Novel Piperine compound AB05 (N-5-(3,4-dimethoxyphenyl)-2E,4E pentadienylpiperidine) inhibits H1N1 influenza virus propagation in vitro

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Abstract. Pandemic H1N1 influenza virus respiratory illness has become an inevitable global health concern. With antigenic drift, it becomes necessary to have drugs over tailor-made H1N1 vaccine every year. In the current study, we screened many Piperine derivative in which, N-5-(3,4-dimethoxyphenyl)-2E,4E-pentadienylpiperidine (AB05) and was further studied for anti-H1N1 influenza virus activity and compared with other stains in-vitro on MDCK cell line. Initial cytotoxic doses of AB05 for the MDCK cell line were > 25µM. The results showed a dose-dependent reduction of the viral plaque's in the adsorption assay with EC50 of 0.33 µM. The mechanism of AB05 was by inhibition of matured viral release as evaluated by the time of virus addition with incubation of 6-10 hours. With the promising H1N1 virucidal activity of AB05, we included various strains of human influenza virus to screen AB05 inhibition of Neuraminidase (NA). The result showed 70% NA inhibition in WSN (H1N1), 90% in H3N2 & Influenza B and 49% in Tamiflu resistant H1N1). Further our In silco docking studies substantiated experimental results by showing the difference in binding and cooperation between H1N1 and N3N2. Together these observations illustrate that Piperine derivative AB05 is a promising lead molecule which needs further evaluation in animal models.

INTRODUCTION

The outbreak of H1N1 influenza infection in Saudi Arabia has become an inevitable pediatric infectious disease, which requires huge public health attention and treatment strategy (Alsufiani et al., 2014; Altayep et al., 2017). The pandemic outbreaks of H1N1 recorded in the European Union (EU) and other parts of the world as per the WHO records known to have a potential impact on the health, social and economic conditions of the society (Stoto, 2017). Further influenza has been recognized as priority research in the Priority Medicines Report in many of the developed and developing countries. The highlighted priority research areas of H1N1 influenza include low vaccine uptake and production capacity, expensive antiviral agents, updated influenza virus vaccine and antiviral agents screening.

The influenza virus is known for high mutation rate and complementary matching of the circulating virus and vaccine strains
must be the same for successful vaccination. Further the available antiviral agents for influenza are costly and many times not available in adequate quantity (Al-Qahtani et al., 2017). Since the vulnerable population are the pediatric age group (0-4 years) the toxicity of the antiviral drugs amounts to the morbidity and adds to the burden of disease management. In recent years there are many natural product lead molecules which have shown potential antimicrobial activities. Piperine compounds are one such group with the profound biological activities. Though these compounds are known to possess antibacterial and anti-parasitic activities but not much has been known for their virucidal action. Hence in the current investigation, we have selected the Piperine compound to be screened for anti-influenza virus activity owing to the worldwide disease burden of H1N1 influenza virus infections.

The conventional approach to drug design and development is based on the dissection of the pathogen using biochemical, immunological and microbiological methods (Wang et al., 2017). Research into new drug development is particularly important, as current Influenza vaccines are very slow and expensive to produce. Moreover, it has to be reformulated every year. Each year the influenza virus changes its genome and different strains become dominant, due to the high mutation rate of the virus (Pauly et al., 2017; Tewawong et al., 2017).

The sequencing of the influenza genome and recombinant DNA technology will accelerate the generation of the new antiviral drug by allowing to substitute new antigens into a previously developed strain (Piralla et al., 2017). The availability of the complete genome sequences in combination with novel advanced technologies, such as bioinformatics, microarrays, and proteomics, has revolutionized the approach to drug development and provided a new impetus to microbial research. The genomic revolution allows the design of the drug starting from the prediction of all antigens in silico, independently of their abundance and without the need to culture the pathogen (Zhu et al., 2017; Skowronski et al., 2017).

In the past two decades many naturally occurring compounds which are obtained from food, herbs, and small molecule libraries. They are found to be good therapeutic agent to treat wide variety of ailments and diseases reported in alternative or traditional medical practices (Hu et al., 2005). Further, their isolation, characterization, and usefulness fascinate several researchers to find out novel and efficient methods for in-vitro synthesis. Finally, it paves the way for the synthesis of analogs of those particular components, however, which may or may not be biologically active when compared to the naturally occurring ones. Piperine [(E, E)-1-piperoylpiperidine], a dieneamide with a molecular formula-C_{17}H_{19}N, isolated mainly from black pepper (Piper nigrum L) (Khan et al., 2016). The current study is focused on the antiviral activity of one of the derivative of AB05 against four H1N1 influenza virus isolates.

