Antiviral Activity of Enisamium Against Influenza Viruses in Differentiated Normal Human Bronchial Epithelial Cells

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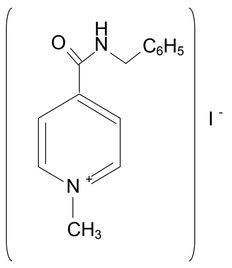
BACKGROUND

Influenza is an acute respiratory illness caused by influenza A and B viruses that occurs as annual epidemics with significant morbidity and mortality and occasional pandemics. Although vaccines have proven effective in mitigating the impact of influenza epidemics, due to possible antigenic mismatch and significant time required to develop a clinically available vaccine against a new virus strain, effective antiviral therapies are essential for immediate intervention against influenza infections. Currently, only a single class of antiviral drugs (e.g. neuraminidase [NA] inhibitors oseltamivir [Tamiflu®, Roche] and zanamivir [Relenza®, GlaxoSmithKline]) is recommended for prophylaxis and treatment of influenza in adults and children worldwide.

Extensive efforts are focused on identifying new treatment options and drug targets to eliminate the pathogenic properties of influenza virus. To this end, some drugs currently marketed in the countries of the former Soviet Union were reported to exert antiviral activity against influenza A and B viruses. One of these drugs is enisamium iodide, or Amizon® is licensed and marketed in Russia, Ukraine, Belarus, Kazakhstan and Uzbekistan as an antiviral agent against influenza (Margitich, personal communication).



Clinical studies conducted in Russia revealed clinical efficacy against influenza A and B, and suggested that the active compound, enisamium could be an attractive candidate for the treatment of influenza. However, experimental evidence for the efficacy of this isonicotinic acid derivative as an anti-influenza agent is lacking. The aim of the study was to evaluate the putative antiviral activity of enisamium against influenza viruses *in vitro*. Initial studies in MDCK and A549 cells revealed poor permeability of enisamium, 0.08 and 0.87%, respectively and lack of antiviral effect. The uptake of enisamium in differentiated normal human bronchial epithelial (NHBE) was greater than A549s and MDCKs, ranging from 1.7 to 2.1%; therefore, NHBE cultures were evaluated for efficacy and an antiviral effect of enisamium against influenza viruses.

Figure 1. Chemical structure of enisamium [(4-Benzylcarbamoyl)-1-methylpyridin-1-ium] iodide

MATERIALS & METHODS

Cell Culture: MatTeks's EpiAirway System (MatTek, Ashland, MA) differentiated normal human-derived bronchial epithelial cells (NHBE) were used for the study. The cells from a single donor were used for assay consistency. The apical surface of the cells was exposed to a humidified 95% air/5% CO2 environment and the basolateral medium changed and mucin washed every 24-48 hours.

Test Materials: The test article, enisamium, was provided by the Farmak JSC. The positive control oseltamivir carboxylate was obtained from Toronto Research Chemicals, TRC (Toronto, Canada).

Virus Challenge: NHBE cells were inoculated with influenza A viruses by exposure of the apical side to influenza virus. After a 1-hour incubation, the viral inoculum was removed from the cells, the apical side of the cells washed with Phosphate Buffered Saline (PBS).

Test Material Administration: For the positive control, oseltamivir carboxylate, NHBE cultures were exposed on the basal side to oseltamivir for 60 minutes, prior to viral inoculation. Enisamium or control media was added to the in the basal media compartment of the NHBE culture system prior to or post inoculation and incubated for the indicated duration of the experiment.

RESULTS

Antiviral Activity of Enisamium Chloride Against Influenza A and B Viruses in Differentiated NHBE Cells

Drug, dose (μM)[a]	Influenza virus titers ($log_{10}TCID_{50}$/mL ± SD) A/GA/20/06 (H1N1) H275Y	A/Brisbane/59/07 (H1N1)	A/TN/1-560/09 (H1N1)	A/Perth/16/09 (H3N2)	B/Texas/06/11
0	6.9 ± 0.4	5.58 ± 0.1	7.58 ± 0.7	6.7 ± 0.5	6.8 ± 0.4
Oseltamivir[b]	6.0 ± 0.4 (-0.9)	3.08 ± 0.6 (-2.5)[c]	3.75 ± 0.4 (-3.8)	3.0 ± 0.4 (-3.2)	4.9 ± 0.3 (-1.8)
Enisamium					
40	6.5 ± 0.0 (-0.4)	5.67 ± 0.1 (ND)	6.50 ± 0.3 (-1.1)	6.2 ± 0.9 (-0.5)	6.2 ± 0.6 (-0.6)
200	6.2 ± 0.6 (-0.8)	5.50 ± 0.3 (-0.1)	6.75 ± 0.0 (0.8)	6.3 ± 0.1 (-0.3)	5.8 ± 0.5 (-1.0)
600	5.1 ± 0.3 (-1.8)	4.33 ± 1.0 (-1.25)	5.67 ± 0.1 (-1.9)	4.8 ± 0.4 (-1.8)	4.8 ± 0.4 (-1.9)
1000	4.8 ± 0.5 (-2.1)	3.25 ± 0.5 (-2.3)[c]	4.83 ± 0.1 (-2.8)	4.1 ± 0.7 (-2.6)	4.6 ± 0.1 (-2.2)

