Melaleuca Alternifolia Concentrate inhibits entry of influenza virus H1N1 into host cell

Key Laboratory of Tropical Disease Control of Ministry of Education, Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou510080, China

ABSTRACT

Influenza virus causes high morbidity of population infection annually and pandemic spread occasionally as well. *Melaleuca alternifolia* Concentrate (MAC) is an essential oil derived from a native Australian tea tree which has been found to have anti-inflammatory, antiviral and antibacterial effect, etc. The aim of this study was to investigate whether MAC has any effect on inhibiting influenza virus infection *in vitro* and the possible mechanism of it. The antiviral activity of MAC was examined by its inhibition of cytopathic effect of the virus. The immunofluorescence, direct enzyme linked immunosorbent assay, electron microscopy and in silico prediction were performed to evaluate the interaction between MAC and the viral haemagglutinin. The results showed that MAC at concentration lower than 0.25% did not have any cytotoxic effect on MDCK cells. Applied MAC in cell monolayer before or after virus infection in MDCK cells could not confer the protection of cellular viability and induce the reduction of virus HA titer. While the influenza virus was incubated with MAC for one hour, no cytopathic effect of MDCK cell was found after the virus infection and there was no immunofluorescence signal was detected in the host cell. Electron microscopy showed that the virus treated with MAC remained structural integrity. The computatial simulations predicted that MAC inhibits entry of influenza virus into host cell by destroying the structural the viral HA protein *in vitro*. In conclusion, we have preliminary proved that MAC has the effect on anti-influenza virus by interfere with viral entry into the target cells.

Keywords: *Melaleuca alternifolia* Concentrate (MAC), influenza virus, haemagglutinin, uncoating.

Introduction

Influenza is an infectious disease caused by influenza virus which is a RNA virus of the family Orthomyxoviridae. Influenza spreads around the world in seasonal epidemics, with an estimated three to five million cases of severe illness and 250,000 to 500,000 people death per year [1]. While four major influenza pandemics had occurred in the 20th century and caused more than 20-50 million of deaths, influenza virus infection remains one of the leading causes for the modality and mortality[2,3]. A new H1N1 influenza A virus, which is also called the 2009 pandemic A/H1N1 influenza virus, had spread throughout the world and caused serious influenza pandemic in 2009[4,5]. While over 17,000 deaths caused by 2009 pandemic A/H1N1 influenza virus infection has been reported since its identification in Mexico in April 2009 [6]. However, 2009 pandemic A/H1N1 influenza virus, like other influenza virus A strains, developed resistant to adamantanes. too. Though, right now, the neuraminidase (NA) inhibitors, oseltamivir, which can interfere with the enzymatic activity of the neuraminidase (NA) of the influenza virus, has been mainly used for the treatment of influenza patients, the 2009 pandemic A/H1N1 influenza virus has been reported to be resistant to it [7,8]. It has been recently reported that over 160 sporadic viral isolates of 2009 pandemic A/H1N1 influenza virus show resistance to oseltamivir due to the NA H275Y genotype mutation [8,9]. On the other hand, though the vaccines against 2009 pandemic A/H1N1 influenza virus infection have been developed and used in clinical practices, the safety of theses vaccines remains one of the major public concerns in most of countries[10,11,12,13]. The deaths and serious side effects of vaccines against 2009 pandemic A/H1N1 influenza virus have been reported[14]. Since the influenza pandemics remain one of the most serious public health threats, a new, safe and effective drug against its infection is in urgent needing.

Herbal extracts have been reported to have an important role in controlling virus infection, by serving as immuno-modulators during influenza virus infection[15]or blocking the interaction of virus with target cells, or having virucidal activity through direct interaction with the virus[16,17]. Most importantly, accumulating evidence has suggested that treatment of herbal extracts might be able to reduce the risk of drug-resistant virus emerging[18]. *Melaleuca alternifolia* Concentrate (MAC), which is an essential oil derived from the leaves or terminal branches of a native Australian tea tree, *Melaleuca alternifolia*, is a heterogeneous mixture of approximately 100

chemically defined components that mainly contain terpinen-4-ol (56%-58%), gamma terpinene (20.65%), and alpha terpinene (9.8%)[19]. The ability of MAC to induce anti-inflammatory effect[20,21] and inhibit infection of various microbial species, such as bacteria[22,23], viruses[24,25] and fungi[26,27] makes it a promising candidate for the therapeutic development for H1N1 influenza virus infection.

