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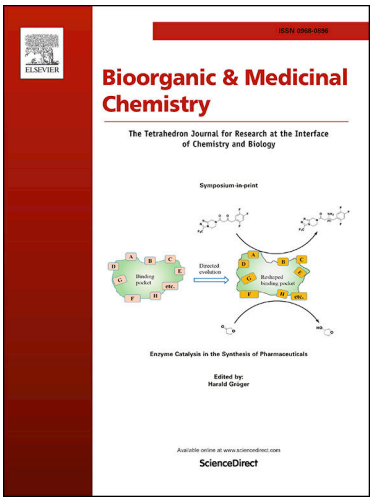

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Design, Synthesis and Biological Evaluation of Substituted Flavones and Aurones as Potential Anti-Influenza Agents

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ABSTRACT

We designed a series of substituted flavones and aurones as non-competitive H1N1 neuraminidase (NA) inhibitors and anti-influenza agents. The molecular docking studies showed that the designed flavones and aurones occupied 150-cavity and 430-cavity of H1N1-NA. We then synthesized these compounds and evaluated these for cytotoxicity, reduction in H1N1 virus yield, H1N1-NA inhibition and kinetics of inhibition. The virus yield reduction assay and H1N1-NA inhibition assay demonstrated that the compound **1f** (4-methoxyflavone) had the lowest EC_{50} of 9.36 nM and IC_{50} of 8.74 μM respectively. Moreover, kinetic studies illustrated that compounds **1f** and **2f** had non-competitive inhibition mechanism.

Keywords: Flavone, Aurones, H1N1, Neuraminidase, Non-competitive, Influenza.

1. INTRODUCTION

Influenza A virus (IAV), a single-stranded (−) RNA virus that triggers annual seasonal influenza epidemics and occasional global pandemics, is the leading cause of morbidity and mortality worldwide. IAV is classified according to the antigenicity of their envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA). HA regulates viral binding to host cell sialic acid (SA) receptors and aids the fusion of viral and endosomal membranes to allow entry of the viral genome into host cells. NA mediates release of the newly formed virus particle from the host cell by cleaving the glycosidic linkage between SA-linked host cell-surface receptor and HA, facilitating the spread of infection to other host cells.[1] The distinct antigenic properties of different HA and NA molecules are used to classify influenza type A viruses into subtypes: eighteen for HA (H1-H18) and eleven for NA (N1-N11).[2] Among various circulating subtypes of IAV, influenza A (H1N1) virus has repeatedly caused pandemics in 1918, 1977 and 2009.[3] In 1918, the most lethal pandemic in history, resulted in around 50 million deaths worldwide.[4] The pandemic form of influenza is expected to reappear.[5]

The current anti-influenza drugs, neuraminidase (NA) inhibitors such as oseltamivir (OMV) and zanamivir (ZMV) , are transition state analogs of sialic acid (SA) [6], and both the drugs have comparable structures and hence similar binding interactions with NA. However, mutations in the NA enzyme have conferred resistance to these drugs.[7] Thus, search for molecules with newer scaffolds and different binding pattern in NA is imperative. Non-competitive inhibitors inhibit the enzyme by binding allosterically to the target enzyme. Thus, non-competitive inhibitors by their alternate binding mode could avoid the problem of resistance caused due to mutations. The alternate binding sites in NA could be 150-cavity and 430-cavity that are present adjacent to SA/OMV binding site. Compounds binding to 150-cavity have shown

antiviral activity against OMV resistant strains.[8] Furthermore, compounds projecting towards 430-cavity have demonstrated potent inhibition of various NA subtypes.[9] In our endeavour to search for NA inhibitors with alternate binding mode, we had reported a series of chalcones binding to 150-cavity as non-competitive inhibitors of H1N1-NA and anti-influenza agents. [10] In our attempt to further optimize the activity, we have selected flavone and aurone scaffolds that are the cyclized derivatives of chalcone, since rigidification by cyclization has been a common approach to increase the activity of a lead compound.[11] Interestingly, several plant-based flavones and aurones are reported to show H1N1-NA inhibition.[12]

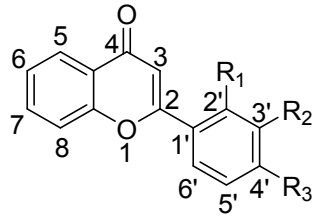
Considering the above facts, we have designed a series of substituted flavones and aurones [nine flavones (**1a**-**1i**) and nine aurones (**2a**-**2i**)] and studied their binding mode using computational studies. Furthermore, we have synthesized sixteen (eight flavones and eight aurones) compounds and assessed nine (six flavones and three aurones) of these compounds *in-vitro* for cytotoxicity, reduction of H1N1 virus yield, H1N1-NA inhibition. Additionally, the binding mechanism of the compounds to NA has been explored by kinetic experiments.