Table 1. [a]NHBE cells were inoculated with influenza viruses at a MOI of 0.01 PFU/cell. After an adsorption period of 1 hour, enisamium chloride was added to the basal chamber at the listed dose and incubated for 24 hours at 37°C under 5% CO_2. Viruses released into the apical compartment of NHBE cells were harvested at 24 hours post-inoculation.

[b]Concentration of oseltamivir carboxylate was 1 μM.

[c] Virus titers were determined in MDCK cells by the $TCID_{50}$ assay and expressed as $log_{10}TCID_{50}$/ml. The difference in the mean virus titers (expressed as $log_{10}TCID_{50}$/mL) between virus-infected untreated and enisamium-treated NHBE cells is shown in parenthesis.

Inhibitory Activity of Enisamium Against Different MOIs of A/Brisbane/59/2007 (H1N1) Influenza Virus

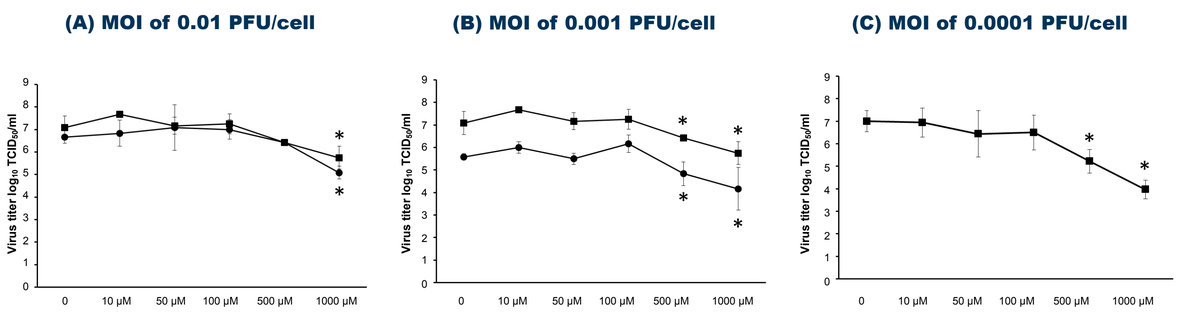


Figure 2. Differentiated NHBE cells were infected with influenza A/Brisbane/59/2007 (H1N1) virus at MOIs of 0.01 PFU/cell (A), 0.001 PFU/cell (B), and 0.0001 PFU/cell (C). Enisamium (1000, 500, 100, 50, and 10 μM) was added to the basal chamber 1 hour after virus inoculation. Apical washes were collected 24 (black circles) and 48 hours (black squares) post-infection (p.i.) and titrated by the $TCID_{50}$ assay. The assay's limit of detection (0.75 $log_{10}TCID_{50}$/mL).

Intracellular Uptake Kinetics of Enisamium

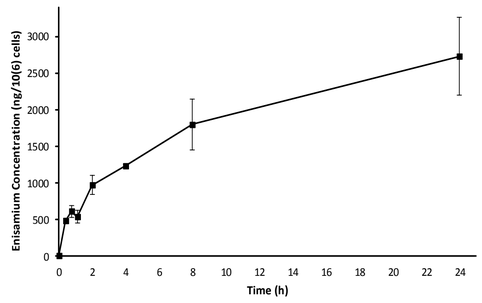


Figure 3. Uninfected NHBEs were treated with 1000 μM of enisamium and cells harvested at 0.25, 0.5, 1, 2, 4, 8, and 24 hours post-exposure. Concentrations in cells presented as enisamium chloride.

Permeability of Enisamium in Differentiated NHBE Cells

Drug, dose (μM)[a]	n[b]	Concentration in NHBE cells (mean ± SD, ng)[c]	Permeability (%)
10	3	36.8 ± 3.8	1.4
50	3	213 ± 34	1.6
100	3	410 ± 77	1.6
500	3	1727 ± 108	1.3
1000	3	3009 ± 132	1.1

Table 2. [a]Enisamium at indicated doses was incubated with differentiated NHBE cells at 37°C under 5% CO_2 for 24 hours in serum-free Bronchial Epithelial Cell Growth Medium.