The haemagglutinin (HA) on the surface of influenza virus particles is a major viral membrane glycoprotein molecule, which is synthesized in the infected cell as a single polypeptide chain precursor (HA0) with a length of approximately 560 amino acid residues and subsequently cleaved into two subunits of HA1 and HA2 by an endoprotease[28,29]. The crystallographic structure of the HA has a long tightly intertwined fibrous stem domain at its membrane-proximal base, a globular head which is containing the sialic acid receptor binding site (RBS)and five antigenic sites surround the receptor-binding site[30]. The mature HA on the viral surface is a trimeric rod-shaped molecule with the carboxy terminus inserted into the viral membrane and the hydrophilic end forming the spike of the viral surface [31,32,33]. Although the amino acid sequence of different virus strain identity can be less than 50%, the structure and functions of these HAs are highly conserved[29].

The major function of the HA is as the receptor-binding ligand, leading to endocytosis of the virus into the host cell and subsequent membrane-fusion events in the infected cell [29,34]. Influenza virus initiates infection by binding to sialic acids on the surface of target cells. After endocytosis, the endosomes become lower pH value mainly because of the activity of the Vacuolar-type H+-ATPase (V-ATPase)[35]. In the acid environment of the endosome, the HA molecule is cleaved into HA1 and HA2 subunits and then undergoes a conformational change which resulting in the exposure of the fusion peptide at the N-terminus of the HA2 subunit [36,37]. The fusion peptides insert into the endosomal membrane, while the transmembrane domains remain anchored in the viral membrane. Finally, the fusion peptide brings the endosomal membrane and the viral membrane into juxtaposition, leading to fusion. Subsequently, a pore is opened up by this structural change of more than one hemagglutinin molecule and then the contents of the virion are released into the

cytoplasm of the cell. This completes the uncoating process[38].

The purpose of this study was to determine the antiviral effect against 2009 pandemic A/H1N1 influenza virus using *in vitro* test of cytopathic effect (CPE) inhibition of MAC. As previously described, terpinen-4-ol was the main component of MAC; here we also assess the feasibility and sensitivity of interaction of terpinen-4-ol with the viral haemagglutinin protein through in silico prediction for confirming the drug target and the characterization of the protein changing after treatment with MAC.

Materials and methods

Bio-safety

All experiments involving pathogenic influenza A viruses were performed in a bio-safety level 2 (BSL2) laboratory of Zhongshan School of Medicine of Sun Yat-sen University, Guangzhou, China.

Cells and virus

Madin-Darby canine kidney (MDCK) cells maintained by our laboratory were grown in Dulbecco's modified Eagle's medium (DMEM , Invitrogen Corporation, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Scientific HyClone product line, Logan, Utah, USA) at 37°C, 5% CO_2 (Heracell 150i, Thermo Scientific, Langenselbold, Germany). No antibiotics or anti-mycotic agents were used in cell or virus culture. 2009 H1N1 pandemic influenza virus strain provided as a gift from Guangdong Centers for Disease Control and Prevention was propagated from clinical isolates and maintained in our laboratory. The virus strain was propagated in MDCK cells that were cultured in 0.02% TPCK- trypsin (Amresco Inc., Solon, Ohio, USA) at 37°C, 5% CO_2. The supernatant containing virus particles in MDCK cell culture was collected when 75%-100% CPE was observed. The virus was stored at -80°C in aliquots until use.

Melaleuca Alternifolia Concentrate (MAC)

Hundred percent MAC (batch 270409) was provided by NeuMedix Biotechnology Pty Ltd, Australia. Preliminary experiments established the optimal solubility into dimethyl sulfoxide (DMSO) (Beijing Dingguo Changsheng

Biotechnology Co. Ltd., Beijing, China) and the concentration of stock solution was 10% (v/v). For testing, the MAC stock solution was diluted by serum free DMEM media for working solutions with various concentrations.

Virus titrations

The virus strain was titrated by standard Tissue Culture Infectious $Dose_{50}$ ($TCID_{50}$) assay in MDCK cells. Briefly, MDCK cells were seeded in 96-well culture plates (about 5×10^4 cell/well) in DMEM with 10% fetal bovine serum (FBS) for 12-24 hours at 37°C with 5%CO_2. After cell propagation, growth medium was removed and 10 fold serial dilutions of the GZ01/09 virus suspension in DMEM media with 1μg/ml TPCK-trypsin were added to the wells. The plate was incubated at 37°C with 5%CO_2, and morphological changes on the MDCK cells were observed microscopically every 12 hours. The final CPE was recorded after 72 hours. TCID50 was calculated by counting all the wells with +-4+ CPE as being positive. TCID50 was calculated by the Reed-Muench method[39].

