SCREENING FOR LEAD COMPOUNDS AND HERBAL EXTRACTS WITH POTENTIAL ANTI-INFLUENZA VIRAL ACTIVITY

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Abstract. Nonstructural protein 1 (NS1) of the highly pathogenic avian influenza virus (H5N1) contains a conserved RNA binding domain (RBD) that inhibits antiviral functions of host-innate immune response. Dimerization of NS1 forms a central groove and binds to double stranded (ds) RNA. This region might serve as a potential drug target. In this study, three dimensional structure model of NS1 RBD protein was constructed and virtual screening was performed to identify lead compounds that bound within and around the central groove. The virtual screening showed that 5 compounds bound within the central groove with binding energy ranging between -16.05 and -17.36 Kcal/mol. Two commercially available compounds, estradiol and veratridine, were selected for using in an *in vitro* screening assay. The results showed that neither of the compounds could inhibit the association between dsRNA and NS1 RBD protein. In addition, 34 herbal extracts were examined for their inhibitory effects. Five of them were able to inhibit association between NS1 RBD and dsRNA in electrophoresis mobility shift assay. Four herbs, *Terminalia belirica, Salacia chinensis, Zingiber montanum* and *Peltophorum pterocarpum*, could reduce >50% of infectivity of H5N1 in a cell-based assay, and it is worth further studying their potential use as source of antiviral drugs.

Keywords: virtual screening, antiviral compounds, NS1 protein, influenza A virus, herbs

INTRODUCTION

Highly pathogenic avian influenza virus (HP-AIV) subtype H5N1 is a highly contagious agent that causes a severe systemic disease in avian species. The disease commonly known as avian influenza or bird flu has spread widely from Asia to Europe, Middle East and Africa in 2005 (Cattoli *et al*, 2006; Melville and Shortridge, 2006). The H5N1 HP-AIV belongs to the family Orthomyxoviridae, genus *Influenzavirus A* and generally circulates among

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domestic and wild birds (Bragstad *et al*, 2007; Reperant *et al*, 2011; Watanabe *et al*, 2011). Additionally, the virus can also infect a variety of animal species including human, dog, cat, tiger and ferret (Songserm *et al*, 2006; Vahlenkamp *et al*, 2008; Zhou *et al*, 2009). Infections in human and other animal mostly results from consuming or handling dead infected birds as well as direct contact with high amounts of virus in contaminated fluid. The infection can cause serious respiratory illness among human population and is fatal in high-risk patients, with mortality rate in humans of over 50% (Neumann *et al*, 2010).

Antiviral drugs have been used for treatment and prevention of influenza A viruses including the HP-AIV. Currently, antiviral drugs are available for prophylactic uses in unvaccinated high-risk individuals as well as hospital personnel as prevention strategies, and therapeutic uses in patients with clinical signs to minimize the symptoms and reduce viral shedding (Adisasmito *et al*, 2010). Currently, available antiviral drugs are M2 ion channel blockers, amantadine and rimantadine, and neuraminidase inhibitors, zanamivir and oseltamivir. However, point mutations within the target proteins result in mutants resistant to these drugs (Boivin *et al*, 2002; Hay *et al*, 1986; Bright *et al*, 2005; Smee *et al*, 2009; Hauge *et al*, 2009). Therefore, searching for new drugs is essential for the influenza prevention and treatment, especially, against the novel virus strains.

Nonstructural protein 1 (NS1) is a multifunctional protein expressed in large amounts in H5N1 HP-AIV infected cells (Lin *et al*, 2007). NS1 is detected predominantly in nuclei of infected cells but undetectable in virions. It is one of the most important virulence factors and essential for viral replication (Hale *et al*, 2008). NS1 inhibits host cellular gene expression while promoting viral protein synthesis (Li *et al*, 2006; Ma *et al*, 2010; Spesock *et al*, 2011; Twu *et al*, 2007; Zhou *et al*, 2010) as well as antagonizing cellular innate immune response (Basu *et al*, 2009; Ngamurulert *et al*, 2009). It is a 26 kDa protein and is divided into 2 domains, N-terminal RNA binding domain (RBD) and C-terminal effector domain (ED) (Bornholdt and Prasad, 2008). RBD is composed of the first 73 amino acids and is highly conserved among influenza A viruses. Dimerization of RBD creates an RNA binding site around a central groove (Yin *et al*, 2007). Residues, Thr5, Pro31, Asp34, Arg35, Arg38, Lys41, Gly45, Arg46 and Thr49 of RBD are highly conserved and responsible for binding double-stranded (ds) RNA. Thus, both central groove and RNA binding surface might serve as a potential new drug target of HP-AIV H5N1.

