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Anti-influenza virus activities of 4-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene)amino]-*N*-(4,6-dimethyl-2-pyrimidin-2-yl)benzenesulphonamide and its derivatives

Periyasamy Selvam[1]*, Narayanan Murugesh[2], Markandavel Chandramohan[3], Robert W Sidwell[4], Miles K Wandersee[4] and Donald F Smee[4]

[1]Arumigu Kalasalingam College of Pharmacy, Krishnankoil-626 190, India
[2]Institute of Pharmacology, Madurai Medical College, Madurai-625 020, India
[3]Bharat Ratna Kamarajar Liver Hospital and Research Centre, Madurai-625 001, India
[4]Institute of Antiviral Research, Utah State University, Logan, UT, USA

*Corresponding author: Tel: +91 4563 227389; Fax: +91 4563 265 772; E-mail: periyasamyselvam2001@yahoo.co.in

4-[(1,2-Dihydro-2-oxo-3H-indol-3-ylidene)amino]-*N*-(4,6-dimethyl-2-pyrimidinyl)-benzenesulphonamide (SPIII-5H) and related compounds were tested for antiviral activity against influenza A (H1N1, H3N2, and H5N1) and B viruses in Madin Darby canine kidney (MDCK) cell culture. Among the compounds tested, SPIII-5H and four derivatives (5-chloro [SPIII-5Cl], 5-bromo [SPIII-5Br], 5-methyl [SPIII-5Me] and N-acetyl [SPIII-NA]) showed similar antiviral potencies, with only the 5-fluoro (SPIII-5F) derivative being ineffective. Fifty percent effective concentration (EC_{50}) values were determined in cytopathic effect (CPE) inhibition assays quantified by neutral red dye uptake. By this method, the active compounds were inhibitory to the H1N1 strain of influenza A at 2.7–5.2 μg/ml, to the H3N2 strain of influenza A at 13.8–26.0 μg/ml, to the H5N1 strain of influenza A at 3.1–6.3 μg/ml and to influenza B at 7.7–11.5 μg/ml. Confirmatory virus yield reduction studies against influenza A (H1N1) virus demonstrated antiviral activity (90% inhibition) at concentrations of 2–10 μg/ml. No cytotoxic effects were evident in actively growing uninfected cells or stationary monolayers at 100 μg/ml. Potencies of the compounds were similar to those of ribavirin, but much less than those of oseltamivir carboxylate against the various viruses. Time-of-addition studies indicated the compounds inhibited an early step in the virus replication cycle, probably virus adsorption/penetration, and no virucidal activity was evident. The basic molecule is amenable to diverse chemical modifications, which may improve water solubility and antiviral potency.

Keywords: antiviral, influenza A, influenza B, isatin, sulphadimidine

Introduction

Isatin (2,3-dioxoindole), a versatile lead molecule for potential bioactive agents and certain derivatives was reported to possess anticancer activity (Popp *et al*., 1969). One such compound, methisazone (N-methylisatin-β-thiosemicarbazone), was one of the first clinically used synthetic antiviral agents for the treatment of smallpox (Bauer *et al*., 1960; Smee and Sidwell, 2003), and N,N-disubstituted thiosemicarbazone derivatives of isatin were inhibitory to HIV-1 replication (Teitz *et al*., 1994). In studies performed by our group, some novel isatin derivatives were synthesized and evaluated for antiviral, anticancer and antibacterial activities (Selvam *et al*., 2001; Selvam *et al*., 2004); some of these compounds showed significant inhibitory effects against HIV-1 replication. More recently, this class of compounds was reported to inhibit orthopoxvirus replication *in vitro* (Selvam *et al*., 2006). In view of the broad-spectrum antiviral activities of the isatin derivatives, the present work was conducted to evaluate the efficacy of some novel 4-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene)amino]-*N*-(4,6-dimethyl-2-pyrimidinyl) benzenesulphonamides (Figure 1) against influenza A and B viruses in Madin Darby canine kidney (MDCK) cells.