2. RESULTS AND DISCUSSION

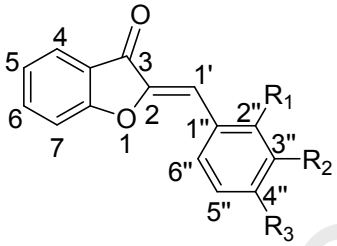
2.1. Computational studies

The docking validation studies could acceptably replicate (RMSD 0.5236) the binding pose of the co-crystallized ligand *viz.* ZMV in the H1N1-NA X-ray crystal structure (PDB ID: **3B7E**)[13]. We designed several substituted flavones and aurones (**Table 1**) by varying substituents on the phenyl ring that provide diverse lipophilic and electronic properties to the designed molecules.

Table 1: Designed substituted flavones (2-(substitutedphenyl)-4H-chromen-4-one) and aurones ((2*Z*) 2-(1-(substitutedphenyl)methylene)benzofuran-3(2H)-one)



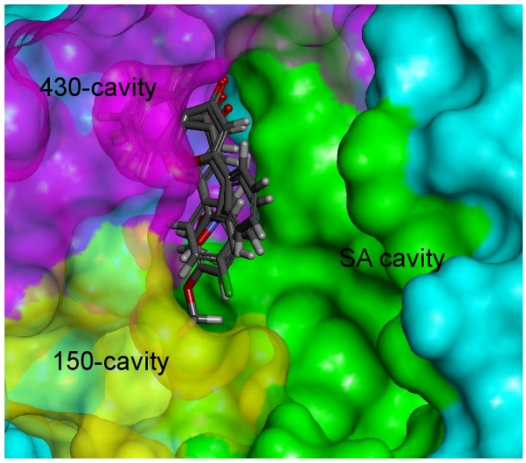
Substituted Flavones (1a-1i)



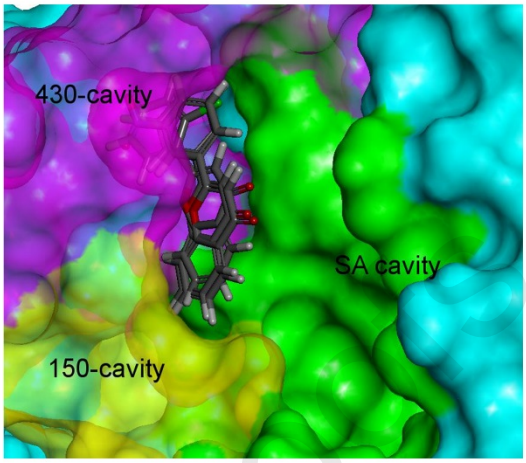
Substituted Aurones (2a-2i)

Sr. No.	Compound	R_1	R_2	R_3
1.	**1a**	Cl	H	H
2.	**1b**	H	Cl	H
3.	**1c**	H	H	Cl
4.	**1d**	OCH_3	H	H
5.	**1e**	H	OCH_3	H
6.	**1f**	H	H	OCH_3
7.	**1g**	NO_2	H	H
8.	**1h**	H	NO_2	H
9.	**1i**	H	H	NO_2
10.	**2a**	Cl	H	H
11.	**2b**	H	Cl	H
12.	**2c**	H	H	Cl
13.	**2d**	OCH_3	H	H
14.	**2e**	H	OCH_3	H
15.	**2f**	H	H	OCH_3
16.	**2g**	NO_2	H	H
17.	**2h**	H	NO_2	H
18.	**2i**	H	H	NO_2

These designed flavones and aurones were then docked in H1N1-NA catalytic site with a view to examine the effect of various substituents on the binding mode. Docking studies with the designed flavones and aurones revealed that all compounds dwelled in 150-cavity and 430-cavity (**fig. 1**) of the catalytic site comparable to previously reported chalcones.[10] This showed that the binding pattern was not altered due to cyclization of chalcone to flavone and aurone that had alternate binding mode similar to chalcones.