The purpose of this study was to analyze the region of RBD, especially the area within and around the NS1 central groove, as a new target of anti-AIV H5N1 drugs. Three dimensional (3 D) structure model of NS1 RBD of AIV H5N1 A/chicken/Thailand/KU14/2004 was constructed. Then, molecular docking and virtual screening were performed to identify lead compounds that bind within and around the central groove of the NS1 RBD. In addition, RNA binding assay and electrophoresis mobility shift assay (EMSA) were developed to identify NS1 RBD inhibitors.

MATERIALS AND METHODS

Cloning of full length NS gene and RNA binding domain of NS1

Total RNA was extracted from allantoic fluid of embryonated eggs infected with HP-AIV H5N1 (A/chicken/Thailand/KU14/2004, KU14) using Trizol reagent®

(Invitrogen, Carlsbad, CA). Then, RNA was reverse transcribed using a pair of universal primers (Hoffmann *et al*, 2001) and SuperScriptIII enzyme (Invitrogen, Carlsbad, CA). For amplification, specific primers for the NS1 gene were designed to contain *Bam*HI and *Sal*I restriction enzyme sites: NS1-*Bam*HI_F5′-ACTTGGATCC-GATTCCAACACTGTGTC-3′ and NS1-*Sal*I_R 5′-CAGTGTCGACTCAAACTTCT-GACTCAATTG-3′ (Manasteinkij *et al*, 2008). PCR cycle was performed for at a total of 35 cycles comprising 94ºC for 30 seconds, 55ºC for 30 seconds, and 72ºC for 45 seconds, with a final heating at 72ºC for 7 minutes.

PCR amplicons were separated in 1% agarose gel and purified using QIAEXII kit (Qiagen, Hilden, Germany) before being ligated to plasmid pGEM_Teasy (Promega, Madison, WI). The ligation product was transfected into *E. coli* strain DH5± (NEB, Madison, WI) and positive colonies were selected by PCR colony screening. Subsequently, recombinant plasmid pGEM_NS1 was purified using QIAprep® (Qiagen, Hilden, Germany). Integrity of the nucleotide sequence was confirmed by DNA sequencing and analysis (Macrogen, Seoul, Korea).

To test for RNA binding function of NS1, sequence encoding the 72 amino acids of the RNA binding domain of NS1 gene was amplified from pGEM_NS1 using primers NS1-*Bam*HI_F (5′-ACTT-GGATCCGATTCCAACACTGTGTC-3′) and RBD-NS1-*Sal*I_R (5′-ATATGTC-GACTTAAGACTCCTCCTCCAG-3′) using PCR conditions described above.

Modeling of 3D structure of NS1 RBD

Expasy tool (http://expasy.org/tools/) was used to translate the DNA sequence into amino acid sequence. Then, NS1 RBD amino acid sequence was submitted to search for homologous proteins using the protein Blast Software (www.ncbi.nlm.nih.gov). The 3D structure of PDB protein (PDB accession code: 2Z0A) was created using Modeller 9v1 software (Sali and Blundell, 1993). Hydrogen atoms and specified axis of rotation were added in the 3D structure for further analysis by using virtual screening.

Virtual screening for inhibitor compounds

Lead compounds or ligands were prepared using the data from National Cancer Institute (NCI) database (http://dtp.nci.nih.gov/branches/dscb/repo_open.html) containing 1,990 compounds that already have defined atomic orbitals and charges in the structures. AutoDock3.0.5 software (Morris *et al*, 1998) was used to perform virtual screening between NS1 RBD protein and compounds from the NCI database. Their binding energies were calculated using genetic algorithm (GA) while the movement of atoms in the compounds and site chains of the target protein were restricted. Grid box was created to cover RNA binding site of NS1 RBD. The screening results were visualized by PyMOL software version 0.99 (DeLano, 2002).

NS1 RBD expression and purification

Transformed *E. coli* BL21 containing pQE_NS1 were grown in LB-broth with ampicillin at 37ºC. Expression of protein was induced with 0.2 mM IPTG at 16ºC overnight. Cells were collected by centrifugation at 1,300*g* for 20 minutes, and the pellet was washed once with the cleaning buffer (0.01 m Tris, 0.1 m NaCl, pH8.0) and resuspended with lysis buffer containing 0.25 mg/ml lysozyme. Cells were disrupted by sonication for 20 minutes with 10 second pulse and 5 second pauses using an ultrasonicator (Sonicator® ultrasonic processor XL, Heat Systems,

New York, NY). Soluble protein was collected by centrifugation at 11,000*g* for 20 minutes. NS1 RBD recombinant protein was purified by affinity chromatography using Protino® Ni-IAD Resin (Macherey-NaGel, Düren, Germany). NS1 RBD protein was eluted with 100 mM imidazole and analyzed using SDS-PAGE and western blot techniques (Sariya *et al*, 2011).