Materials and methods

Antiviral compound, viruses and cells

4-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene)amino]-*N*-(4,6-dimethyl-2-pyrimidin-2-yl)benzenesulphonamide (SPIII-5H) and five derivatives (5-fluoro [SPIII-5F], 5-chloro [SPIII-Cl], 5-bromo [SPIII-Br], 5-methyl [SPIII-5ME], and N-acetyl [SPIII-NA]) were prepared by combining the isatin (2,3-dioxoindole) and its derivatives (5-fluoro, 5-chloro, 5-bromo, 5-methyl and N-acetyl) with

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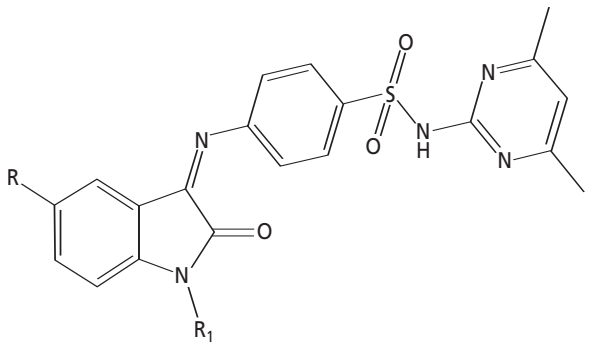
Figure 1. Structure of 4-[(1,2-dihydro-2-oxo-3H-indol-3-ylidene)amino]-*N*-(4,6-dimethyl-2-pyrimidinyl)-benzene sulphonamide (SPIII-5H) and its derivatives



Compounds	R	R_1
SPIII-5Br	Br	H
SPIII-5Cl	Cl	H
SPIII-5F	F	H
SPIII-5H	H	H
SPIII-5Me	CH_3	H
SPIII-NA	H	$COCH_3$

sulphadimidine in the presence of glacial acetic acid (Selvam *et al*., 2001). Oseltamivir carboxylate (GS 4071, from R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ, USA) and ribavirin (ICN Pharmaceuticals, Costa Mesa, CA, USA) were used as positive controls for these experiments. Cyanovirin-N-(CV-N) was kindly provided by Barry O'Keefe (National Cancer Institute, Frederick, MD, USA). Influenza A/New Caledonia/20/99 (H1N1), influenza A/Panama/2007/99 (H3N2), and influenza B/Shanghai/361/02 viruses were obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA). Influenza A/Duck/MN/1525/81 (H5N1) was provided by Robert Webster (St. Jude Children's Research Hospital, Memphis, TN, USA). The viruses were propagated in MDCK cells (obtained from the American Type Culture Collection, Manassas, VA, USA) in serum-free medium (MEM with 0.18% sodium bicarbonate) supplemented with 10 μg/ml trypsin (Sigma, St. Louis, MO, USA), 1 μg/ml EDTA and 50 μg/ml gentamicin.

Antiviral and cytotoxicity assays

The inhibitory effects of the compounds on influenza virus replication were determined by cytopathic effect inhibition (CPE) assays in MDCK cell monolayers conducted in 96-well microplates (Sidwell and Smee, 2000; Smee *et al*., 2002). Compounds in half-log_{10} dilution increments were applied to cells 5–10 min before adding virus, using three wells for infection and two wells for toxicity controls. Fifty cell culture infectious virus doses (50 $CCID_{50}$) of virus were then added, and the plates were incubated for 3 days when inhibitor-free cell cultures were completely destroyed by virus. At this time, the mean percentage of cell viability in each set of three infected wells or set of two toxicity control wells was quantified by a neutral red dye uptake method (Smee *et al*., 2002), using 0.011% final concentration of the dye for 2 h. An Excel spreadsheet was developed for converting optical density readings to percentages of untreated control values. Concentrations of compounds reducing viral CPE by 50% (EC_{50} values) were calculated by plotting concentration versus percent inhibition on semilog_{10} graph paper. As no toxicity was apparent to the uninfected stationary cell monolayers at the highest (100 μg/ml) concentration, separate assays were performed for toxicity determinations using rapidly dividing cells. Actively dividing cells are more susceptible to toxicity than stationary monolayers of cells. For this assay, MDCK cells in 24-well plates were seeded at 2.5×10^4 cells per well and allowed to attach overnight. Compounds in half-log_{10} dilutions were applied to cells in growth medium (MEM with 0.18% sodium bicarbonate and 5% fetal bovine serum) using four wells per compound concentration, and the plates were incubated for 3 days. At that time neutral red was added for 2 h and the plates were processed as previously described (Smee *et al*., 2002). In order to quantify neutral red, 0.1 ml of medium from each extracted well was transferred to a 96-well plate, so that optical density could be read with an ELISA plate reader. Fifty percent cell inhibitory concentrations (IC_{50} values) were then calculated by using a similar method to the EC_{50} values. A selectivity index (SI value, which is equal to IC_{50} divided by EC_{50}), was calculated for each set of data. Three independent assays were performed to derive mean values ±standard deviation.