**MATERIALS AND METHODS**

**Viruses and cell culture**

Influenza virus strains A/WSN/33 (H1N1), A/Virginia/2009 (H1N1), A/PR/8/34 (H1N1), A/Aichi/2/68 (H3N2) and B/Florida/4/2006 virus stocks purchased from the American Type Culture Collection (ATCC) were used in this study. Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen), 2 mM L-glutamine (Gibco), and a nonessential amino acid mixture (NEAA; 0.1 mM; Gibco).

**Cytotoxicity assay**

The cytotoxicity assay was performed as described previously elsewhere (Alsufiani et al., 2014). Briefly, MDCK cells in 96-well plates were treated with serial dilutions of AB05 and then incubated at 37°C for 72 h. We added 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Molecular Probes, USA) in order to
determine the cytotoxicity. The absorbance was measured at 570 nm using a microplate reader. The CC50 of AB05 was defined as the induction of 50% cell death (Hosseini et al., 2012).

**EC50 assay**
The EC50 was determined as described previously (Hsieh et al., 2012). Briefly, 9× TCID50 A/WSN/33 virus were challenged with MDCK cells in the presence of serial dilutions of AB05. Post 72 h, the infected cells were morphologically examined for cytopathic effect (CPE) and fixed with 4% paraformaldehyde (PFA) and stained with crystal violet. The cell density was measured using a VICTOR3 Multilabel Plate Reader (PerkinElmer, USA). The EC50 was defined as the concentration that inhibited 50% of virus-induced CPE (Kodama et al., 1996).

**Plaque Inhibition assay**
MDCK cells (6×10^5 cells/well) were seeded into 6-well plates and incubated for 24 h at 37°C. After washing the cells with 1% PBS, followed by infection with Influenza A virus (WSN) of 50 PFU/well. After 1 h of viral adsorption at 37°C, the cells were washed twice with 1% PBS. The cells were then overlaid with DMEM containing 0.3% agarose with the indicated concentrations of compound AB05 (N-5-(3, 4-dimethoxyphenyl)-2E, 4E-pentadienylpiperidine) (1 µM, 2 µM, 3 µM, and 4 µM). After incubation for 48 h at 37°C under 5% CO2, the cells were fixed with 10% formaldehyde followed by staining with 1% crystal violet. The numbers of plaques were counted, and the antiviral activity of the compounds was calculated with respect to the virus control (Derksen et al., 2016).

**Time-of-addition assay**
MDCK cells in six-well plates were infected with influenza virus A/WSN/33 (MOI = 0.01) for 1 h. After infection, the medium was discarded and cells were washed with PBS three times. Then, AB05 (3 µM) added to cells at -2 h, 0 h, 2 h, 4 h, 6 h, 8 h, and 10 h after infection. The supernatants were harvested at 12 h post-infection and viral titers were determined by plaque-forming assay (Furuta et al., 2005).

**Neuraminidase inhibition assays**
The Neuraminidase (NA) activity of influenza A and B viruses was measured by a fluorescence-based assay using the fluorogenic substrate MUNANA (Sigma-Aldrich, St Louis, MO), based on the method described previously (Alsufiani et al., 2014). Two-fold virus dilutions were prepared in enzyme buffer [32.5 mM of 2-(N-morpholino)ethanesulfonic acid (MES), 4 mM of calcium chloride, pH 6.5] and added (100 µL/well) in duplicate to a flat-bottom 96-well opaque black plate (Corning, Tewksbury, MA). After pre-incubation for 20–30 min at 37°C, the MUNANA substrate (separately pre-incubated for 20–30 min at 37°C) was added to all wells (50 µL/well) to achieve a final concentration of 100 µM. Immediately after adding the MUNANA substrate, the plate was transferred to a 37°C pre-warmed Synergy-2 multi-mode microplate reader (BioTek Instruments, Winooski, VT) using excitation and emission wavelengths of 365 and 460 nm, respectively. The fluorescence signal from the enzyme buffer and MUNANA substrate alone in the absence of enzyme was subtracted as background from the signals obtained in the other wells. The drug concentration required to inhibit 50% of the NA enzymatic activity (IC50) was determined by plotting the percent inhibition of NA activity as a function of the compound concentration calculated from the dose-response curve (Woods et al., 1993; Marathe et al., 2013; Fage et al., 2017).

