the TCID<sub>50</sub> value decreased significantly in viral samples obtained by pre-treating GAsV XX strain (MOI=0.1) with 80  $\mu$ M of AP21 before infection (Fig. 6). Compared with infected group, pre-incubation with 80  $\mu$ M of AP21 reduced GAsV viral titers over 10-fold at 12, 24, 36, and 48 h post-infection.



**Fig. 4:** Characterization of the antiviral activity of AP21 using IFA. GAsV XX strain (MOI=0.1) was pre-incubated with AP21 of 0  $\mu$ M, 5  $\mu$ M, 20  $\mu$ M, or 80  $\mu$ M at 37°C for 1h before inoculation onto LMH cells grown in 96-well cell culture plates. After incubation of the peptide-virus mixture with the cells at 37°C for 2h, the cells were washed with PBS and cultured for another 48h in DMEM/F12 media containing 2% FBS at 5% CO<sub>2</sub>. Immunofluorescence staining was carried out to locate positive infections together with DAPI. The results were observed under a fluorescence microscope and images were merged. Mock-infected LMH cells were used as controls.

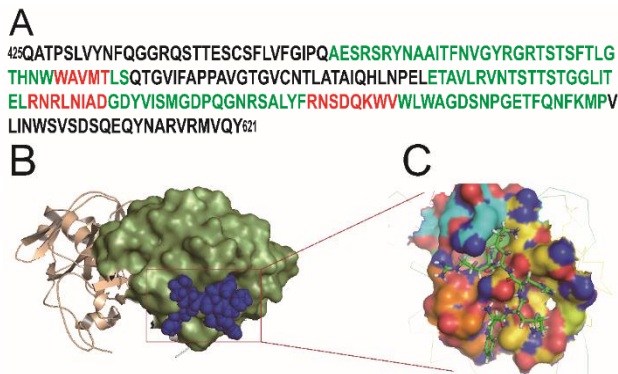


**Fig. 5:** Characterization of the antiviral activity of AP21 using Western blot. Peptide AP21 of 0, 20, or 80  $\mu$ M was pre-incubated with GAsV XX strain (MOI=0.1) at 37°C for 1h before incubation with LMH cells grown in 24-well cell culture plates. After incubation of the peptide-virus mixture with the cells at 37°C for 2h, the cells were washed with PBS and cultured for another 48h in DMEM/F12 media containing 2% FBS at 5% CO<sub>2</sub>. Then, the cells were washed with PBS and mixed with 100  $\mu$ L RIPA lysis buffer. The protein was transferred onto a PVDF membrane and sequentially incubated with 5% skimmed milk, GAsV-specific monoclonal antibody 2B4 or anti-beta-actin monoclonal antibody, and HRP-conjugated goat anti-mouse IgG at 37°C for 1h. The signal was detected using ECL substrate and the image was captured using the Chemiluminescent Imaging System.



**Fig. 6:** Changes in viral titers. Peptide AP21 of 0 or 80  $\mu$ M was incubated with GAsV XX strain (MOI=0.1) at 37°C for 1h before incubation with LMH cells grown in 24-well cell culture plates for 2h. After washing the cells with PBS, DMEM/F12 media containing 2% FBS was added to the wells. The cells were cultured at 5% CO<sub>2</sub> for 12, 24, 36, and 48h before harvesting the virus by repeated freeze-thawing and centrifugation. Supernatants from each group were tested using IPMA to determine the viral titer (TCID<sub>50</sub>). \*denotes the significant change in viral titers (P<0.05).

**Location of the binding sites of the peptide on capsid spike:** A complex of AP21 and capsid spike was obtained by molecular docking using SYBYL software. Peptide AP21 was projected onto the structure of capsid spike and the PyMOL program was used to visualize the positions of the binding sites of AP21 on capsid spike. It was shown that the capsid spike exist as a homodimer and the identified binding sites were located on the surface of capsid spike (Fig. 7). And the amino acids that AP21 bound to were not continued but spatial, consisting of 487-491aa, 545-552aa, and 572-579aa.