Confirmatory virus yield reduction assays were performed by replicating 50 $CCID_{50}$ of influenza A/New Caledonia/20/99 (H1N1) per well in 96-well microplates for 3 days in the presence of varying half-log_{10} concentrations of the active compounds. The virus-containing samples were stored in the plates at −80°C prior to titration. Virus yields at each concentration were later determined by titration of samples on fresh monolayers of MDCK cells by endpoint dilution method (Reed and Muench, 1938) in 96-well plates using four microwells per dilution.

In order to understand the mode of action of this class of compounds against influenza virus, the parent compound (SPIII-5H) at 100 μg/ml was applied to cells in 24-well microplates at varying times pre- and post-virus exposure. A virus challenge dose of 5,000 $CCID_{50}$ per well (which was 100-fold more virus than used for the CPE assays described above) was applied for 18 h to assess virus production from a single virus replication cycle. Virus yields were determined in 96-well microplates for each treatment regimen in the same manner described above. When the compound was removed from the cell culture medium prior

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Table 1. Inhibitory effects of isatin compounds, oseltamivir carboxylate, and ribavirin on the replication of influenza A and B viruses in Madin Darby canine kidney cells

Compound	R	R[1]	Virus strain	EC_{50}, μg/ml*	SI[†]
SPIII-5H	H	H	A (H1N1)	3.4 ±1.1	>29
			A (H3N2)	26.0 ±14.4	>3.8
			A (H5N1)	3.7 ±1.9	>27
			B	7.7 ±2.0	>13
SPIII-5F	F	H	A (H1N1)	>100	0
			A (H3N2)	>100	0
			A (H5N1)	>100	0
			B	>100	0
SPIII-5Cl	Cl	H	A (H1N1)	3.9 ±1.7	>26
			A (H3N2)	23.3 ±16.0	>4.3
			A (H5N1)	4.0 ±2.0	>25
			B	8.8 ±5.3	>11
SPIII-5Br	Br	H	A (H1N1)	2.7 ±1.3	>37
			A (H3N2)	13.8 ±9.8	>7.3
			A (H5N1)	4.1 ±2.3	>24
			B	10.7 ±6.4	>9.3
SPIII-5Me	Methyl	H	A (H1N1)	3.2 ±2.3	>31
			A (H3N2)	17.7 ±10.8	>5.6
			A (H5N1)	3.1 ±2.6	>32
			B	11.5 ±9.1	>8.7
SPIII-NA	H	N-acetyl	A (H1N1)	5.2 ±1.9	>19
			A (H3N2)	25.7 ±5.5	>3.9
			A (H5N1)	6.3 ±6.0	>16
			B	11.1 ±8.6	>9.0
Oseltamivir carboxylate	–	–	A (H1N1)	0.022 ±0.012	>4,545
			A (H3N2)	0.032 ±0.02	>3,125
			A (H5N1)	0.010 ±0.006	>10,000
			B	0.93 ±1.0	>108
Ribavirin	–	–	A (H1N1)	3.7 ±1.2	>27
			A (H3N2)	5.9 ±1.9	>17
			A (H5N1)	4.3 ±1.7	>23
			B	5.5 ±2.5	>18

*Fifty percent effective (virus-inhibitory) concentration (EC_{50}) determined in cytopathic effect inhibition assays quantified by neutral red dye uptake. Mean values ±SD are from three independent experiments. [†]Selectivity index (SI – 50% cell-inhibitory concentration [IC_{50}] divided by EC_{50}). In assays performed using actively dividing cells, the IC_{50} for each isatin derivative or oseltamivir carboxylate was >100 μg/ml. SIs for ribavirin were based on effects in confluent monolayers (>100 μg/ml inhibition), since the drug acts as a cytostatic agent (Muller *et al.*, 1977), inhibiting actively dividing cell proliferation at approximately the same concentration as it inhibits virus replication.