430-cavity
SA cavity
150-cavity

a


430-cavity
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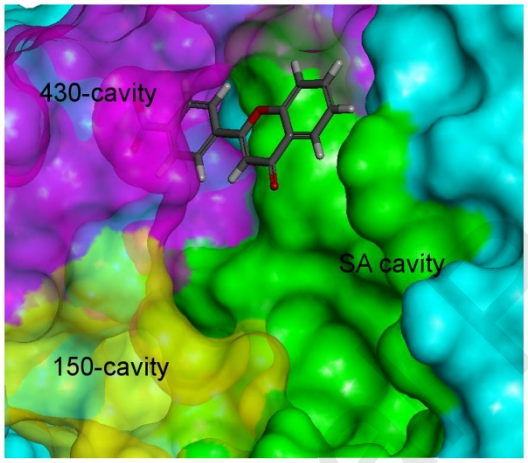
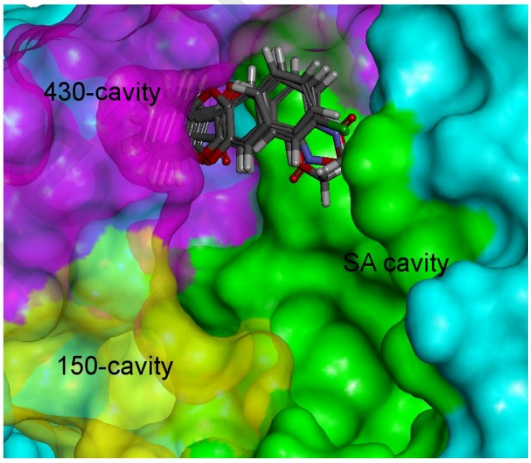
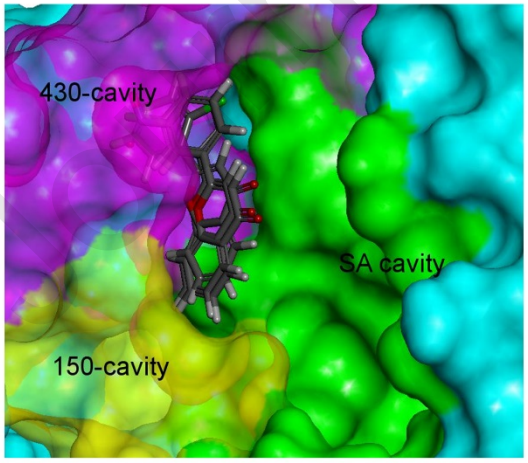
b


430-cavity
SA cavity
150-cavity

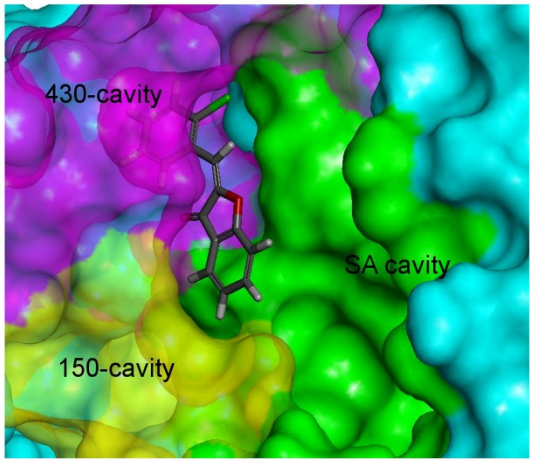
c


430-cavity
SA cavity
150-cavity

d


430-cavity
SA cavity
150-cavity

e


430-cavity
SA cavity
150-cavity

f

Fig. 1 Docked conformation of compounds (a) **1c-1g**, (b) **1a**, **1b** & **1h**, (c) **1i**, (d) **2b-2e**, **2g** & **2h**, (e) **2f** & **2i**, (f) **2a** in H1N1-NA

The docked conformation of compounds **1c-1g** revealed that the chromenone ring of these compounds had hydrophobic interactions with the sidechains of Trp403, Ile427, Pro431, Lys432 (**fig. 1a**). However, the compounds **1a**, **1b**, **1h** had reverse orientation compared to compounds **1c-1g** owing to the electrostatic interaction and hydrophobic interaction of chromenone ring with Arg118 and Val149, respectively (**fig. 1b**). The compound **1i** had different docked pose than other designed flavones, in which the *p*-nitro group on its phenyl ring had electrostatic interaction with Trp403 and Arg428, while its chromenone ring had hydrophobic interaction with Pro326 (**fig. 1c**).