MTT assay to determine the cellular viability of MDCK cells

The cellular viability of MDCK cells was measured quantitatively by the reduction of formazan dye using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China) assay. Briefly, confluent MDCK cell monolayer in 96-well culture plates was washed with sterile PBS and incubated for 3 hours at 37°C after 40μl/well of MTT solution (5 mg/mL) was added into each well. When a purple precipitate was clearly visible, the liquid was carefully withdrawn without touching the sediment or the cells. DMSO at 100μl /well was added to dissolve the purple formazan, and the absorbance at A490 was read with an Absorbance Microplate Reader (Gene Co. Ltd., Hong Kong, China).

Bioimaging in 96 well plates

The influence to entering host cell of the influenza virus by MAC treatment was determined by an immunofluorescence assay on MDCK cells in a 96 well plate. Briefly, MDCK cells were plated in a sterile 96-well plate about 10 000 cells/well.

The influenza virus suspension treated with MAC of final concentration of 0.010% for 0.5 and 1 h at room temperature, virus suspension and maintain media for cell control were inoculated to the cell monolayer respectively, for 5 hours in order for sufficient viral protein synthesis in the host cell. The cells were incubated at room temperature in the 3.7 % formaldehyde 10 minutes for fixation; 0.1% Triton X-100 5 minutes for permeabilization and 3% fetal bovine serum 30 minutes for blocking. The influenza virus was stained with influenza A m1 (matrix protein 1) antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, California, U.S.A.) followed by Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) (Molecular Probes, Invitrogen, Carlsbad, CA). Finally, 50 µl per well of Fluoroshield™ with DAPI (4', 6-Diamidino-2-phenylindole) (Sigma-Aldrich, Inc., St. Louis, MO, USA) was added and analyzed using an imaging instrument (Leica DMI4000B, Meyer Instruments, Inc., Houston, TX, USA).

Electron Microscopy observation of the influenza virus morphology

MDCK cells with or without treatment with MAC were observed under an inverted microscope. The concentration and the treatment time of MAC were indicated in figure legends. Each 10 µl of MAC-treated and untreated virus suspension was placed on a clean slide. Two copper grids were applied to float on the drops of virus suspensions using fine, clean forceps for 2 min. The bulk of the fluid was removed with the edge of the copper grid vertically on a strip of filter paper. Air dry the copper grid for 1 min. The copper grids were applied to float on a drop of 2% potassium phosphotungstate, using fine clean forceps, for 1 min. The bulk of the fluid was removed with the edge of the copper grid vertically on a strip of filter paper. Air dried the grid and examined in the Electron Microscope.

Molecular dynamics simulations

The structure of HA (PDB: 3AL4)(40) were used in the docking calculations. Program Autodock 4(50) with Lamarckian genetic algorithm is used to carry out the molecular docking. To evaluate the binding energies between the ligand and receptor, AutoGrid program was used to generate the grid map with 80X80X80 points spaced equil at 0.375 Å is using. The AMBER11 simulation suite(51) was used in molecular dynamics(MD) simulations and data analysis.

Binding free energy calculation

The binding free energies (ΔGbind) were calculated using the MM-GBSA approach(52) inside the AMBER program. The first step of MM-GBSA method is the generationof multiple snapshots from an MD trajectory of the protein-ligand complex and a total 0f 50 snapshots were taken from the last 5 ns trajectory with an interval of 100 ps. For each snapshot, the free energy is calculated for each molecular species (complex, receptor, and ligand) using the following equation(53).