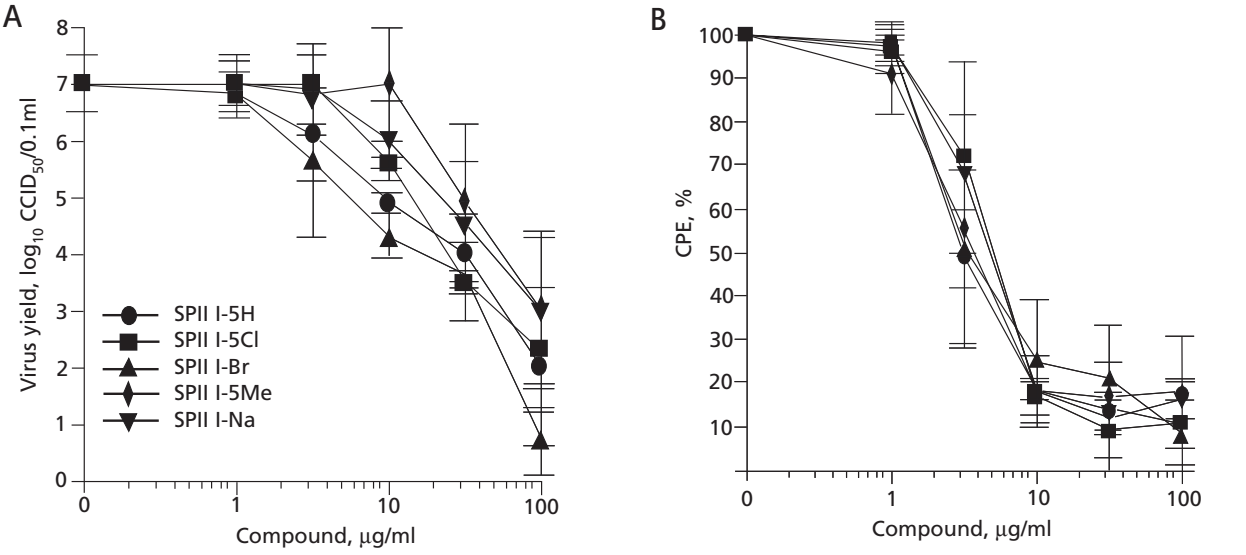
to the virus replication period (18 h), the cells were rinsed three times with medium to remove residual compound. Statistical differences in virus yield were analysed by using the one-tailed Mann–Whitney *U* test.

Results

Antiviral activity and cytotoxicity of isatin derivatives

The results of the viral CPE inhibition and cytotoxicity studies are reported in Table 1. Compound SPIII-5F was inactive against influenza A and B viruses at the highest concentration tested (100 μg/ml). The other five compounds (SPIII-5H, SPIII-5Cl, SPIII-5Br, SPIII-5Me, and SPIII-NA) were active and had nearly the same 50% inhibitory effects against the individual influenza viruses tested. These compounds were inhibitory to influenza A (H1N1) at 2.7–5.2 μg/ml, to influenza A (H3N2) at 13.8–26.0 μg/ml, to influenza A (H5N1) at 3.1–6.3 μg/ml and to influenza B at 7.7–11.5 μg/ml. Thus, the compounds exhibited the least potency against the H3N2 virus. Compared to each other, no compound appeared to be significantly superior in activity to another among the active substances tested. Oseltamivir carboxylate, a neuraminidase

Figure 2. Virus yield reduction **(A)** and CPE inhibition **(B)** of influenza A/New Caledonia/20/99 (H1N1) virus resulting from treatment of infected cells for 3 days with isatin derivatives



After quantifying viral cytopathic effect (CPE), virus yields were determined in subsequent assays using new Madin Darby canine kidney cell monolayers. Virus yield values represent means of two independent assays, whereas CPE results are the means of three independent assays.

inhibitor used as the positive control compound, exhibited greater antiviral potency with activity seen at 0.01–0.032 μg/ml against influenza A viruses and 0.93 μg/ml against influenza B virus, with activities comparable to the previously published work (Smee *et al*., 2001). A second positive control, ribavirin, was effective against the strains of influenza virus at concentrations of 3.7–5.9 μg/ml.