[b]Number of wells assessed in the study.

[c]The cellular extracts were harvested and the concentration of enisamium determined using LC-MS-MS. Concentration is expressed as ng per 10^6 cells.

Effect of Time-of-Enisamium Addition on Inhibition of Influenza Virus Replication in NHBE Cells

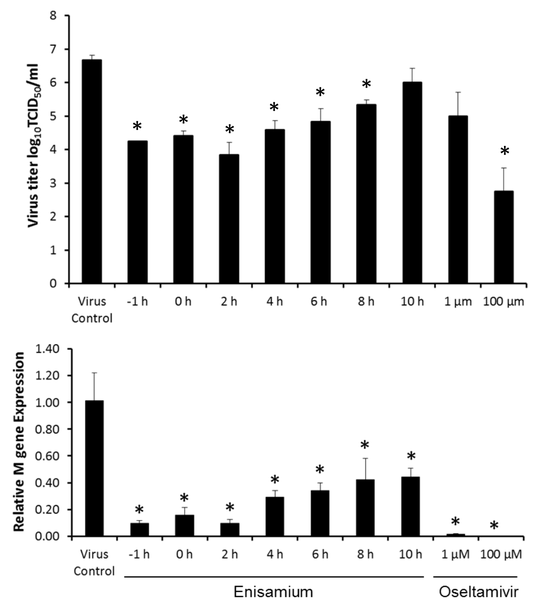


Figure 4. Differentiated NHBE cells were infected with influenza A/Brisbane/59/2007 (H1N1) virus at an MOI of 1.0 PFU/cell and exposed to enisamium at 2000 μM. Enisamium was added to the basal chamber at -1, 0, 2, 4, 6, 8, and 10 hours after virus inoculation and remained for the duration of the 24 hours. The time of viral inoculation is indicated as 0 hours. Supernatants were collected 24 hours p.i. from the upper chamber and titrated by the $TCID_{50}$ assay (A). Matrix gene expression is relative to the virus control and gene expression was normalized to those of β-actin mRNA in the corresponding sample (B).

Effect of Pre-incubating NHBE Cells with Enisamium Prior to Viral Inoculation

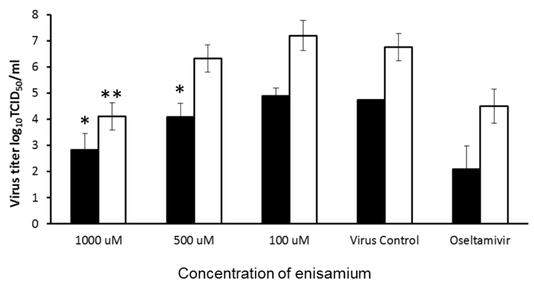


Figure 5. Differentiated NHBE cells were pre-treated with enisamium at 1000, 500, or 100 μM 24 hours prior to inoculation. After 24 hours NHBE cells were inoculated with influenza A/Brisbane/59/2007 (H1N1) virus at an MOI of 0.001 PFU/cell and enisamium was maintained in the basal culture media throughout the experiment. Oseltamivir carboxylate (1 μM) was added to the basal compartment 1 hour before virus inoculation and maintained throughout the experiment. The time of viral inoculation is indicated as 0 hours. Supernatants were collected 24 hours (black bars) and 48 hours (white bars) p.i.

CONCLUSIONS

- In a dose-dependent fashion, enisamium decreased replication of all influenza viruses tested, including a seasonal H1N1 carrying the H275Y NA mutation, and thus showing efficacy against oseltamivir-resistant virus.
- Antiviral efficacy of enisamium was virus dose-dependent and efficacy more pronounced at lower infectious doses.
- Enisamium is most effective when added within 8 hours after infection.
- Intracellular concentration of enisamium achieved at 1000 μM at the time of infection correlated with enisamium antiviral efficacy.

We have reported antiviral activity of an anti-influenza compound, enisamium, supporting the reported clinical efficacy. Although the mechanism of action of enisamium has yet to be identified, data presented here indicates that enisamium targets viral replication of influenza viruses. When enisamium was added before or shortly after infection, the reduction in M-gene expression and viral titers would suggest enisamium inhibits viral RNA synthesis. Interestingly, viral titers continued to increase in the presence of enisamium; therefore, enisamium suppressed influenza virus replication but did not completely inhibit it. Further exploratory work is required to provide a better understanding of the mechanism of action.

ACKNOWLEDGEMENTS

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