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Identification of Entry Inhibitors for Avian Influenza H5N1 virus

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ABSTRACT

Background. Influenza pandemics occur when a new strain emerges to which the human population has no immunity. The rapid spread of the avian influenza A (H5N1) virus has put more humans at risk of infection and has raised the concern of a new pandemic. In addition, the virus has caused human fatalities in Asia and Europe. There is no vaccine against this virus. Moreover, appearance of neuraminidase inhibitor (NAI) resistant H5N1 strains highlights the need for new therapeutics. Viral entry is an attractive target for the development of new influenza inhibitors since it is an essential step in virus replication. The entry of influenza virus into susceptible cells is mediated by the viral envelope protein, hemagglutinin (HA). In order to discover potential new influenza virus therapeutics, we identified and partially characterized inhibitors of the HA mediated entry process.

Methods. HA (H5) from avian influenza H5N1 (Quinghai strain) was used to generate pseudotype virus (HIV/HA). HIV/HA was used in a high throughput screen (HTS) to identify small molecule inhibitors of HA (H5) mediated entry under BSL2 conditions.

Results. We have so far screened approximately 40,000 discrete compounds and identified 141 primary hits. The Z' factor for the HTS was 0.5±0.2. Primary hits were counter screened with pseudotype virus expressing an unrelated glycoprotein (VSV-G) and infectious H1N1 virus for their specificity. They were evaluated for their potency, and cytotoxicity with resynthesized compounds. Only 36 of the primary hits specifically inhibited the HA mediated entry process. The final hit rate from the HTS was 0.09%. All the 36 hit compounds exhibited IC_{90} values of ≤25µM. Structurally, the HA inhibitors can be represented as clusters of ≥2 members each and singletons.

Conclusion. We have identified new compounds with potential to be developed as antiviral agents for the prevention and treatment of influenza infection. Compounds may also be useful as molecular probes for the study of influenza virus entry into host cells.

INTRODUCTION

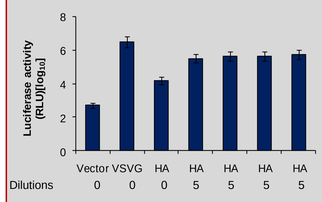
Influenza A viruses cause recurrent epidemics with substantial human morbidity and mortality, and are also associated with pandemics. Pandemics occur when a new influenza virus strain emerges, by re-assortment of genes from two or more distinct types of influenza viruses (antigenic shift), and to which the human population has no immunity. New influenza viruses emerge from the aquatic bird reservoir, and acquire human-to-human transmission to cause influenza pandemics. The influenza H1N1 virus of the 1918–1919 influenza pandemic obtained all of its eight genes from the avian reservoir, whereas the Asian H2N2 and the Hong Kong H3N2 pandemic influenza strains emerged by re-assortment between the circulating human influenza virus and an avian H2 or H3 influenza virus. We are now faced with concerns regarding the emergence and spread of the highly pathogenic avian influenza A/H5N1 virus and its potential to evolve into a pandemic strain. In addition, current spread of pandemic influenza A/H1N1 viruses (Swine flu) highlights the urgency of developing effective countermeasures against these viruses. Since the first recorded direct bird-to-human transmission of avian A/H5N1 virus in 1997, the World Health Organization (WHO) has recorded 436 confirmed H5N1 cases with 262 fatalities since 2003 (1-3). Vaccines, currently the primary strategy for protection against influenza infection, are only effective if they match the circulating virus type(s). Since the timing and subtype of the next influenza pandemic cannot be predicted, a 'pandemic vaccine' cannot be developed in advance against new emerging strain(s) (4). Anti-viral drugs offer the promise of inhibiting influenza regardless of its genetic variations. However, the efficacies of the currently approved anti-virals have been restricted by the emergence of drug-resistant viruses, including the emergence of oseltamivir (Tamiflu) resistant seasonal influenza A/H1N1 and avian influenza H5N1 viruses (5, 6). These drug resistant viruses have highlighted the need for developing novel therapeutics with reduced drug resistance potential.

We report here the identification of a new class of influenza A virus inhibitors that target the viral envelope protein hemagglutinin (HA). HA mediates the binding and entry of the influenza virus into the host cell. Virus entry is an attractive target for influenza inhibitors because inhibition of this step can block the propagation of virus at an early stage, minimizing the chance for the virus to evolve and acquire drug resistance. Entry inhibitors have been developed for several important viruses and the FDA has approved the use of the peptidic HIV entry inhibitor, enfuvirtide (7). Entry inhibitors of paramyxoviruses have also been identified that interfere with the formation of this fusion hairpin structure (8). HA is a class I envelope protein, and mediates influenza virus entry through receptor binding and fusion of the virus with host cells. Like other class I viral envelope proteins, it also undergoes a series of conformational rearrangements during fusion. Our plan for developing new anti-influenza therapeutics is to target HA. We have performed a high throughput screen (HTS) to identify HA specific inhibitors. While HA displays significant inter and intra-subtype variation (>30%) [8], the receptor binding and fusion domains remain highly conserved. Targeting these two highly conserved sites will inhibit influenza virus infection with a low incidence of resistance development.