None of the isatin derivatives caused cytotoxic effects (as measured by neutral red uptake) to either confluent cell monolayers or actively dividing MDCK cells up to 100 μg/ml. Higher concentrations of compounds were not tested in the assays due to solubility limitations. Because of the low cytotoxicity profile exhibited by the compounds, the selectivity indices for the active isatin compounds were favourable for all but the H3N2 virus.

To confirm the antiviral activity seen with the active isatin compounds, virus yield reduction assays were conducted. Dose-response reductions in virus titre occurred with these inhibitors, with 90% inhibition (one $\log_{10}$ reduction) seen at concentrations of 2–10 μg/ml (Figure 2A). The activities of compounds on virus-induced cytopathic effect as quantified by neutral red dye uptake at each concentration are depicted in Figure 2B. Minimal viral CPE was observed at the 10–100 μg/ml concentrations, which corresponded with dramatic reductions in virus yield.

Mode of antiviral action of isatin compounds

The parent compound SPIII-5H was selected for further evaluation to study the mode of antiviral action of this class of compounds. In an initial experiment, exposure of the cells to 100 μg/ml of the SPIII-5H was done at varying starting times relative to virus exposure and for varying lengths of cell incubation (Figure 3A). Treatment with SPIII-5H that was terminated before virus exposure (–1 to 0 h) and treatment starting 3 h after virus exposure (3–18 h) were largely ineffective. Treatment starting 1 h before virus exposure and terminating 3 h after exposure was just as effective as treatment from 1 h before to 18 h after virus exposure. These results indicate that an early event in the virus life cycle was inhibited by SPIII-5H. A follow-up experiment was performed to determine whether short-term early treatments were effective with the other active SPIII compounds. Treatments (100 μg/ml) were given only for 3 h from –1 to 2 h relative to virus infection. The compounds performed similar to SPIII-5H in reducing virus titre in culture, with a mean $\log_{10}$ virus titre for all of the compounds considered together of 4.5 ±0.2 compared with 6.7 ±0.3 for the virus control ($P<0.05$).

To further narrow down the incubation time required for virus inhibition, exposure of virus plus SPIII-5H to cells was conducted for as short as 15 min (Figure 3B). A positive control compound known to block virus adsorption in virus-neutralizing manner, CV-N (O'Keefe *et al*., 2003), was tested in parallel. In this experiment, SPIII-5H and virus were exposed to cells for as little as 15 min and caused a significant reduction in virus yield, although the potency of inhibition was much less than that of CV-N.

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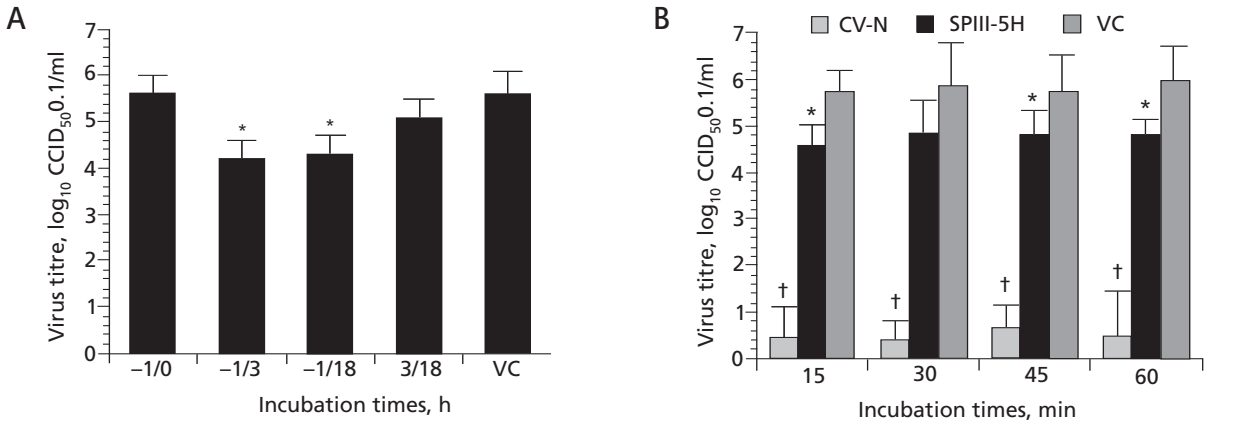
Figure 3. Effects of varying treatment starting times and lengths of incubation on the efficacy of SPIII-5H (100 μg/ml) against an influenza A/New Caledonia/20/99 (H1N1) virus infection in MDCK cells



