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# Susceptibility of recent Canadian influenza A and B virus isolates to different neuraminidase inhibitors

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**Abstract**

Forty-two influenza A and 23 influenza B isolates collected from untreated subjects during the 1999–2000 influenza season in Canada were tested for their susceptibility to three neuraminidase inhibitors (zanamivir, oseltamivir carboxylate and RWJ-270201 or BCX-1812) using a chemiluminescent neuraminidase assay. Influenza B isolates were less susceptible than A viruses to all tested drugs. RWJ-270201 was the most potent drug against both influenza A(H3N2) (mean $IC_{50}$: 0.60 nM) and B (mean $IC_{50}$: 0.87 nM) viruses. Oseltamivir carboxylate was more active than zanamivir for influenza A(H3N2) isolates (mean $IC_{50}$: 0.73 vs. 2.09 nM) whereas it was less potent against B viruses (mean $IC_{50}$: 11.53 vs. 4.15 nM). 

*Keywords:* Influenza B; Inhibitors; Virus; Influenza A

Despite the availability of inactivated trivalent vaccines, influenza is still associated with significant morbidity and mortality worldwide. Amantadine, the only antiviral available until recently for the treatment of influenza infections, has been hampered by its narrow spectrum of activity (effective against influenza A viruses only), frequent side-effects and rapid and frequent emergence of viral resistance (Couch, 2000). A new class of antiviral agents, the inhibitors of the influenza neuraminidase (NA), has recently been developed (Gubareva et al., 2000). The first agents to be commercialized, zanamivir and oseltamivir, have shown excellent in vitro activities against influenza A and B viruses which translated into significant clinical benefit (Hayden et al., 1997; Mist Study Group, 1998; Treanor et al., 2000; Boivin et al., 2000). So far, resistance to the neuraminidase inhibitors has been infrequent, as determined in some clinical trials (Barnett et al., 2000; Boivin et al., 2000; Couch, 2000; Hayden et al., 2000). We now report on the susceptibility of recent influenza A and B field isolates recovered mainly from untreated subjects against three neuraminidase inhibitors: zanamivir, oseltamivir carboxylate and the cyclopentane derivative RWJ-270201 (BCX-1812).

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Nasal or pharyngeal swabs were obtained from patients consulting with an influenza-like illness of < 72-h duration in six major Canadian cities (Vancouver, Edmonton, Toronto, Montréal, Québec City, Halifax) from the end of November 1999 through April 2000. Viruses were grown once on Madin–Darby canine kidney (MDCK) cells, then typed and subtyped using monoclonal antibodies and a multiplex PCR assay, respectively as previously described (Boivin et al., 2001). Susceptibility of some representative clinical isolates and vaccine strains to RWJ-270201 reported as the $IC_{50}$ was first assessed using two different NA inhibition assays, e.g. the fluorometric test using MUN as substrate (Barnett et al., 2000) and a chemiluminescent test using the NA-STAR substrate (Tropix, Bedford, MA). The latter assay was performed as previously described (Buxton et al., 2000) except that the final concentration of the substrate was 100 μM (instead of 60 μM) and the chemiluminescence was read using a ML2200 counter (Dynatech Laboratories, Chantilly, VA). All viruses were re-grown in phenol red-free medium before titration and testing in the chemiluminescent assay to avoid a color quenching effect. Subsequently, all clinical isolates were evaluated for their susceptibility to zanamivir and oseltamivir carboxylate (both drugs were synthesized at Glaxo Wellcome, Stevenage, UK) and to RWJ-270201 (synthesized at R.W. Johnson Pharmaceutical Research Institute, Raritan, NJ) by the use of the chemiluminescent assay. All statistical analyses were performed with SAS systems (version 6.12; SAS Institute, Cary, NC) and procedures.

Representative clinical isolates and vaccine strains of influenza A (H1N1), A (H3N2) and B viruses were first titrated and then $IC_{50}$ values against RWJ-270201 were determined by both the fluorometric and the chemiluminescent NA inhibition assays. As shown in Table 1, although RWJ-270201 $IC_{50}$ values were generally slightly higher when evaluated by the chemiluminescent assay (especially for influenza A strains), there was no significant difference in the mean $IC_{50}$ values between the two assays ($P = 0.10$, ANOVA for repeated measures). The intra-/interassay variabilities of the fluorometric and chemiluminescent assays were 22/37% and 20/35%, respectively, using a larger set of isolates from previous years.

The 1999–2000 influenza isolates including 38 A (H3N2), four A (H1N1) and 23 B viruses, as well as four vaccine strains, were evaluated for their susceptibility to zanamivir, oseltamivir carboxylate and RWJ-270201 using the chemiluminescent NA inhibition assay (Table 2). Influenza B clinical isolates were found significantly less susceptible than influenza A (H3N2) isolates when tested against the three drugs ($P < 0.01$ for all comparisons, ANOVA). Influenza A (H1N1) isolates were more susceptible than influenza A (H3N2) viruses when evaluated against two of the three drugs (zanamivir and RWJ-270201) although no statistical analysis was performed due to the small number of influenza A (H1N1) isolates available. RWJ-270201 $IC_{50}$ values of influenza A (H3N2) isolates were significantly lower than those of oseltamivir carboxylate and zanamivir ($P < 0.01$ for both comparisons, ANOVA for repeated measures). Similarly, RWJ-270201 $IC_{50}$ values of influenza B isolates were significantly lower than those of the two other compounds ($P < 0.01$ for both comparisons,