**Computational modeling and docking**
The amino acid sequence of neuraminidases from H1N1 or H3N2 were retrieved from Uniprot database (https://www.uniprot.org/). Protein BLAST against PDB database predicted reasonable templates for both H1N1 and H3N2 NA (PDBid: 4b7q and 3tia respectively). Templates were retrieved from PDB databank (https://www.rcsb.org/). Modeller9.19 was used to model the three dimensional structure of NA using the above-mentioned respective templates. For computational docking studies, AB05 was prepared using ChemSketch, other known NA inhibitors were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). All
the molecules including receptor (neuraminidase) were prepared according to autodock format using tools provided by MGL tools. Computational docking was performed using Autodock-VINA and results were analyzed using Discovery Studio visualizer from BIOVIA.

RESULTS

Initial cytotoxic assessment of all the five different piperine derivatives was undertaken in the MDCK cell line. Almost all the five compounds showed no cytotoxicity by both morphological assessment and MTT assay up to a concentration of 25 µM and above. Further, all the five different piperine derivative compounds were screened for anti-influenza virus activity against A/WSN/33 strain by neutralization assay (Table 1). The structure of AB01-AB04 was shown in supplementary Figure 1. The selection of the strain A/WSN/33 for initial screening is due to its virulence and as well as for its endemic nature (H1N1). The piperine derivative which inhibited the virus propagation by 50% as evidenced by cytopathic effect (CPE) which was observed with cell culture was selected for further antiviral investigations. However, one compound N-5-(3, 4-dimethoxyphenyl)-2E, 4E-pentadienyl piperidine also known as AB05 showed significant inhibition A/WSN/33 strain. The CC50 of AB05 was 25.1 ± 0.5387 µM and selective index 76. The chemical structure is depicted in Figure 1.

The AB05 was later escalated to be screened for other type of Influenza A virus (H3N2), and Influenza B virus of the influenza strains and oseltamivir was used as a control. The observed result showed that the AB05 compound reduced the plaque numbers in a dose-dependent manner similar to oseltamivir and the results are given in Figure 2. We next analyzed the efficiency of AB05 against A/WSN/33 (H1N1), 50 PFU / well by using plaque reduction assays. Various concentrations of AB05 (N-5-(3, 4-dimethoxyphenyl)-2E, 4E-pentadienyl piperidine) (1 µM, 2 µM, 3 µM, and 4 µM) was used to cover the infected monolayer with medium and it was incubated for 48 h. We observed that the plaque numbers diminished in a dose-dependent manner and the EC50 values were 0.33 µM as revealed in Figure 3.

The time of addition assay was conducted to elucidate the antiviral mechanism of AB05 in a single infectious cycle using influenza A/WSN/33-infected in MDCK cells. Figure 4a plots the time of the addition of AB05 during the course of the viral infection. After 12 h of post-infection, the supernatant was collected to determine the viral titer using a plaque assay. The results showed that the AB05 reduced the viral titers at the late stage (6 to 10 h) by blocking the release of progeny

Table 1. Anti-Influenza virus strains (A/WSN/33/H1N1) activities of Piperine derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (µM)</th>
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<tbody>
<tr>
<td>AB01</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>AB02</td>
<td>0.83 ± 0.16</td>
</tr>
<tr>
<td>AB03</td>
<td>0.78 ± 0.45</td>
</tr>
<tr>
<td>AB04</td>
<td>1.69 ± 0.95</td>
</tr>
<tr>
<td>AB05</td>
<td>0.33 ± 0.03</td>
</tr>
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Data are presented as means ± standard deviations of results from at least two independent experiments.

Figure 1. Structure of N-5-(3, 4-dimethoxyphenyl)-2E, 4E-pentadienyl piperidine (AB05).
Figure 2. a. Influenza A virus H3N2, b. Influenza B virus-MDCK cells were incubated with the influenza virus at about 100 PFU / well. After adsorption of the virus for 1 h at 37°C, the cells were then overlaid with DMEM containing 0.3% agarose with the 1- 4 nM of oseltamivir and 1- 4 µM of AB05 compound concentrations. After incubation for 48 h, the plaque numbers were diminished in a dose-dependent manner.