***In vitro* transcription and RNA labeling**

The 110 bp long digoxygenin (DIG) labeled single stranded RNA (ss) were transcribed *in vitro*. *In vitro* transcription was performed in a reaction volume containing 100 ng DNA template, transcription buffer, DIG RNA labeling mixture, 10 mM DTT, and 20 U of T7 or T3 RNA polymerase (Roche, Mannheim, Germany) for sense and antisense ssRNA, respectively. DIG-11-UTP labeling efficiency was verified by dot blot hybridization compared with the DIG-labeled probe control (Roche, Mannheim, Germany). The amount of RNA was measured with a spectrophotometer (Beckman Coulter, Brea, CA). To generate double stranded RNA probe, sense and antisense RNA were mixed together, incubated at 95ºC for 5 minutes and subsequently left at room temperature for 30 minutes for annealing.

RNA binding assay

The binding reaction between NS1 RBD protein and DIG-dsRNA was examined at different protein concentrations (0.05, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 µM). RNA binding reaction composed of 10 X Tris-glycine, 80% glycerol, 0.1 M DTT, RNaseOUT (Invitrogen, Carlsbad, CA), 500 ng/µl tRNA (Roche, Mannheim, Germany), 1 µM NS1 RBD protein and each selected lead compound and incubated at 30ºC for 10 minutes, followed by addition of 1 µM DIG-dsRNA and further incubated at 30ºC for 20 minutes. The reaction was kept on ice until used.

EMSA

Electrophoresis was performed using 12% nondenaturing PAGE in 1 X TBE running buffer (90 mM Tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0), at 100 V, for 5 hours. EMSA results were analyzed by northern blot technique as follows. The RNA and RNA-protein complex were transferred on to a positively charged nylon membrane (PALL, East Hills, NY). Blotting was performed at 80 V, 200 mA, for 2 hours. Thereafter, the membrane was heated at 120ºC for 30 minutes and then washed in the washing buffer [0.1 M maleic acid, 0.15 M NaCl, 0.3% (v/v) Tween20, pH 7.5] before incubating in the blocking buffer (Roche, Mannheim, Germany) with shaking for 30 minutes. After removing the blocking buffer, antibody solution containing anti-digoxygenin AP Fab fragment (Roche, Mannheim, Germany) was allowed to react with the RNA probes on the membrane at room temperature for 1 hour. After washing, the chemiluminescent substrate (Roche, Mannheim, Germany) was added onto the membrane to detect the position of the RNA probes and positive signal was visualized by exposing to x-ray film.

Herbal extraction

Thirty-four natural Thai herbs used in this study are listed in Table 1. Ten grams of the crushed herb were extracted in 180 ml of hexane using a Soxhlet extractor for 8 hours. Thereafter, hexane was removed by self-evaporation in a chemical fume hood. The herbal extract was dissolved in 1 ml of DMSO (Sigma, St Louis, MO) and stored at -20ºC until used.

Cell-based assay for antiviral drug screening

Cell-based assay was modified from previous published protocol (Noah *et al*,

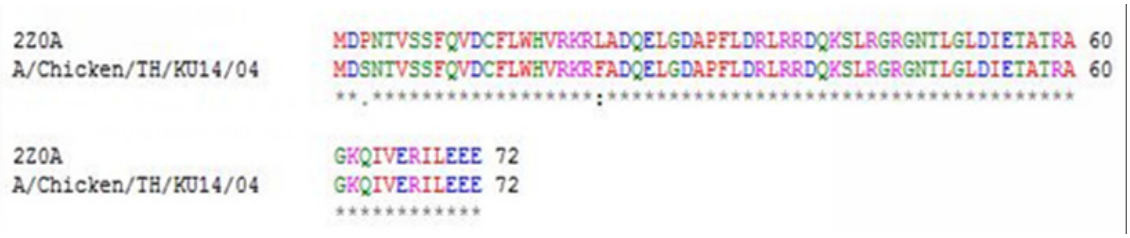


Fig 1–Comparison between the first 72 amino acids of NS1 protein (NS1RBD) of influenza virus A/crow/Kyoto/T1/2004 (H5N1) (2Z0A) and that of A/chicken/TH/KU14/04 NS1 RBD. Star indicates the mismatched amino acid residuces.