The compounds **2b-2e**, **2g** and **2h** are docked (**fig. 1d**) such that the benzofuranone ring of these compounds occupied hydrophobic hole (Trp403, Ile427, Pro431, Lys432). Whereas, the binding conformations of compounds **2f** and **2i** had an opposite orientation compared to compounds **2b-2e**, **2g** and **2h** in which the *p*-methoxy group on the phenyl ring of **2f** occupied hydrophobic hole (Trp403, Ile427, Pro431, Lys432) while the *p*-nitro group on the phenyl ring of **2i** had electrostatic interaction with Trp403 and Arg428 (**fig. 1e**). Compound **2a** had an altered docked conformation in comparison with remaining designed aurones due to electrostatic interaction of its benzofuranone ring with Arg118 (**fig. 1f**). The detailed study of these non-bonded interactions of designed flavone and aurones with catalytic site residues is summarized in **Table S1** (see supplementary data)**.**

2.2. Chemistry

The target compounds **1a-1i** (substituted flavones) and **2a-2i** (substituted aurones) were synthesized via oxidative cyclisation of substituted 2′-hydroxychalcone using iodine in DMSO

[14] and mercuric acetate in pyridine [15] respectively using Scheme 1 (see supplementary data). The suitable 2′-hydroxychalcones were formerly prepared via the classical Claisen–Schmidt condensation [16] reaction between 2′-hydroxyacetophenone and appropriately substituted benzaldehydes in basic conditions using NaOH (20 % w/v aqueous solution, 5 ml).[17] The presence of *methoxy* / *chloro* group on B-ring of 2′-hydroxychalcone is favoured as compound **1a-1f** and **2a-2f** were obtained in comparatively higher yield than compounds **1h-1i** and **2h-2i** which have *nitro* substituent on the B-ring. The structures of the synthesized compounds were characterized by nuclear magnetic resonance spectroscopy (^{1}H NMR), infrared spectroscopy (IR) and mass spectroscopy (MS). In the ^{1}H NMR spectra of the synthesized compounds, chromenone ring proton (H-3) of flavone and vinylic proton (H-1′) of aurone appeared as singlets in the range of δ 6.95-7.28 and δ 6.91-7.20 respectively. The cyclic C-O stretch in flavone and aurone was observed near 1370 cm^{-1} and 1300 cm^{-1} respectively. The carbonyl C=O stretch in flavone was characterised by a band near 1700 cm^{-1}, while in aurone the band was near 1650 cm^{-1}. The IR band for endocyclic C=C stretch in flavone was near 1600 cm^{-1}, whereas the exocyclic C=C was near 1660 cm^{-1}. ^{13}C-NMR chemical shift for vinylic carbon (C-1′) of the synthesized aurones were in range of 107.29 - 113.23 [18] which confirmed the formation of thermodynamically more stable *Z* form of aurone. Mass spectra of all synthesized flavones and aurones gave the predicted $[M+H]^+$ peak corresponding to their estimated molecular mass.

2.3. *In-vitro* evaluation

The MTT-Formazan assay was used to evaluate the cytotoxicity of the synthesized compounds[19]. The concentration that led to a 50% reduction in cell viability (CC_{50}) was used to ascertain the cytotoxicity. Compounds **2b** and **2e** with CC_{50} values of 26.90 and 7.81 μM respectively, showed cytotoxicity. The anti-influenza effect of non-cytotoxic compounds was

tested for reduction of H1N1 virus yield in MDCK cells. Oseltamivir acid (OMVA) and quercetin (QR, reported as the most potent natural non-competitive inhibitor) [20] were used as the standard for competitive inhibition and non-competitive inhibition respectively. The concentration that resulted in 50% reduction in log_2HA titre (EC_{50}) was measured and used to determine the antiviral effect, **fig. 2**.