Figure 3. Inhibition of Influenza A virus.
MDCK cells were incubated with influenza virus (A/WSN/33 (H1N1) and Tamiflu resistant H1N1 at about 50 PFU / well. After adsorption of the virus for 1 h at 37°C, the cells were then overlaid with DMEM containing 0.3% agarose with the indicated compound concentrations. After incubation for 48 h, the plaque numbers were diminished in a dose-dependent manner and reduced size in Tamiflu resistant H1N1.
Figure 4. Time of addition assay.

MDCK cells were infected with influenza virus A/WSN/33 (H1N1) (MOI = 0.01). At post-infection, the medium was discarded and cells were washed with PBS three times. AB05 (3 µM) was added at 0 h, 2 h, 4 h, 6 h, 8 h, and 10 h after infection. At 12 h post-infection, the supernatants were collected and infectious titers were determined by plaque assay. Statistical significance was assessed by a comparison between the AB05-treated group and the virus control group by using a student’s t-test analysis (**, p-value, 0.01 and ***, p-value, 0.05).

Virions (Figure 4b). These results suggested that the antiviral mechanism of AB05 was through inhibition of the late stage of the influenza virus which is interfering with the virus release step.

The NA assay using MUN as substrate was based on classical methods, with the exception that water was used in place of the DMSO for diluting the inhibitors and the concentration of MUN used was 20 mM. The purified NA was titrated by serial twofold dilutions in 32.5 mM Mes, pH 6.5, and 4 mM CaCl₂ and the concentration of NA against NA activity was plotted graphically to determine the enzyme concentration to be used in subsequent inhibition assays (data not shown). An exact concentration of the enzyme stock was determined by using Tamiflu (5 nM) (Oseltamivir) as an active site titrant. 4-Methylumbelliferone was quantified by fluorometric determination using a Synergy 2 multi-mode microplate reader with an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Wells were read at a rate of 1.0 s per well. The inhibited effects were compared with the untreated virus along with Oseltamivir.

The NA inhibition assay result expressed that the AB05 inhibited various types of Influenza viruses with the IC₅₀ ranges from 1–3.5 µM. AB05 inhibited the NA activity of 70% of WSN (IC₅₀ 3.5±0.88 µM), 90% of H₃N₂ (IC₅₀ 1.5±0.356) and Influenza B (IC₅₀ 1.2±0.325) and 49% of Tamiflu resistant H₁N₁ (IC₅₀ 65±5) (Figure 5).

In order to assess the interaction efficacy of AB05 with NA, we modelled the three dimensional structure of NA from A/WSN/33 (H1N1), and A/Aichi/2/68 (H3N2) (Figure 6 a and c). As there is a difference in the inhibitory activity of AB05 between H1N1 and H3N2, we wanted to check the binding efficacy of AB05 with NA modelled from both the strains. The Homology modeling predicted the three dimensional structure of NA from both the strains (Fig. X a-d). Structural superimposition indicates the root mean square difference between modelled NA structures from H3N2 and H1N1 is less than 2Å (1.50). Modelling shows the active site or the known NA inhibitor-binding site in the NA structure (Fig. 6 b and d).

We then performed computational docking of AB05 along with other known NA inhibitors to the known inhibitor-binding site at the NA (Figure 7 a and c). Results indicate AB05 binds efficiently with both NA modelled from H1N1, and H3N2 strains. Binding modes predicted by computational docking of other known inhibitors including Laninamivir (H3N2) or Zanamivir (H1N1) corroborates with the experimental models. Based on the docking studies, AB05 binds with the highest efficiency towards H3N2 when compared to
Figure 5. AB05 inhibits NA enzymatic activity.
AB05 inhibited the enzymatic activity of NA derived from WSN (H1N1), Tamiflu Resistant (H1N1), H3N2, and Influenza B viruses. Data points are presented as means ± SD of triplicated experiments.

Figure 6. Structural modeling of neuraminidase.
(a) Homology modeling of NA protein from I3N2. (b) Dorsal view of inhibitor binding site. (c & d) Modelled structure of NA from H1N1 and its inhibitor binding site respectively.
all other known inhibitors or NA from H1N1 (Fig. 7 b and d).

DISCUSSION

Globally, seasonal influenza causes between 250,000 and 500,000 deaths every year (WHO, 2009). Influenza is not only a disease of great medical importance but also of economic importance. At present, vaccination and antiviral drugs are principle strategies to prevent influenza. Available anti-influenza drugs include inhibitors of NA (e.g. oseltamivir and zanamivir) (Huang et al., 2008), M2 proton channel (amantadine and rimantadine) (Wang et al., 1993), and RNA-dependent RNA polymerase (RdRp) (favipiravir) (Baranovich et al., 2013). However, influenza vaccines must be reformulated each year due to the constant antigenic evolution of influenza. Additionally, NA inhibitors and M2 ion-channel inhibitors have limited efficacies as drug resistance develops over the period of time (Hurt et al., 2009; Seibert et al., 2010).