$$\Delta G_{bind}=G_{com}-G_{rec}-G_{lig} \quad (46)$$

$$\Delta G_{bind}=\Delta E_{mm}+\Delta G_{solv}-T\Delta S \quad (47)$$

$$\Delta G_{mm}=\Delta E_{elec}+\Delta E_{vdw}+\Delta E_{ini} \quad (48)$$

$$\Delta G_{solv}=\Delta G_{GB}+\Delta G_{np} \quad (49)$$

$$\Delta G_{np}=\gamma\Delta SASA+b \quad (50)$$

where G_{comp}, G_{rec}, and G_{lig} were the free energies for the complex, receptor, and ligand, respectively. ΔE_{mm} was the molecular mechanics energy of the molecule expressed as the sum of the internal energy of the molecule plus the electrostatics and van der Waals interactions; ΔG_{solv} was the solvation free energy of the molecule; T was the absolute temperature; and ΔS is entropy of the molecule. ΔE_{elec} was the Coulomb interaction, ΔE_{vdw} was the van der Waals interaction, and ΔE_{ini} was the sum of the bond, angle, and dihedral energies; in this case, $\Delta E_{ini} = 0$. ΔG_{GB} is polar solvation contribution calculated by solving the GB equation(54) for MM_GBSA method. ΔG_{np} was the nonpolar sovation term γ was the surface tension that was set to 0.0072 kal/(mol $Å^2$) and b was a constant that was set to 0. SASA is the solvent accessible surface area ($Å^2$) that was estimated using the MOLSURF algorithm. The solvent probe radius was set to 1.4 Å to define the dielectric boundary around the molecular surface.

The vibrational entropy contributions were estimated by NMODE analysis(55) and 50 snapshots were used in the NMODE analysis. To obtain the contribution of each the binding energy, MM_GBSA was used to decompose the interaction energies to each residue involved in the interaction by only considering molecular mechanics and sovation energies without the contribution of entropies.

Statistical analysis

The cell survival and the ELISA results in each group was expressed as the mean ± S.D. and the data was statistically compared with the relative control group using

one-way analysis of variance (ANOVA), SPSS 17.0 for Windows software. $P < 0.05$ was considered to be statistically significant.

Results and discussion

As an initial step to determine the ant-virus effect of MAC, MAC has any effect on the cellular viability was determined by a MTT assay, which is a colorimetric assay for assessing the viability of cells. Although MAC at concentration higher than 0.050% could induce significant cellular death, it did not have any cytotoxic effect on MDCK cells when lower than 0.025%. In addition, 10% DMSO/DMEM control was set up because there was DMSO in the MAC solution. Interestingly, the absorbance value of the cell incubated in 10% DMSO/DMEM was similar to the cell control (Fig.1). This observation also indicated that the cell death was produced by MAC at a high concentrations but not DMSO, because of the concentration of DMSO in the MAC working solution is far lower than 10%. These pieces of data suggested that MAC at proper concentrations does not have any cytotoxicity. So we choose concentration of 0.020% as a maximum study concentration for further experiments to determine the anti-viral effect of MAC.

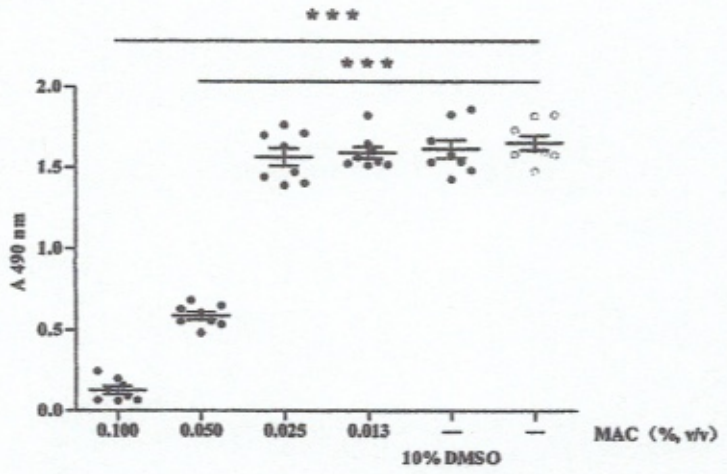

Fig 1. Effects of MAC on the MDCK cell viability. MACs of different concentration were applied to the MDCK cell monolayer, the 10% DMSO/DMEM control and the cell control were set up. After 72hours incubation at 37℃, 5% CO_2, the viability of MDCK cells were determined by a standard MTT assay protocol, as described in details in section of Materials and Methods. The data were presented as means+s.d. ***: p<0.001.

To determine whether MAC could confer the protection capability of influenza virus to the cell, MDCK cells were first treated with 0.020% MAC for 1hour, 2hours, and 4hours, respectively. MAC was then removed by careful sterile PBS wash. The MDCK cell monolayer was then inoculated with 2009 pandemic A/H1N1 influenza virus in 100 TCID50 per well for 1 h. The viability of MDCK cells were then determined by MTT method and the virus production was tested by HA assay, when ++-+++ CPE was observed on the virus control and the cell control was shown no CPE (about 48h–72h). As shown in **Fig.2,** no significant increase of cellular viability of MDCK cells was observed when MDCK cells were pretreated with MAC for one hour, two hours, and four hours **(A1, A2 and A3)** respectively, compared with the virus control and significant lower than the cell control and the ribovirin (ribo) control. It was worth noticing that, the cell survival under A1, A2 and A3 condition remained same. In other words, there was no tendency that the cell survival was increased according to prolonging the time treated with MAC. These data, therefore, indicated that pretreatment of MDCK cells with MAC could not confer any protection of cellular viability.