2007). An aliquot of 100 µl of Madin-Darby canine kidney (MDCK) cells (3 X 10^5 cells/ml) were seeded in 96 well plates and incubated overnight at 37°C under an atmosphere of 5% CO_2. Five herbal extracts with inhibitory effect were diluted from 2, 20, 200 to 4,000 µg in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) before adding into each well containing the monolayer of MDCK cells. Subsequently, 100 $TCID_{50}$ (50% Tissue Culture Infective Dose) of a reverse genetics derived avian influenza virus (Lekcharoensuk *et al*, 2012) were added into each well and the plates were incubated at 37°C for 18 hours. A blank control and an herbal extract that did not inhibit NS1 RBD activity (*Vitexnegundo L.*) were included as negative controls. Each experiment was performed in three replicates. The cells were fixed in absolute methanol and immunoperoxidase monolayer assay was performed as described previously (Lekcharoensuk *et al*, 2010). Numbers of positive foci in each well resulting from viral infection were counted under an inverted microscope (Olympus, Hicksville, NY).

Statistical analysis

Percent of foci reduction was calculated based on the ratio between mean of foci present in drug-treated wells and those without herb treatment. The herbal extracts with >50% reduction are considered to possess inhibitory effect. The difference between mean of the herbal extract with NS1 RBD inhibitory effect and that of *Vitexnegundo L.*-treated group were compared using Posthoc comparison with Tukey's method.

RESULTS

3D structure modeling of NS1 RBD protein

The first 72 amino acid residues at N-termini of the NS1 protein corresponding to NS1 RBD of influenza virus A/chicken/Thailand/KU14/2004 was 97.2% identical to those of A/crow/Kyoto/T1/2004 (H5N1; KyotoT1) with only two mismatches (Fig 1). The 3D structure of KU14 NS1 RBD protein was constructed from the 2Z0A file of Kyoto T1 NS1 RBD using Modeller 9v1 software. The homology model was validated and the absence of residue in disallowed region was confirmed by the application of PROCHECK program (Laskowski *et al*, 1993).

The 3D structure of KU14 NS1 RBD displayed by PyMOL software version 0.99 (Fig 2) showed that the two subunits of KU14 NS1 RBD associate in opposite direction to form a dimer similar to that previously described for A/Vietnam/1203/2004 (H5N1) (Yin *et al*, 2007; Bornholdt and Prasad, 2008). The secondary

Table 1
Thai herbs used in screening for NS1 inhibitors using EMSA.

Scientific names of herbs		
Azadirachta indica	*Saccharum officinarum*	*Strychnos nux-vomica*
Rhinacanthus nasutus	*Myristica fragrans*	*Curcuma xanthorrhiza*
Brucea amarissima	*Ruellia tuberose*	*Terminalia belirica*
Cinnamomum loureiroi	*Curcuma longa*	*Sapium indicum*
Quercus infectoria	*Lawsonia inermis*	*Terminalia chebula*
Salacia chinensis	*Spondias pinnata*	*Caesalpinia sappan*
Mallotus philippensis	*Maclura cochinchinensis*	*Diospyros mollis Griff*
Zingiber montanum	*Pentace burmannica Kurz*	*Garcinia mangostana L*
Peltophorum pterocarpum	*Acacia catechu*	*Citrus hystrix*
Moringaoleifera Lam	*Euphorbia hirta L.*	*Vitex negundo L.*
Plumbago indica L.	*Dracaena lourieri*	*Hibiscus sabdariffa L.*
Mammea siamensis T. Anders		

structure of each NS1 RBD monomer is composed of 3 α-helices, helices 1-3, connected to each other by short loops. Helix 2 of each monomer constitutes the surface of NS1 RBD responsible for RNA binding. The different amino acids between NS1 of KU14 and Kyoto T1, serine at position 3 and phenylalanine at position 23, are not involved in dimerization and RNA binding. The surface structure of RBD dimer of KU14 NS1 (Fig 2B) shows the central groove of the molecule surrounded by helix 2 from each monomer. Arginine at position 38 responsible for binding with dsRNA (Yin *et al*, 2007) is located in helix 2 of both monomers.