RESULTS

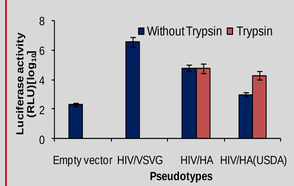
Development of HTS Assay and Pilot Screen

NA treatment of the producer 293T cells is required for optimal infectivity



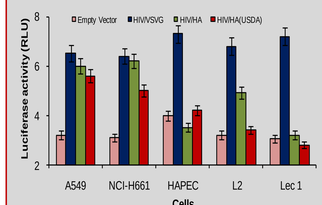
- 293T cells were treated with NA 26h post-transfection
- NA treatment of the producer 293T cells enhances infectivity of HIV/HA(H5).

HIV/HA(H5) does not require trypsin treatment



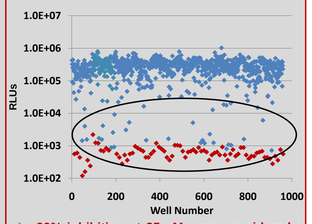
- Trypsin treatment for 30 min at 37°C did not enhance HIV/HA(H5) infectivity
- Trypsin treatment enhanced the infectivity of low pathogenic HIV/HA(USDA)

A549 Cells are used in the HTS screen



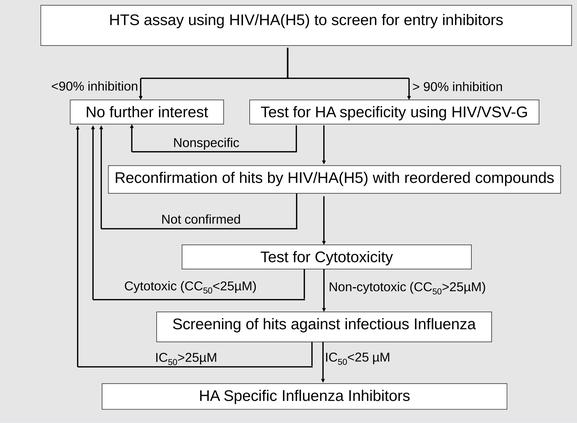
- Human lung cells (A549 and NCI-H661) are very susceptible to infection
- Rat lung cells (L2) and Lec1 cells were not very susceptible to infection

Pilot Screen



- >90% inhibition at 25 µM was considered a 'hit'
- Signal to background ratio of >10³
- Z' factor for the experiments 0.5±0.2

Experimental Design Workflow Diagram

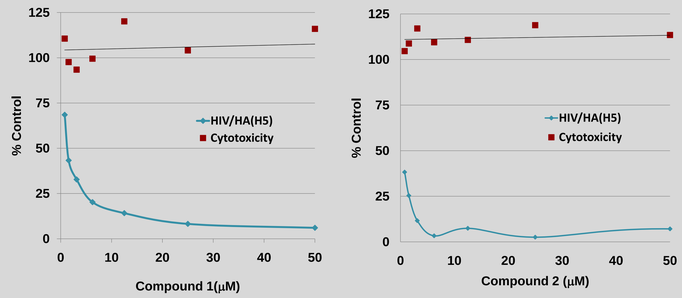


Results of HTS Assay for HA(H5) Inhibitors

Compounds Screened	Number of hits			Specific Hits displaying CC_{50}>25µM[c]
	Hits inhibiting >90% HIV/HA(H5) [%] [a]	Hits inhibiting >50% HIV/VSV-G [%] [a,b]	Specific hits (Primary hits inhibiting HIV/HA(H5) only) [%]	
40,000	2,038 (5.0)	1,897 (4.7)	141(0.35)	36(0.09)

- a HIV/HA(H5) and HIV/VSV-G were generated by transfection of 293T cells with pNL4.3.Luc.R–E– as the HIV-I expression vector and with HA(H5) or VSV-G, respectively.
- b 1,897 primary hits inhibited HIV/VSV-G by more than 90% at 25uM, whereas the RLU values of the controls varied by ≤20%.
- c CC_{50} values were determined using "AlphaScreen SureFire GAPDH Assay Kit "(Perkin Elmer) .

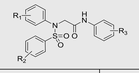
Comparison of Inhibitory Activity and Cell Toxicity of Lead Influenza Compounds



Specificity of Influenza HA(H5) Hits

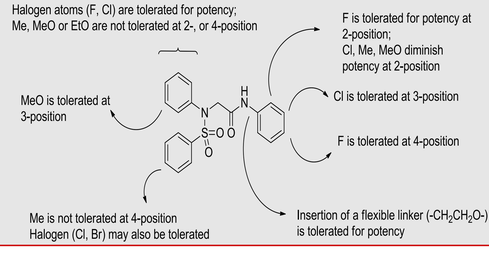
Comp. #	IC_{90} (µM)				IC_{50}(µM)
	HIV/HA(H5)	HIV/VSV-G	HIV/HA(H7)	HIV/MLV	Influenza H1N1 (PR8)
1	26.5	>100	>100	>100	20.2
2	3.9	>100	>100	99.0	5.2
3	22.6	>100	41.6	12.1	16.0
4	20.6	>100	78.4	84.4	24.5
5	13.4	53.4	14.9	23.7	15.1