Because of pretreatment with MAC could not make MDCK cell produce any change for protecting influenza virus infection, we then examined whether treatment of virus but not MDCK cells with MAC could confer any protection of cellular viability. 2009 pandemic A/H1N1 influenza virus were first treated with MAC at a concentration of 0.010% for 0.5 hour and 1 hour, respectively. The mixtures were added to MDCK cells monolayer. The cellular viability and HA titer were tested as mentioned above. As shown in **Fig. 2 (B1 and B2),** although infectivity of the influenza virus treated with MAC for 0.5h was still remained, the virus treated with MAC for 1h presented poor infection to the host cell. Therefore, these data indicated that the influenza virus treated with MAC would dramatically lose its infective ability to the host cell.

Subsequently, the effect of MAC to the influenza virus has entered into MDCK cells was investigated. The influenza virus was inoculated to MDCK cells monolayer with 100 TCID50 per well for 1 hour to make the virus enter the host cells. The supernatant was then removed by sterile PBS wash and instead of 0.020% MAC/DMEM containing TPCK-trypsin 1μg/ml. The cellular viability and HA titer were tested as mentioned above. The results shown as part **C** of **Fig.2,** MAC could not

induce appreciable increase of the cellular viability of MDCK cells compared with the virus control. This indicated that MAC could not prevent influenza virus replication and biosynthesis in the host cell. The new generation of virus produced in the host cell could complete the life cycle and export from the host cell.

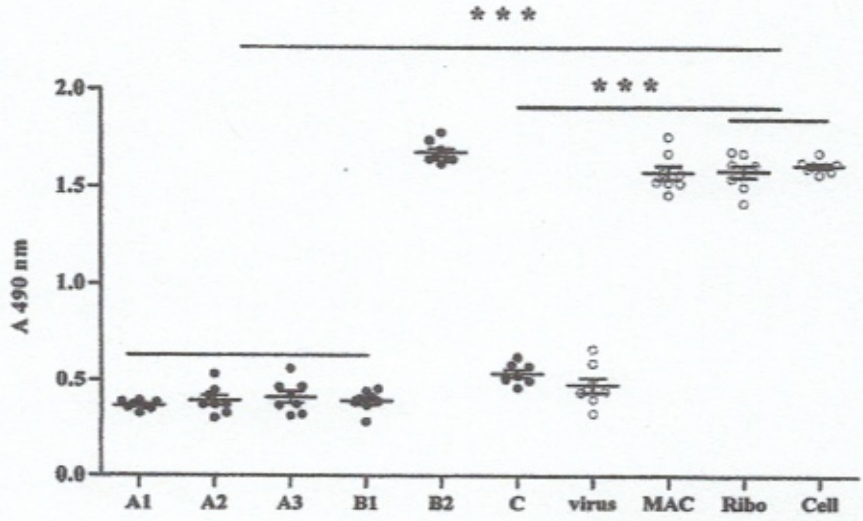

Fig 2. Protection efficacy of MAC against 2009 pandemic A/H1N1 influenza virus infection to MDCK cells. **A:** 0.020% MAC was applied to the MDCK cell monolayer for 1hour **(A1)**, 2hours **(A2)** and 4 hours **(A3)**, respectively. MAC in each MDCK cell culture was then removed by extensive sterile PBS wash and then 2009 pandemic A/H1N1 influenza virus in 100 TCID50 were infected for 1 hour and then instead of the maintain media. **B:** MAC was diluted into 0.010% with 10^3 TCID 50/ml 2009 pandemic A/H1N1 influenza virus suspension for incubation of 0.5 hour and 1 hour (**B1** and **B2**), respectively. The mixtures were added to MDCK cells monolayer for 1 hour and then instead of the maintain media. **C:** 100 TCID50 influenza viruses were inoculated to MDCK cell monolayer for 1 hour. The supernatant was then instead of 0.020% MAC/DMEM. Virus, MAC, ribo and cell represented 2009 H1N1 influenza virus control, 0.020% MAC control, 0.010% ribovirin control and the MDCK cell control, respectively. The viability of MDCK cells were then determined by MTT method when over ++ CPE was observed in the virus control and the cell control was shown no CPE (about 48h). The data were presented mean ±s.d. ***: $p<0.001$.