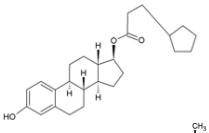
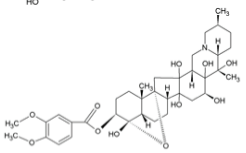
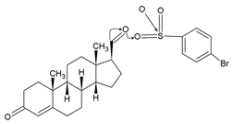
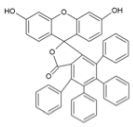
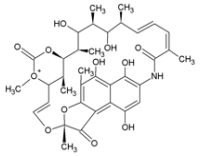
Virtual screening for NS1 specific lead compounds

Of 1,990 compounds in the NCI database, 20 compounds had binding energies < -15.00 Kcal/mol. The top five compounds with the lowest binding energy and possessing the best fit in the central pocket of the NS1 RBD dimer are shown in Table 2. All five compounds were able to bind within the central groove region, which is the dimer-interface area of the NS1 RBD (Fig 3). Compounds contain aromatic rings that could form H-bonds with amino acid residues of the NS1 protein. Amino acid residues of NS1 RBD that could form H-bonds with the compounds, namely, serine at position 42, arginine at positions 19, 38, and 46 and aspartate at position 39, are shown in Fig 4 (stick model). These residues are in the groove area and associate with dsRNA binding function of the NS1 RBD protein. The thin sticks represent the other residues within 5Å radiuses of the amino acids that form H-bond with the compounds.

Screening assay for NS1 RBD inhibitors

The RNA binding assay and EMSA system were developed in order to examine the specificity of the binding reaction prior to being applied for screening for NS1 RBD inhibitors and the optimal condition comprised 1 μM NS1 RBD and 100 nM DIG-labeled dsRNA, respectively. The DIG-labeled dsRNA probe was allowed to react with NS1, trypsin, chymotrypsin and a histidine tagged protein in the RNA binding assay. A reaction containing DIG-labeled dsRNA alone was also included as

Table 2
Structures and chemical formulae of five compounds with the lowest binding energy to NS1 RBD protein.

Compound	Structure	Chemical formula	Amino acid residues of NS1 RBD protein forming H-bond with compound	Binding energy (Kcal/mol)
NCI0035		$C_{26}H_{36}O_3$	Ser42', Arg46'	-16.39
NCI0082		$C_{36}H_{51}NO_{11}$	Arg19(2)	-17.36
NCI0932		$C_{27}H_{33}BrO_5S$	Arg19(2)	-16.05
NCI1212		$C_{44}H_{28}O_5$	Arg38	-16.39
NCI1322		$C_{37}H_{47}NO_{12}$	Asp39(2), Asp3', Ser42'	-17.21

a negative control. Differential migrations of RNA alone and RNA-protein complex were determined by EMSA. DIG-labeled dsRNA specifically bound NS1 RBD protein but did not react with other irrelevant proteins (Fig 5A). Subsequently, the RNA binding assay was performed to test whether the best binding compounds predicted by virtual screening could disrupt the association between NS1 RBD protein and dsRNA *in vitro* and 2 commercially available compounds, estradiol (NCI0035) and veratridine (NCI0082), were chosen. EMSA demonstrated that both compounds at all tested concentrations (10 μM - 1 mM) could not change the migration distance of the RNA probes, indicating that they were unable to inhibit the binding reaction between dsRNA and NS1 RBD protein (Fig 5).

Inhibitory effects of herbal extracts

EMSA was also applied to search for herbal extracts with RBD NS1 inhibitory effect. Out of 34 herbs examined, only 5 herbs, namely, *Curcuma xanthorrhiza, Terminalia belirica, Salacia chinensis, Zingiber montanum* and *Peltophorum pterocarpum*,