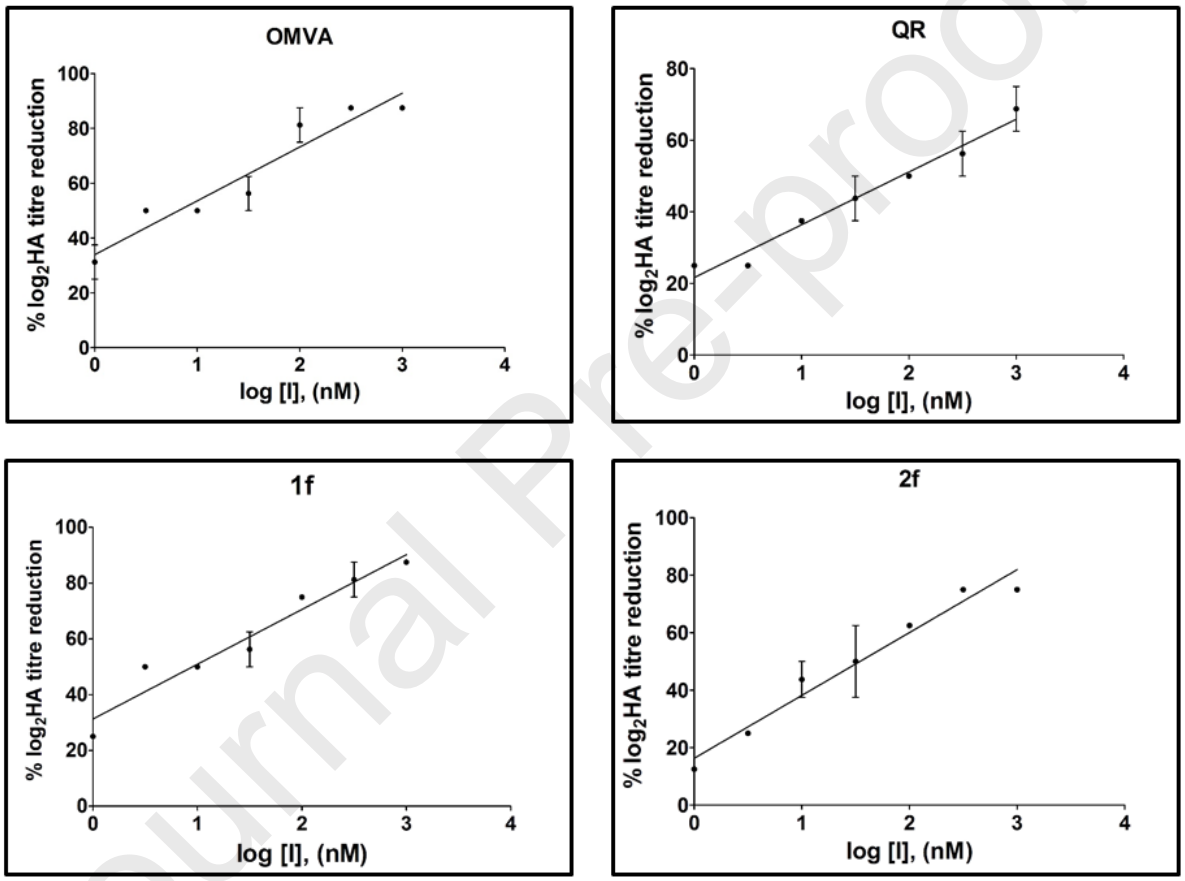


Fig. 2 Effects of Oseltamivir acid (OMVA), Quercetin (QR), compounds **1f** and **2f** on H1N1 virus yield

The virus yield reduction assay demonstrated that the designed flavones and aurones appreciably decreased the viral titer of H1N1 (**Table 2**).

Table 2: *In-vitro* evaluation of tested compounds.

Sr. No.	Compound	CC_{50} (µM)	EC_{50} (nM)	SI	IC_{50} (µM)
1.	**1a**	388±1.03	Not tested	-	Not tested
2.	**1b**	174.3±1.08	47.7±1.06	3654	32.60±1.15
3.	**1c**	115.4±1.07	35.9±1.12	3214	27.65±1.16
4.	**1d**	170.9±1.06	Not tested	-	Not tested
5.	**1e**	263.4±1.21	12.57±1.05	20955	10.76±1.09
6.	**1f**	191.8±1.08	9.36±1.04	20491	8.74±1.15
7.	**1g**	Not synthesized			
8.	**1h**	269.1±1.12	14.12±1.16	19058	15.36±1.20
9.	**1i**	162.5±1.09	11.93±1.07	13621	13.20±1.21
10.	**2a**	338.7±1.06	Not tested	-	Not tested
11.	**2b**	26.9±1.05	Not tested	-	Not tested
12.	**2c**	272.4±1.10	51.39±1.18	5301	45.36±1.13
13.	**2d**	273.2±1.09	Not tested	-	Not tested
14.	**2e**	7.81±1.01	Not tested	-	Not tested
15.	**2f**	169.4±1.07	35.28±1.10	4802	37.50±1.12
16.	**2g**	Not synthesized			
17.	**2h**	396.3±1.06	Not tested	-	Not tested
18.	**2i**	213.0±1.10	46.32±1.12	4598	41.00±1.19
19.	**QR**	253.8±1.08	84.46±1.03	3007	7.75±1.13
20.	**OMVA**	713.4±1.07	7.1±1.16	100478	6.48±1.13 x 10^{-4}

All compounds were examined in a set of duplicated experiment; CC_{50} (mean ±std error of mean) values represent the concentration that showed 50 % cytotoxicity; EC_{50} (mean ±std error of mean) values represent the concentration that resulted in 50 % $\log_2$HA viral titre reduction; SI = Selectivity Index was generated by the ratio of CC_{50} and EC_{50}; IC_{50} (mean ±std error of mean) values of compounds represent the concentration that caused 50% enzyme activity loss.