SAR of Sulfonamide Anti-Influenza Inhibitors



Compound	R_1	R_2	R_3	HIV/HA (H5) IC_{90} (µM)	CC_{50} (µM)
6	2, 4-di-Cl	4-H	2-F	5.3	>25
7	3-OMe	4-H	3,5-di-Cl	18.5	31.5
8	2,5-di-OMe	4-Me	4-Cl	>100	>100
9	3,4-di-Cl	4-H	4-H	20	42
10	2, 4-di-Cl	4-H	4-F	25	50.5
11	4-OMe	4-H	2-Me	No inhibition	>100
12	4-OMe	4-H	4-F	No inhibition	>100
13	4-OMe	4-H	2,6-di-OMe	No inhibition	>100
14	2-OMe	4-H	2,3-di-Me	No inhibition	>100
15	2-Me, 4-Cl	4-Me	3-OMe	>100	43.4
16	4-Cl	4-Me	4-OMe	No inhibition	>100
17	3-Cl	4-H	2,6-di-Me	No inhibition	>100
18	3-Cl	4-H	2,4-di-OMe	No inhibition	>100
19	3-Cl	4-H	2-Cl	>100	90.3
20	2-Cl	4-H	2,6-di-Me	No inhibition	>100
21	2-Cl	4-Me	2,5-di-OMe	No inhibition	>100
22	2-Cl	4-Me	3-Me	No inhibition	>100
23	2-Cl	4-Me	2,4-di-OMe	No inhibition	>100

Preliminary SAR Sulfonamide Scaffold



SUMMARY

- Developed a HTS assay using HIV/HA(H5) to screen for HA (H5) inhibitors
- Identified 36 HA(H5) specific inhibitors with IC_{90}<25uM and CC_{50} >25uM
- All 36 compounds inhibited cell culture grown influenza virus (H1N1)(PR8)
- The HA inhibitors identified included multiple clusters of chemically related structures, as well as singletons.
- Compound 2 having a sulfonamide scaffold was chosen as lead compound and limited SAR studies were performed
- Preliminary SARs have suggested that halogen groups (F, Cl) are tolerated on the phenyl ring attached to the sulfonamide N atom, while Me, MeO or EtO is not tolerated at 2-, or 4-position.
- F is tolerated at 2-, or 4-position of the phenyl ring attached to the amide moiety, and Cl is tolerated at the 3-position of this phenyl ring.
- Preliminary SAR further suggested that these sulfonamide compounds bind into a hydrophobic binding site on influenza HA
- Additional SAR, spectrum and mechanism of action studies are currently being conducted.

METHODS

Viruses: Pseudotype were produced by co-transfecting 12 µg of construct containing appropriate virus envelope glycoprotein with 12 µg pNL4-3-Luc-R−-E− HIV vector into 293T cells (90% confluent) in 10 cm plates with Lipofectamine 2000 (Invitrogen) according to the supplier's protocol.

Cell culture grown influenza H1N1(PR8) viruses were propagated and titrated in MDCK cells over 3 days at 37°C in the presence of 1µg/ml tosylsulfonyl phenylalanyl-chloromethylketone (TPCK)-treated trypsin (Sigma-Aldrich) following standard protocol (9).

Chemical Libraries. The chemical libraries screened represent broad and well-balanced collections of over 152,500 compounds. They were purchased from Chembridge (San Diego, CA) and Timtec (Newark, DE), diluted in 96-well master plates at 2.5 mM in dimethyl sulfoxide (DMSO), and stored at -20°C. Compounds were selected in the molecular weight range of 200-500 Da. They have favorable cLogP values (calculated logarithm of n-octanol/water partition coefficient), and encompass over 200 chemotypes.

HTS of combinatorial chemical libraries: HTS of combinatorial chemical libraries using pseudotype virus was performed in 96 well plates. Low passage A549 cell monolayers were infected with 100µl of pseudotype virus containing 8µg/ml polybrene in the presence of 25uM (final concentration) test compounds. After 5 h, the inoculum was removed, fresh media was added and the plates were incubated for 72 h at 37ºC and 5% CO_2. Infection was quantified using the Britelite Plus™ assay system (Perkin Elmer) in a Wallac EnVision 2102 Multilabel Reader (Perkin Elmer, MA). The percent inhibition was calculated as: 100 x [Relative Luciferase Unit (RLU) in the presence of compound - RLU of negative control / RLU of positive control (without any inhibitor) - RLU of negative control].

Cell Viability Assay. AlphaScreen SureFire GAPDH Assay Kit (Perkin Elmer) was used to test cell viability by measuring endogenous cellular GAPDH in cell lysates according to manufacturer's protocol.

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