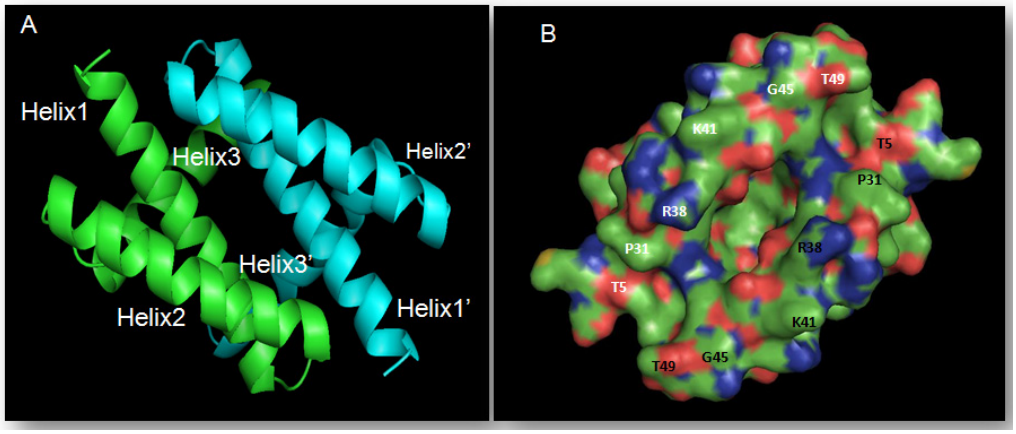


Fig 2–Three dimensional structure of NS1 RBD protein of influenza virus A/chicken/TH/KU14/04. (A) Ribbon diagram demonstrating the secondary structure of NS1 RBD dimer of KU14. Each monomer folds into 3 helices connecting by short loops. (B) Space filling model showing protein surface structure of the RBD dimer. Two amino acid residues responsible for dsRNA binding are Arg 38 and Lys 41. A groove in the center of the protein molecule is visible.

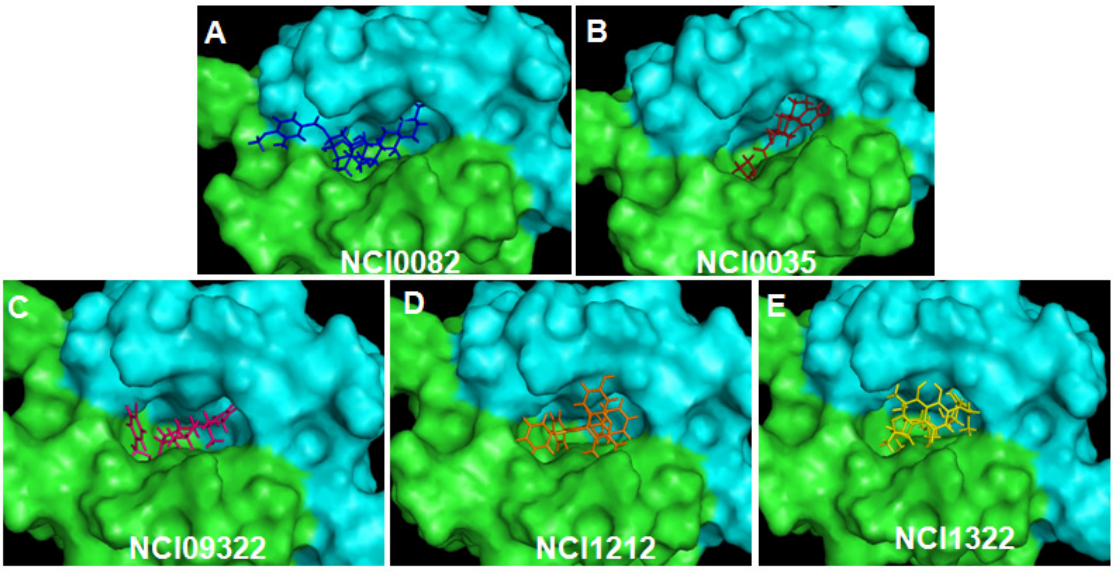


Fig 3–Three dimensional structure of NS1 RBD protein of KU14 complex with each of the five compounds with the lowest binding energy. The compounds bind within the central groove and interact with the dimer interface of the RBD. The structures of the compounds are given in Table 2.

at a concentration of 10 mg/ml were able to inhibit the binding between NS1 RBD and the labeled dsRNA probe leading to alteration of probe mobility (Fig 6). These herbal extracts were further tested for their ability to inhibit viral infectivity in a cell culture system. Four of the herbal extracts (*Terminalia belirica, Salacia*

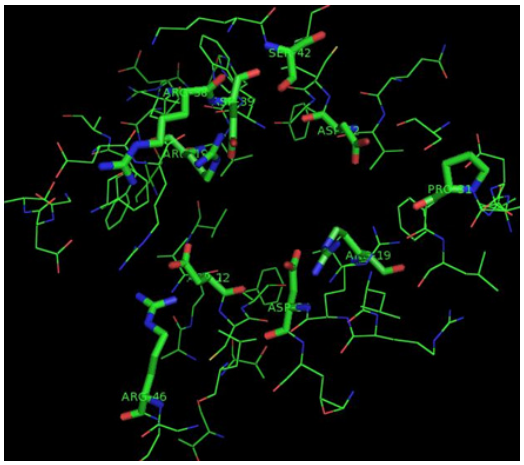


Fig 4–Amino acid residues within and around dsRNA binding groove of NS1 RBD protein. Thick sticks represent the residues that are able to form H-bond with the 5 compounds listed in Table 2. The other residues (thin sticks) represent amino acids that are within 5Å distance of the H-bond forming residues.