Compound **1f** (flavone with *p*-methoxy group on phenyl ring) had highest activity with EC_{50} value of 9.36 nM, comparable with the commercially used drug, OMVA (EC_{50} = 7.1 nM) while the compound **2c** (aurone with *p*-chloro group on phenyl ring) had the lowest activity with EC_{50} of 51.39 nM. The high selectivity index values of these compounds indicated that they decreased the

virus replication without adverse effect on the living host cells. H1N1-NA inhibition by the compounds was determined by calculating the concentration required to inhibit 50% of the enzyme activity (IC_{50}). The IC_{50} values of the evaluated flavones ranged from 8.74 to 32.60 μM, while aurones ranged from 37.50 to 45.36 μM (**Table 2**), **fig. 3**. A detailed analysis of H1N1-NA inhibition indicated that among tested flavones, *para*-substitution on the phenyl ring is preferred over *meta*-substitution since compounds **1c**, **1f**, **1i** showed relatively better H1N1-NA inhibition compared to their meta-isomers **1b**, **1e**, **1h**. In view of this, we tested only *para*-substituted aurones for H1N1 viral titre reduction and H1N1-NA inhibition. Furthermore, *methoxy* substitution on phenyl rings of flavone and aurone is favoured over *nitro* and *chloro* substitution as *methoxy* substituted compounds **1e**, **1f** and **2f** showed higher H1N1-NA inhibition than the *nitro* substituted (**1h**, **1i**, **2i**) and *chloro* substituted (**1b**, **1c**, **2c**) compounds. A similar trend in SAR is observed for H1N1 viral titre reduction assay. Overall, the substituted flavones exhibited better anti-influenza activity over substituted aurones.