Given the results that MAC could inhibit 2009 pandemic A/H1N1 influenza virus infection when the MAC was applied before the virus enter MDCK cell, but could not prevent replication and biosynthesis of the virus in the host cell, MAC appear to inhibit entry of influenza virus into the host cell. To assess the efficacy of MAC anti influenza virus further, an immunofluorescence assay was performed. The influenza viruses were treated with MAC of final concentration 0.010% for 0.5 hour and 1hour, respectively, and the virus control and the cell control were set up. The

primary antibody and the fluorochrome labeled secondary antibody produced cytoplasm staining patterns in MDCK cells infected by the influenza virus treated with MAC for 0.5 hour and the virus without treated, whereas only robust nuclear staining was detected by DAPI in MDCK cells infected by the influenza virus treated with MAC for 1 hour and the cell control (**Fig. 3**). In addition, integrity of the virus particle after incubation with MAC was tested by Electron Microscopy. No matter the influenza virus treated with MAC or not, the numerous entire virus particles could easily be visualized in the images. The change of the general structure of the virion could not be observed (**Fig 4**). This result demonstrated that MAC could not lysis the virion.

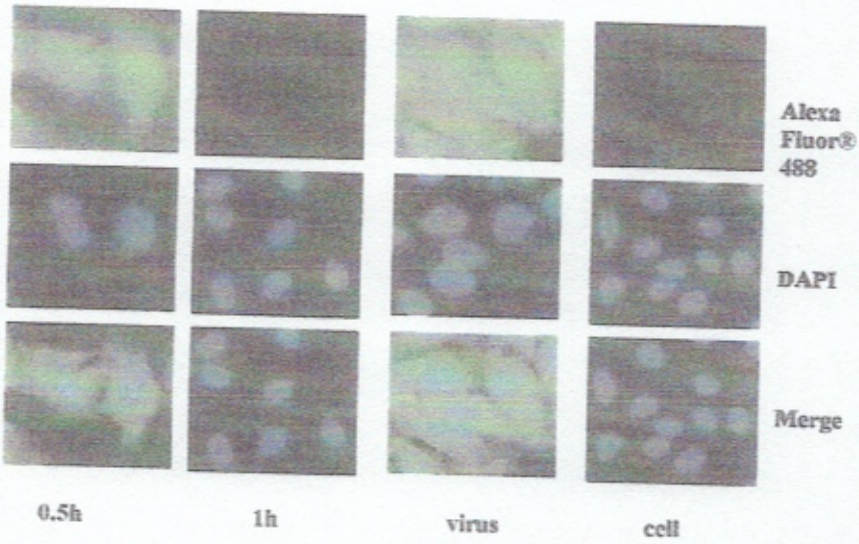

Fig 3. Treatment with MAC prevents the influenza virus entering the host cell. 0.010% MAC in the virus suspension incubation for 0.5h (0.5h) and 1 h (1h), the influenza virus untreated (virus) were inoculated respectively to MDCK cell monolayer in a 96 well plate for 5 hours, at the same time a cell control (cell) was set up. The virus was stained intracellularly with influenza A M1 antibody and Alexa Fluor® 488 Goat Anti-Mouse IgG. Double immunofluorescence staining graphs indicated the location of the virus was in the cytoplasm. Noted that the left second line, which showed the virus treated with MAC for 1 h, there was no green immunofluorescence served as negative control and in contrast to the virus control showing green immunofluorescence.

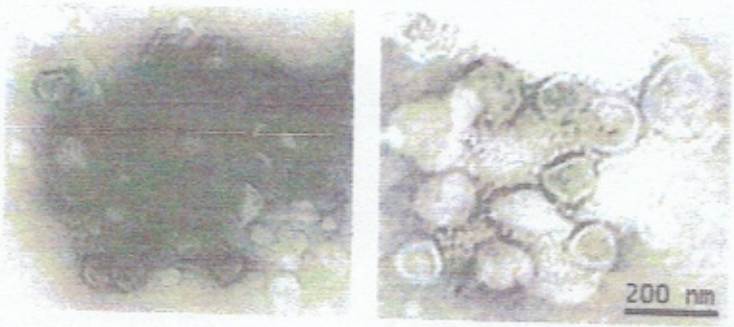

Fig 4. The role of MAC on destroying the structural integrity of the virion. The influenza virus was treated with MAC of 0.010% final concentration for 1 hour (left) and the virus control (right) was set up. Numerous intact virions could easily be visualized with close density of virion in both groups. The images shown here is one of the representative views of 10 replicates.