**Fig. 7:** Analysis of the binding sites of AP21 on capsid spike. With the crystal structure of the capsid spike of TAsV-2 (PDB ID: 3TS3) as template structure, the three-dimensional structure of the capsid spike of GAsV XX strain was obtained using SWISS-MODEL. Molecular docking between AP21 and capsid spike was performed using SYBYL software and a complex of the two molecules was obtained by projecting peptide onto the structure of capsid spike. PyMOL software was used to visualize potential binding sites of the antiviral peptide and their structural information on capsid spike. A. amino acid sequence of the capsid spike of GAsV XX strain. Green area represents the potential receptor binding pockets on capsid spike used for molecular docking. Red area represents the binding sites of AP21. B. Complex of the peptide ligand AP21 and capsid spike shown in a cartoon mode. Capsid spike existed in the form of a homodimer. The purple and the green each represents the monomer of capsid spike. C. Binding of the peptide ligand AP21 with capsid spike shown in a surface mode.

## DISCUSSION

GAsV-associated gout disease has led to significant economic losses and remains a serious threat to the goose industry (Yang *et al.*, 2018; Xu *et al.*, 2024). Since peptides are easy to synthesize, less immunogenic, and stable in their physicochemical properties, they have been used to inhibit protein-protein interactions and viral infections in the treatment many infectious diseases (Lok *et al.*, 2012; Zhou *et al.*, 2019; Sun *et al.*, 2021). Due to the absence of effective vaccines and drugs to control GAsV, there is a critical need for small peptides that can potentially inhibit GAsV infection to manage outbreaks. Instead of deriving peptide ligands from bait studies where a synthetic peptide or phage-displayed peptide is tested for interaction with a specific target, researchers now could artificially design peptides that best fit the active site of the protein target using computer-based virtual screening (Malik *et al.*, 2017). In addition, the chemical structure of the peptides (linear or cyclic) could be modified to be more compatible with that of the target using molecular docking. Previous studies have shown that rationally-designed peptides exerted antiviral activities against a number of viruses, including dengue virus, influenza virus, herpes simplex virus, and

human immunodeficiency virus (Vilas Boas *et al.*, 2019; Lee *et al.*, 2023; Soler *et al.*, 2023). More recently, two peptides were computationally verified to possess high binding affinity to the heptad repeat 1 (HR1) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein out of the 17 peptides experimentally validated to be capable of inhibiting SARS-CoV-1 (Efaz *et al.*, 2021). The FlexX docking algorithm embedded in SYBYL molecular modelling package is a fast docking method that allows sufficient flexibility of ligands while keeping the target protein rigid (Mouchlis *et al.*, 2010). Previously, we found that the FlexX/SYBYL program could give a better correlation between the evaluation result and the equilibrium dissociation constant (KD) of peptide against CSFV E2 protein than GalaxyPepDock (Yu *et al.*, 2018). And the peptides designed using FlexX docking method better served the purification of PCV-2 capsid protein and the preparation of capsid protein-based nanoparticle vaccines (Hao *et al.*, 2019; Wang *et al.*, 2020b). In this study, FlexX docking was applied to design and screen peptide ligands that could be well compatible to the active sites of the capsid spike of GAsV XX strain. Firstly, the structure of the capsid spike of GAsV XX strain was built by homology modelling with SWISS-MODEL using the crystal structure of TAsV-2 capsid spike as template. Potential receptor binding pockets (455-493aa and 524-598aa) on the capsid spike of GAsV XX strain were determined according to the location of the receptor binding pockets found on the surface of TAsV-2 capsid spike. Secondly, these areas were specifically focused on molecular docking with the designed virtual peptide library under the multi-channel surface mode using SYBYL software. Lastly, thirty peptides targeting the pocket area of the capsid spike of GAsV XX strain were selected based on the Consensus Score (CScore), which was reported to provide more reliable binding mode and binding affinity between a ligand and a target protein by integrating the strengths of several scoring functions (Ouyang *et al.*, 2012). To ensure the synthesis and characterization of high-affinity peptides, a CScore value greater than 5.0 was set to be the threshold.