(A) SPIII-5H alone. **(B)** SPIII-5H compared to cyanovirin-N-(CV-N). **P*<0.05, †*P*<0.01 relative to virus control. Starting times for applying SPIII-5H and ending times of treatment (start/end) are indicated for **(A)** relative to virus exposure (starting at 0 h). In **(B)** the compound and virus were exposed to cells only for the times indicated. Values are means ±SD for two independent experiments, each with triplicate samples assayed. MDCK, Madin Darby canine kidney; VC, virus control (no compound present).

These results suggest that the SPIII series of compounds acts by inhibition of virus adsorption/penetration.

To rule out virucidal activity, the active SPIII compounds and influenza A/New Caledonia/20/99 virus were incubated together for 30 min at room temperature, then plated in 10-fold dilution increments. Under these conditions where the compounds were diluted beyond their activity level, no differences in virus titre between compound-treated and untreated viruses were seen (data not shown).

Discussion

Isatin derivatives have been reported to exhibit antiviral activity against a variety of pathogenic viruses (Bauer, 1972; Levinson, 1973). N-Methyl isatin-β-4′:4′-diethyl thiosemicarbazone was found to inhibit Moloney leukaemia virus replication (Teitz *et al.*, 1984). Schiff and Mannich bases of isatin derivatives were synthesized and evaluated for antiviral activity. Some of the derivatives showed significant activity against the replication of HIV-1 (Pandeya *et al.*, 1999a, 1999b, 1999c, 1999d, 2000a, 2000b) and orthopoxviruses (Selvam *et al.*, 2006). These studies prompted us to investigate the potential of this series of compounds against influenza viruses, where isatin compounds SPIII-5H, SPIII-5Cl, SPIII-5Br, SPIII-5Me, and SPIII-NA were indeed found to be active against the replication of influenza A (H1N1, H3N2, and H5N1) and influenza B viruses in infected MDCK cells, although the 5-flouro derivative (SPIII-5F) was inactive. Overall, compounds SPIII-5H, SPIII-5Cl, SPIII-5Br, SPIII-5Me, and SPIII-NA were judged to be essentially equivalent in activity in these assays. The introduction of fluorine at the 5 position of the molecule abolished antiviral activity. The antiviral potencies of the active compounds against one type of influenza virus were very similar to the effects observed against the other types of influenza virus. The antiviral activity of these substances against other unrelated viruses is under investigation. The potencies of the isatin compounds were much less than that of oseltamivir carboxylate, but they are in the same potency range as ribavirin.

The isatin compounds were tested further and found to exhibit antiviral activity when exposed to cells very early in the virus replication cycle. None of the compounds tested compared in potency with CV-N, which directly binds to viral hemagglutinin to neutralize virus infectivity (O'Keefe *et al.* 2003). Based upon structural similarity and equivalent antiviral activity among the five active isatin derivatives, the compounds appear to act similarly as virus inhibitors.

The reported degree of inhibition of SPIII-5H at 100 μM on virus yield was much greater in Figure 2 than in Figure 3. The data in Figure 2 represent virus produced over 3 days (or multiple cycles of replication), whereas results of Figure 3 were for an 18 h replication period. Thus, the comparative results suggest a cumulative effect on virus production over multiple cycles of replication.

The particular mechanism of how the active compounds interact with cells and/or virus to block virus infectivity is not understood. Interference with the interaction of viral hemagglutinin with the cell receptor is a potential target, based upon what is known about influenza virus entry into cells (Skehel and Wiley, 2000) and the action of other antiviral substances that inhibit virus adsorption to cells (Cianci and Krystal, 1998). No biochemical or fluorescent

imaging experiments were carried out to try and define the step of virus replication being inhibited. Such studies will be useful once we identify more potent inhibitors that merit such investigation.

This series of compounds suffers from low water solubility, and the antiviral potency is moderate, particularly against the H3N2 virus. To improve these characteristics will require the synthesis and evaluation of additional analogs in this series. Many structural modifications are possible to the basic molecular structure, and are being considered for synthesis.

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