Given the results that MAC could inhibit 2009 pandemic A/H1N1 influenza virus infection when the MAC was applied before the virus enter MDCK cell, but could not prevent replication and biosynthesis of the virus in the host cell. MAC appear to inhibit entry of influenza virus into the host cell. This produce including two key steps: the first is virus attaches to cell-surface via the receptor site on the HA protein and then internalized within endosomes; the next step involves fusion between the viral envelope and endosomal membrane, mediated by the conformational change in the HA protein, triggering uncoating. Viral nucleocapsids are then released into the cellular cytoplasm for Transcription and Translation. Since the two steps are all mediated by HA, the demonstration here might be explained that MAC could prevent influenza virus or the viral genome enter the host cells by interaction with the viral haemagglutinin protein. To ascertain whether the explanation was reliable, the interaction between MAC and the viral haemagglutinin was accessed by mains of molecular dynamics.

MAC has been complete chemically defined and demonstrated that its anti microbe activity was principally attributed to terpinen-4-ol (T4), the main active component[24,25,26,40,41,42]. Actually, Terpinen-4-ol is the main anti microbe bioactive component of essential oil derived from several aromatic plants [43,44,45]. On account of this, the interaction of Terpinen-4-ol and the influenza virus haemagglutinin protein was predicted in silico to confirm the exact target and active characteristics of it.

The root mean-square deviation (RMSD) values were used to measure the conformational stability of the T4-HA complex during the MD simulations. From the

RMSD curves in Figure 5B. It suggested that T4-HA complex obtained from MD simulations is relatively stable. There are three repeated RMSDs of the complex during the MD simulation and all of them are under 0.3nm and the variations are within 0.1nm.

Comparison of the neutral-pH and fusion-pH structure indicated that at fusion pH the membrane-distal domains of HA dissociate, and extensive structural reorganization occurs that involves extrusion of the "fusion peptide" from the interior of the neutral-pH structural. In its position in the fusion-pH structure (56), the fusion peptide is at the N terminus of a new 100-Å-long triple-helical coiled-coil, while the C-terminal membrane anchor is repositioned at the same end of the refolded molecule. Occupation of the membrane fusion site can stabilizes the neutral pH structure through intersubunit and intrasubunit interactions that presumably inhibit the conformational rearrangements required for membrance fusion. Therefore, we concluded that the T4 can stabilize the neutral-pH conformation of HA.

From the MD simulation we can find T4 form two firm hydrogen bond with HA (Figure 5C). The time dependence of distances for these hydrogen bonds is shown in Figure. 5D. It can be seen from Figure 6 that there are two firm hydrogen bonds residues I-56_2 and N-60_2 with averaged 2 Å between T4 and HA, which play an important role in the stability of the complex.