Natural products, which is derived from the traditional medicine, have been found to exert antiviral effects against the influenza virus (Newman & Cragg, 2010). One of the commonly used spices is fruits of black pepper (Piper nigrum) which has been used to treat asthma, colon toxins, obesity, sinus, congestion, chronic indigestion, and fever (Council of Scientific and Industrial Research, 1989). Concerning the antiviral effect, the compound of Piperine was found to inhibit Coxsackievirus type B3 (CVB3) (Mair et al., 2014). At this stage, the current study demonstrated that the antiviral activity of Piperine have the potential herbal remedy to treat and boost the immunity along with other therapies against the influenza viruses.

The present study evaluated the antiviral efficacy of Piperine and observed that the compound selected (AB05) showed inhibition
Supplementary Figure

1) AB01

N-5-(4-methoxyphenyl)-2E, 4E-pentadienoylpiperidine

2) AB02

N-5-(phenyl)- 2E, 4E- pentadienoylpiperidine

3) AB03

N-5-(2,3,4-trimethoxyphenyl)-2E, 4E-pentadienoylpiperidine

4) AB04

N-5-(4-hydroxyphenyl)-2E,4E-pentadienoylpiperidine

of the various influenza viral strains at different time points and concentrations. After MTT assay at very low concentration, antiviral activity showed and found to be associated with decreased viral growth. AB05 has been tested on Influenza A virus (H1N1 and H3N2) and Influenza B virus for its antiviral activity and the potential effect of AB05 were associated with growth inhibition on different concentration similar to oseltamivir. MDCK cells were infected with influenza virus was further treated with AB05 of 3 µM in a time-dependent manner after the last 12 h infection. We observed a significant
reduction in the late stage of influenza virus interfering with virus release step and plaque in comparison to the untreated group. Significant reduction in NA enzyme activity was observed on the treatment of AB05 on cell line infected with different influenza viruses which slow down the viral proliferation.

Computational modelling and docking of AB05 with neuraminidase shows reasonable binding affinities towards both NA (H3N2), and NA (H1N1). However, AB05 shows very high affinity towards NA (H3N2) compared to all other known NA inhibitors or with NA (H1N1). It is in line with our experimental results where AB05 shows 90% inhibition in H3N2 when compared to 70% inhibition in H1N1. This suggests that although there is no significant structural difference between the modelled NA of H1N1 and N3N2 (RMS < 1.50), there could be subtle structural changes, probably near the active site, that could contribute to the strong affinity of AB05 towards H3N2 compared to H1N1. Our results were in agreement with similar in silico docking studies done elsewhere (Deepak et al., 2012) with only difference that we blinded our screening without the results of the docking studies.

Piperidine-based derivatives were identified as novel and potent inhibitors of the influenza virus through structural modification of the original compound (Guoxin et al., 2014). A series of N-substituted piperidine derivatives was able to block the HA and M2 proton channel of Influenza A Virus (Stephen et al., 1999; Deepak et al., 2012). An N-substituted piperine CL-385319 is interfering with the fusogenic function of the Influenza viral hemagglutinin (Liu et al., 2011). The AB05 were not neutralized by the viral Hemagglutinin (data not showed). The AB05 were N-substituted with methoxyphenyl inhibits the viral NA and oseltamivir-resistant NA, rather than other N-substitution piperine-based derivatives which inhibit the viral Hemagglutinin. A novel methoxyphenyl substituted piperidine AB05 exhibit a potent anti-influenza and promising lead molecule which is well substantiated by the in silico docking studies to be further escalated to animal models.

CONCLUSION
The AB05 has shown varied activities against different strains of the Influenza virus. It may be noted that the piperine compound works mechanistically by inhibiting the virus release. It has also shown the NA inhibition, which allow these compounds to work at different stages of the virus replicative cycle. Together these results suggest that AB05 can be promoted to the next stage of animal studies for an ideal anti-influenza viral drug. Further, other piperine derived (in general) compounds can be screened for anti-influenza virus activity to minimize the risk of antiviral drug resistance.

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Conflicts of interest
The authors declare that they have no conflicts or competing interests.

REFERENCE