Table 3
Percent reduction of avian influenza virus infected foci when treated with herbal extracts.

Herb	Concentrations (µg/ml)	% reduction
Curcuma xanthorrhiza	200	44.4
	4,000	42.3
Terminalia belirica	200	37.2
	4,000	60.7
Salacia chinensis	200	17.3
	4,000	67.3
Zingiber montanum	200	52.6
	4,000	64.3
Peltophorum pterocarpum	200	21.4
	4,000	58.7
Vitex negundo L.	200	14.3
	4,000	24.5

chinensis, *Zingiber montanum* and *Peltophorum pterocarpum*) had inhibitory effect as they reduced the infected foci > 50% at 4 mg/ml (Table 3). Additionally, *Zingiber montanum* could inhibit 52.6% of avian influenza virus infection at a lower concentration (200 µg/ml) compared to other herbs. The herbal extract that could not inhibit the binding of NS1 RBD and the dsRNA probe, *Vitexnegundo L.*, did not show any inhibitory effect at all concentrations used.

DISCUSSION

The screening assay for NS1 RNA binding inhibitors was successfully developed by using combination of dsRNA-protein binding assay and EMSA. One key composition of the assay is the NS1 protein. In this study, the N-terminal 72 amino acid RBD of NS1 was used instead of the full-length NS1 protein because cloning and expression of the full-length NS1 gene resulted in water insoluble protein of 26kDa (Manastienkij *et al*, 2008). The denatured and re-folding forms of the NS1 protein could not interact with the dsRNA in the RNA binding assay. Indeed, an X-ray crystallographic study of NS1 protein of AIV H5N1 demonstrated that mutation of arginine at position 38 and lysine at position 41 could prevent the protein accumulations (Bornholdt and Prasad, 2008). However, arginine and lysine in both positions are necessary for dsRNA binding reaction. Therefore, RNA binding domain (NS1 RBD), a 10 kDa protein with α-helix homodimer structure, was cloned and heterologously expressed in *E. coli* in a water soluble native state that could bind to dsRNA (Chien *et al*, 2004) allowing EMSA to be performed. As a result, signal detected from the DIG-labeled dsRNA probes was shifted slower in the binding reaction containing NS1 RBD and RNA.