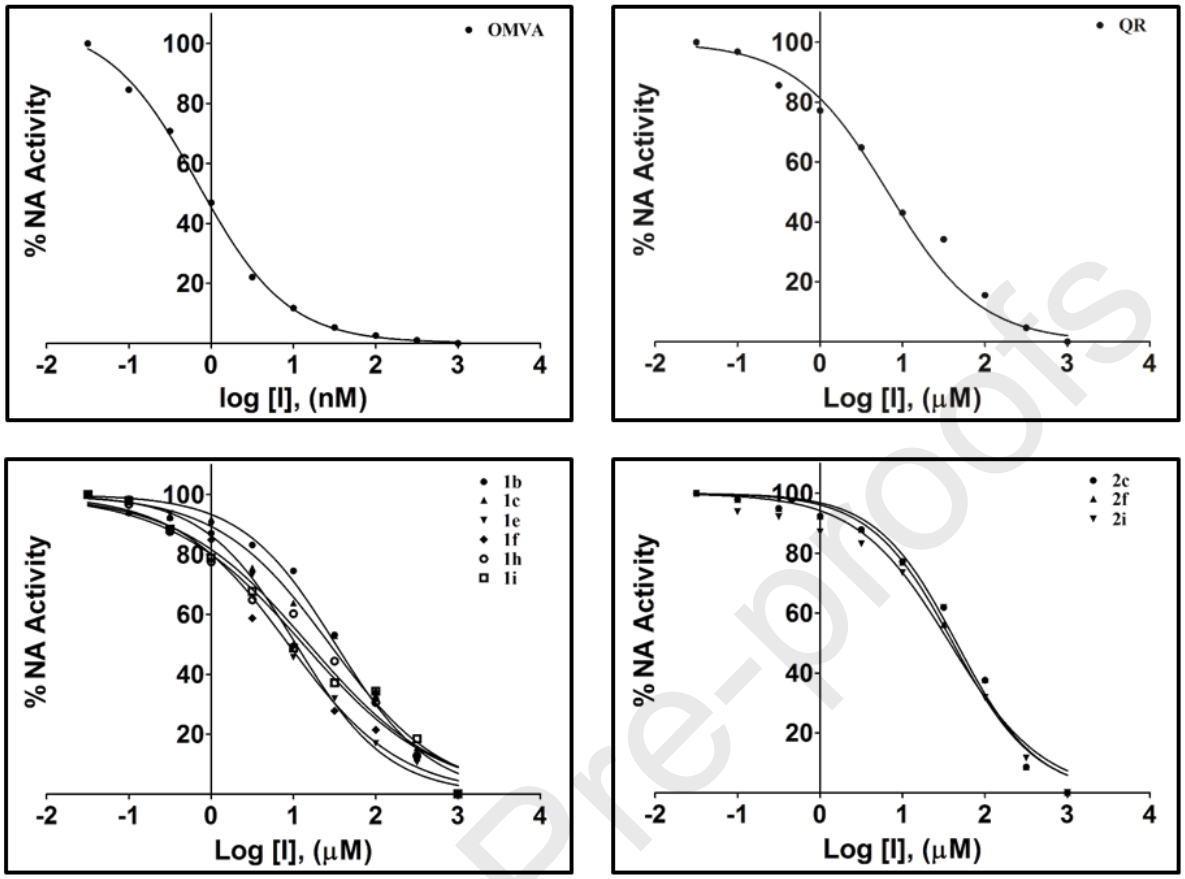


Fig. 3 Effects of Oseltamivir acid (**OMVA**), compounds **1b-1c**, **1e-1f**, **1h-1i**, **2c**, **2f**, **2i** and Quercetin (**QR**) on H1N1-NA for the hydrolysis of substrate

The virus yield reduction assay and NA inhibition studies showed that the tested flavonoids were more active in suppressing viral replication than inhibiting the NA enzyme alone. These results indicate that our tested flavonoids may have supplementary unknown mechanism controlling virus replication.

Enzyme kinetics study was performed on compounds **1f** and **2f**, the most active (based on H1N1-NA inhibition) substituted flavone and aurone, respectively, to verify the non-competitive inhibition of designed flavonoids, as exhibited by docking studies. **OMVA** and **QR** were used as

standard for competitive and non-competitive inhibition, respectively. **OMVA**, structurally similar to SA, showed competitive inhibition.

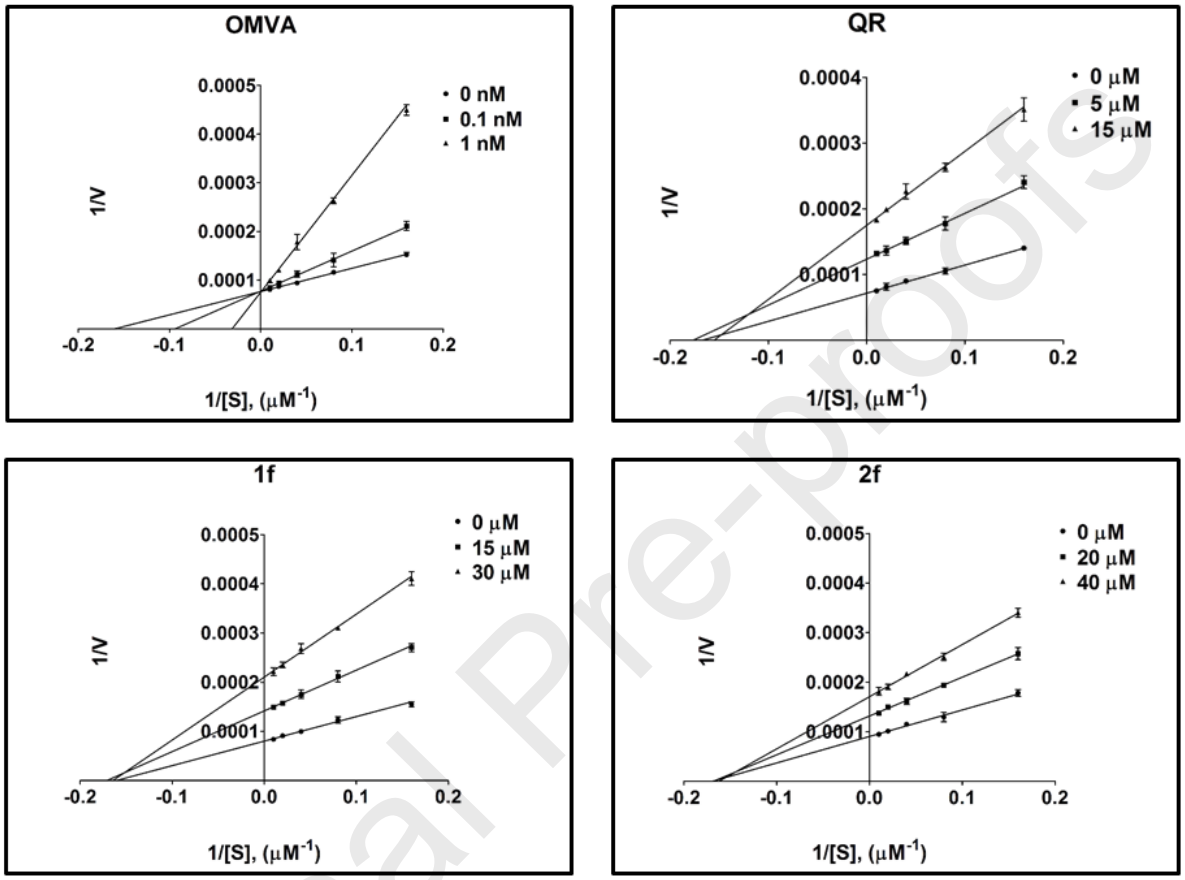


Fig. 4 Lineweaver–Burk plots for the inhibition of Oseltamivir acid (**OMVA**), Quercetin (**QR**), compounds **1f** and **2f** on H1N1-NA for the hydrolysis of substrate in the presence of increasing concentrations of inhibitors for lines from bottom to top