For quick evaluation of the antiviral effect of the peptide ligands, an immunostaining method IPMA was used which offered instant results that could be visualized directly under a light microscope (Zhu *et al.*, 2019; Jin *et al.*, 2022). Inhibitory effect of the peptide on virus infection was confirmed by the reduction of numbers of immunostained cells after pre-incubation of GAsV XX strain with peptide ligands before infection. The tested peptides displayed varied degrees of inhibition on virus infection. AP19, AP21, and AP30 significantly decreased the number of GAsV-infected LMH cells, respectively. Cytotoxicity analysis revealed that there was no significant toxicity of AP21 and AP30 to LMH cells even at a concentration as high as 100μM for 48h. These results indicated that peptide ligands AP21 and AP30 have potent antiviral activity against GAsV infection in cell culture. Due to the lower cytotoxicity of AP21, its antiviral effect was further characterized. Initially, ELISA showed that peptide AP21 possessed a clear binding with recombinant capsid spike of GAsV XX strain. Then, the antiviral activity of AP21 was further confirmed by pre-incubating GAsV XX strain with different concentrations of the

peptide before infection. IFA showed that the inhibitory effect of peptide AP21 on GAsV infection became significantly obvious when the concentration of AP21 increased from 5 to 20 and 80  $\mu$ M. This was validated by Western blot which showed that the expression of the capsid spike of GAsV XX strain was significantly inhibited when the virus was pre-incubated with 80  $\mu$ M of AP21. In addition, viral titer expressed as TCID<sub>50</sub> decreased over 10-fold in viral samples harvested from pre-incubating GAsV XX strain with 80  $\mu$ M of AP21 before the initial infection. Taken together, these results demonstrated that AP21 could exert its antiviral activity against GAsV infection in a dose-dependent manner.

Previous studies have reported that peptide molecules might exert antiviral effects through interfering with viral entry into host cells or targeting intracellular signaling (Vilas Boas *et al.*, 2019; Li *et al.*, 2020). In this study, peptide AP21 was pre-incubated with GAsV XX strain before inoculation onto LMH cells. It could be inferred from the reduction in viral protein expression and viral infectivity that peptide AP21 might interfere with the attachment of GAsV to LMH cells to inhibit viral infection, although the possibility could not be ruled out that peptide AP21 might influence potential cellular signaling to affect GAsV infection. In addition, it has been reported that viral surface proteins were an important target for the induction of antibodies and protective immunity (Mintaev *et al.*, 2022). Previously, multiple epitopes have been mapped to be located on the surface of the capsid spike of GAsV (Ren *et al.*, 2021). And surface-exposed epitopes have been shown to elicit neutralizing antibodies against HAsV (Bogdanoff *et al.*, 2017; Espinosa *et al.*, 2019). In this study, the binding sites of AP21 on capsid spike were found to be located on the surface of the protein, which might also imply that GAsV interfered with viral attachment to host cells. Analysis of the structure of the complex of peptide AP21 and capsid spike using PyMoL software revealed that the binding sites of AP21 were not linear but spatial, consisting of 487-491aa, 545-552aa, and 572-579aa. These results indicated that peptide AP21 selected by virtual screening was well compatible to the active sites of the capsid spike of GAsV XX strain. Moreover, the binding sites of AP21 were different from the recently-identified epitopes on capsid protein of GAsV, indicating that we have identified novel sites to be targeted for potentially blocking GAsV infections (Ren *et al.*, 2021; Ren *et al.*, 2023).

**Conclusions:** In summary, a virtual screening method was applied for the selection of peptide ligands targeting the capsid spike of GAsV XX strain. The potential antiviral effect of the peptide ligands selected by *in-silico* prediction and molecular docking was examined and confirmed by IPMA, IFA, Western blot, and TCID<sub>50</sub> assay. The results showed that a potent antiviral peptide, AP21, possessed clear binding with recombinant capsid spike and reduced GAsV protein expression and viral infectivity in a dose-dependent manner. Structural analysis revealed that the binding sites of AP21 were located on the surface of capsid spike and were discontinued in sequence. The identification of antiviral peptides based on virtual screening and experimental validation can be an effective way to develop peptide ligands targeting various viral

targets and help reduce risk of viral resistance, which is crucial in response to emerging viral threats.

**Ethics statement:** This article does not contain any study with human or animal subjects performed by any of the authors.

**Author contributions:** QJ conceptualization; WL methodology; PL and YW investigation; QJ and WL validation; QJ, YW and LC software; WL and QJ resources; QJ and PL visualization; QJ and YW software; WL and QJ supervision; QJ and YW writing—original draft; QJ, WL and YW writing—review and editing; QJ, PL, LC and YW funding acquisition; All authors have read and agreed to the published version of the manuscript.

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**Data availability statement:** All data generated or analyzed during this study are included in this published article.

**Conflict of interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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