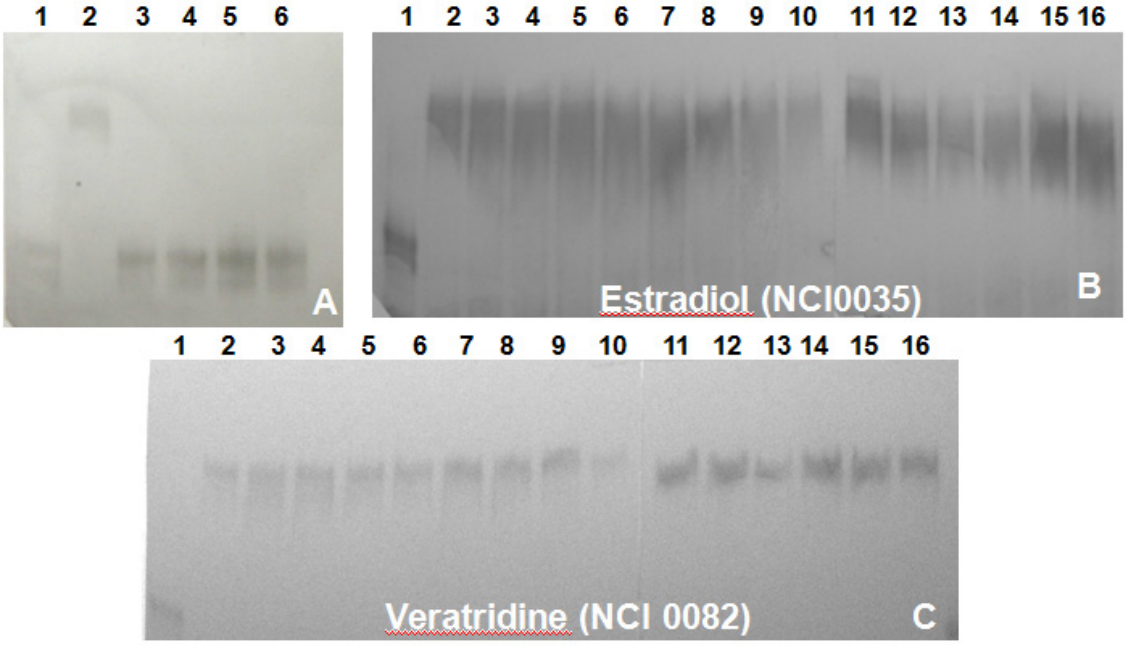


Fig 5–Electrophoresis mobility shift assay of DIG-labeled dsRNA and RBD NS1 protein. A. DIG-labeled dsRNA binding with 1 µM NS1 RBD (lane2) which migrates slower than dsRNA alone (lane1). The dsRNA did not interact with 1 µM trypsin, 1 µM chymotrypsin,1 µM BSA and 1 µM histidine fused protein (lanes3-6). B and C. RNA binding reactions containing fixed amounts of DIG-labeled dsRNA and NS1 RBD protein reacting with different concentrations of either estradiol (NCI0035) or veratridine (NCI0082). Lanes 1-3, dsRNA alone, dsRNA-NS1 RBD complex, and dsRNA-NS1 RBD protein complex with 10% DMSO, respectively. Lanes 4-16, dsRNA-NS1 RBD complex with 10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1,000 µM, respectively of estradiol (B) or veratridine (C).

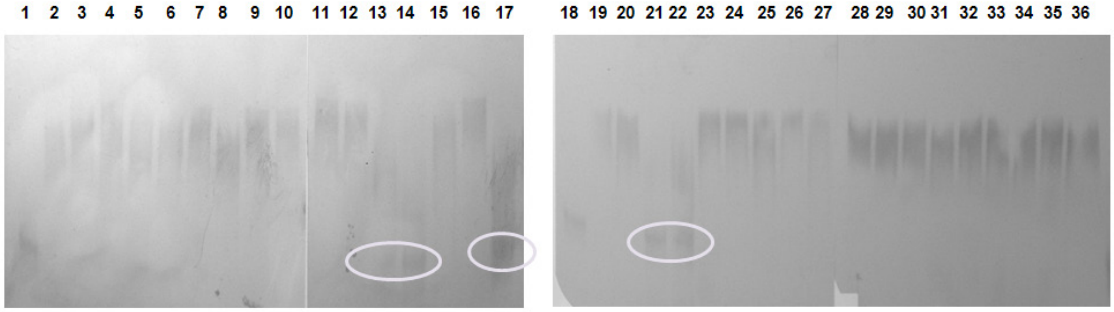


Fig 6–Electrophoretic mobility shift assay of herbal extracts with RNA binding activity to RBD NS1 protein. Lanes 1 and 18,DIG-labeled dsRNA control. Lanes 2-17 and 19-36, 10 mg/ml herbal extracts in the RNA binding reaction composed of DIG-labeled dsRNA and RBD NS1 protein.

This study revealed five lead compounds available from NCI database that could fit within the central groove of the RBD dimer. However estradiol (NCI0032) and veratridine (NCI0082), two commercially available compounds, could not inhibit the binding between dsRNA an NS1 RBD protein. This finding was not artifact as both RNA binding assay and EMSA systems were also used to screen 34 herbal extracts and demonstrated that five of them inhibited binding between the DIG-labeled dsRNA and RBD NS1 protein (Fig 6). The lack of the inhibitory property may be due to the conformational change of the compounds that occurs upon binding to the protein. According to the induced fit theory, both ligand and protein can reorganize themselves in order to adopt the best binding shapes. Most flexible drug-like molecules rarely bind the target protein in their lowest energy state (Perola and Charifson, 2004). Another explanation for the inhibition failure is that the binding of the compounds did not directly interfere with the RNA binding site. Some studies also demonstrated that even though the computational prediction of protein-ligand binding seems to be effective, the outcome of the binding reaction may not appear as expected. For instance, the binding between arabinose-binding protein variants and serotonin, a high-affinity designed ligand, did not occur as predicted since the computational algorithms may not capture all the aspects that are important for the prediction of binding by small molecules (Schreier *et al*, 2009).

In summary, drug-like molecules that could bind within and around the central groove of NS1 RBD were identified by using virtual screening. Even though the selected compounds could not inhibit the RNA binding activity of NS1, they may still be useful as lead molecules to develop chemically modified compounds with higher binding affinity or better inhibitory effect. In addition, the 5 herbal extracts that could inhibit RNA binding activity of NS1 and infection of avian influenza virus in cell culture system are worthy for further study to identify their antiviral active components.

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