Fig. 4 shows the Lineweaver-Burk plot of 1/V versus 1/[S], one obtained in the absence of inhibitor and at two different concentrations of inhibitor. Increasing concentrations of **OMVA** resulted in a family of lines with common y-axis intercept but with a higher value of x-axis intercept, indicating its competitive inhibition. Whereas, increasing concentrations of **QR**, compounds **1f** and **2f**

resulted in families of lines having the same x-axis intercepts along with an increase in the y-axis intercept values, demonstrating their non-competitive inhibition.

3. CONCLUSION

The advent of resistance to the existent anti-influenza agents demands the pursuit of a different strategy in influenza therapy. To achieve this goal, eighteen substituted flavones and aurones were designed by varying substituent on phenyl ring and then docked in H1N1-NA. All the docked compounds occupied the 150-cavity and 430-cavity of the catalytic site. Sixteen of these designed compounds were then synthesised and their structures were confirmed by IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy. Compounds **2b** and **2e** were cytotoxic. The designed compounds suppressed the H1N1 viral titre. Compound **1f** exhibited the highest activity comparable with oseltamivir. Compound **1f** also demonstrated the highest H1N1-NA inhibition however, the activity is lower than oseltamivir. The virus yield reduction and H1N1-NA inhibition studies demonstrated that our designed flavones and aurones are more active in decreasing the H1N1 viral titer than inhibiting the H1N1-NA enzyme alone hinting that our compounds may have an additional unknown mechanism in controlling virus replication. Compounds **1f** and **2f**, the most active substituted flavone and substituted aurone, respectively, displayed non-competitive inhibition.

4. CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

5. ACKNOWLEDGEMENTS

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FIGURE CAPTIONS

Fig. 1 Docked conformation of compounds (a) **1c**-**1g**, (b) **1a**, **1b** & **1h**, (c) **1i**, (d) **2b**-**2e**, **2g** & **2h**, (e) **2f** & **2i**, (f) **2a** in H1N1-NA

Fig. 2 Effects of Oseltamivir acid (OMVA), Quercetin (QR), compounds **1f** and **2f** on H1N1 virus yield

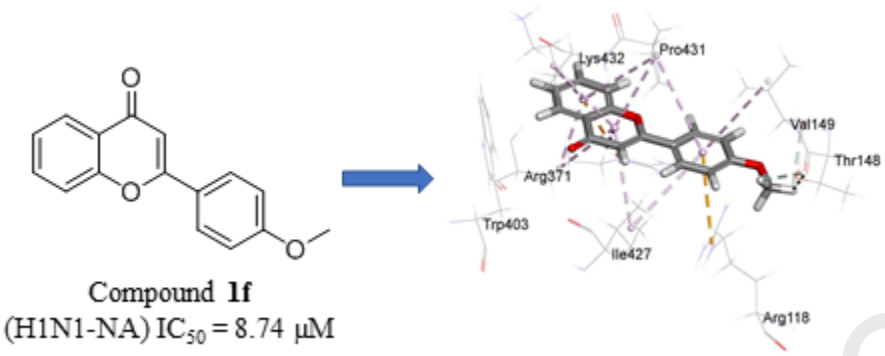
Fig. 3 Effects of Oseltamivir acid (OMVA), compounds **1b**-**1c**, **1e**-**1f**, **1h**-**1i**, **2c**, **2f**, **2i** and Quercetin (QR) on H1N1-NA for the hydrolysis of substrate

Fig. 4 Lineweaver–Burk plots for the inhibition of Oseltamivir acid (OMVA), Quercetin (QR), compounds **1f** and **2f** on H1N1-NA for the hydrolysis of substrate in the presence of increasing concentrations of inhibitors for lines from bottom to top

Highlights

- Substituted flavones and aurones are docked in 150-cavity and 430-cavity of H1N1 neuraminidase
- Substituted flavones and aurones reduce H1N1 viral titre
- Substituted flavones and aurones are non-competitive inhibitors of H1N1 neuraminidase
- Substituted flavones and aurones may be useful as potential anti-influenza agents

Graphical abstract



Compound **1f**
(H1N1-NA) IC_{50} = 8.74 μM