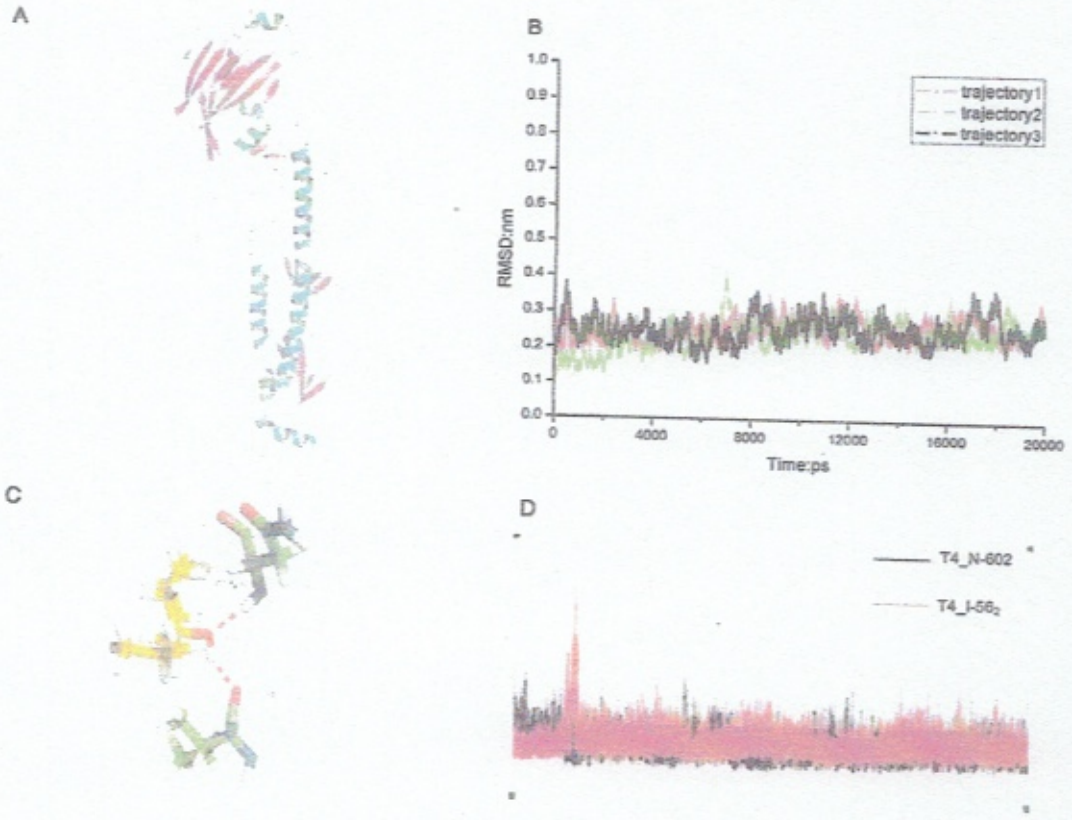

Fig.5 (A)The structures of complex obtained from docking calculations. (B)RMSDs of T4-HA complex compared to their orginal conformations as a function of time. (C)Hydrogen bonds formed between T4 and residues in binding pocket. (D)The time dependence of distance of T4_I-56$_2$(red) and T4_N-60$_2$(black).

An interaction of hydrogen bond was considered to form if the distance between the hydrogen donor and acceptor was less than 3.5 Å. We found that the hydrogen bonds between T4 and residues I-56$_2$ and N-60$_2$ make significant contributions to the binding affinity. Therefore, we believe that the H-bond interaction between the hydroxide radical of T4 and I-56$_2$ and N-60$_2$ stabilizes the T4-HA complex in the stage of MD simulation.

To explore the inhibition mechanisms of T4 with respect to its interaction with HA at the atomic level, the binding free energies were computed by means of the MM_GBSA. In particular, MM_GBSA combine molecular mechanics and continuum solvent models to estimate ligand binding affinities. The MM_GBSA calculation was constructed based a total of 250 snapshots that taken from the 15 ns to 20 ns. Importantly, the calculated binding free energy of the complex was -11.3647 kcal mol^{-1},

that indicated that the T4 bond to HA protein strongly. The results are listed in Table 1. The MD simulation based on the same initiating structure had been repeated for three times.

Table 1. Computed Binding Free Energies for T4 -HA Complexes (kcal/mol)

	trajectory1	trajectory2	trajectory3
ΔE_{ele}	-8.6841	-9.7112	-7.8287
ΔE_{vdw}	-27.7061	-27.6956	-28.3145
ΔE_{ini}	0	0	0
ΔG_{mm}	-36.3902	-37.4068	-36.1432
ΔG_{np}	-3.7628	-3.7310	-3.7499
ΔG_{sol}	10.4921	11.5757	10.9981
ΔG_{GB}	14.2548	15.3067	14.7499
ΔG_{tot}	-25.8981	-25.8310	-25.1450
$-T\Delta S$	14.5903	14.2991	13.8906
ΔG_{bind}	-11.3078	-11.5319	-11.2544

For the complex, the electrostatic energy and the van der Waals energy favorably contributed to the binding free energies. The free energy of T4 binding to HA calculated by MM_GBSA method showed that the binding process is thermodynamically favorable. Therefore, we conclude that T4 binding to membrane fusion site of HA and stablize the conformation of the fusion peptide through this interaction.

In conclusion, understanding how T4 stabilized HA could provide a clue for the development of new influenza fusion inhibitors. The structural and mechanistic insights from the present study provide a valuable foundation for the structure-based design of more potent influenza fusion inhibitors, espcially those for H1N1 influenza virus. We have preliminary proved that MAC has the effect on anti-influenza virus by interfere with viral entry into the target cells.

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