Table 1
Comparison of RWJ-270201 $IC_{50}$ values against influenza viruses using the chemiluminescent and fluorometric assays for neuraminidase activity

| Influenza strains | Chemiluminescence | Fluorometric |
|---|---|---|
| A/Victoria/2/95 (H3N2) | 0.56 | 0.22 |
| A/Sydney/5/97 (H3N2) | 0.82 | 0.48 |
| A/Beijing/262/95 (H1N1) | 0.40 | 0.14 |
| B/Harbin/07/94 | 1.38 | 1.90 |
| A/Clinical-1/99 (H3N2) | 0.60 | 0.20 |
| A/Clinical-2/99 (H3N2) | 0.63 | 0.19 |
| A/Clinical-3/99 (H1N1) | 0.38 | 0.11 |
| B/Clinical-4/99 | 0.87 | 0.74 |
| Mean | 0.71* ($\pm$0.32) | 0.50* ($\pm$0.60) |

* $P$ value = 0.10 (ANOVA for repeated measures).

Table 2
$IC_{50}$ values of clinical isolates recovered from untreated subjects during the 1999–2000 influenza season in Canada and of vaccine strains to neuraminidase inhibitors

| Influenza strains (No. of isolates) | Zanamivir $IC_{50}$ (nM) | | Oseltamivir carb. $IC_{50}$ (nM) | | RWJ-270201 $IC_{50}$ (nM) | |
|---|---|---|---|---|---|---|
| | Mean | Range | Mean | Range | Mean | Range |
| A/H3N2 isolates ($n = 38$) | 2.09 | 1.15–4.22 | 0.73 | 0.32–1.66 | 0.60 | 0.30–0.93 |
| A/H1N1 isolates ($n = 4$) | 1.14 | 0.94–1.32 | 0.90 | 0.71–1.31 | 0.27 | 0.13–0.39 |
| B isolates ($n = 23$) | 4.15 | 2.19–6.34 | 11.53 | 4.93–18.59 | 0.87 | 0.52–1.36 |
| A/Victoria/2/95/H3N2 | 1.60 | 0.85–2.93 | 0.67 | 0.52–0.78 | 0.56 | 0.33–0.72 |
| A/Sydney/5/97/H3N2 | 3.23 | 1.44–4.76 | 1.48 | 0.82–1.97 | 0.82 | 0.62–1.09 |
| A/Beijing/262/95/H1N1 | 0.65 | 0.52–0.81 | 1.53 | 0.97–2.07 | 0.41 | 0.29–0.58 |
| B/Harbin/07/94 | 5.94 | 2.69–9.53 | 10.01 | 6.10–12.80 | 1.38 | 0.97–1.66 |

$IC_{50}$ values of vaccine strains are based on three experiments.

ANOVA for repeated measures). Oseltamivir carboxylate was more active than zanamivir against A (H3N2) isolates ($P < 0.01$) but the opposite was true for B isolates ($P < 0.01$). In a subset of pretherapy (day 1) and day-3 therapy influenza A isolates collected from four patients who received inhaled zanamivir (10 mg BID), no significant difference was found in the mean $IC_{50}$ values over time for zanamivir (1.70 and 1.82 nM), oseltamivir carboxylate (0.75 and 0.78 nM) and RWJ-270201 (0.56 and 0.54 nM). All other tested isolates were from untreated subjects.

Two NA inhibitors of influenza viruses, zanamivir and oseltamivir, have recently been approved in the US and in many other countries for the treatment of influenza A and B infections. RWJ-270201, which is a novel cyclopentane derivative, has been shown to inhibit the growth of different influenza virus subtypes in tissue culture (Smee et al., 2001) and in NA inhibition assays (Bantia et al., 2001) with demonstrated oral efficacy in experimental mouse models (Bantia et al., 2001; Sidwell et al., 2001). In this study, we reported the susceptibility of a panel of recent influenza A and B virus isolates (from 1999 to 2000) recovered mostly from untreated patients against three NA inhibitors: zanamivir, oseltamivir carboxylate and RWJ-270201. Our study differs from previous ones by assessing recent clinical isolates of low passage numbers (instead of highly-passaged vaccine strains) and by using a chemiluminescent instead of a fluorometric assay for measuring viral NA activity. This newly-described chemiluminescence-based assay, which uses a 1,2-dioxetane derivative of sialic acid (NA-STAR) as the substrate, has previously been reported to be more sensitive than the fluorometric method using the MUN substrate in detecting virus NA activity (Buxton et al., 2000). In our study, we did not address the question of sensitivity by testing isolates with low level of NA activity, but we did not find any significant differences in the $IC_{50}$ values for RWJ-270201 when a subset of isolates with good NA activity was tested by the two methods, although more data are needed for comparative purposes across viral types. Because the chemiluminescent assay is more rapid (1 h 15 min versus 2 h per run), as reproducible and more sensitive than the fluorometric assay, it should now be considered the method of choice for assessing NA activity of viral isolates. Furthermore, neuraminidase enzyme assays are preferred to the plaque reduction assay to evaluate NA inhibitors' activity since the latter has been shown to be extremely variable and unpredictive of in vivo susceptibility to zanamivir (Barnett et al., 2000).

Although no clinically-relevant resistance cutoff values have been determined for the different influenza NA inhibitors using NA-based assays, a 10- to 1000-fold shift in drug susceptibility has been reported for laboratory-derived NA mutants using the fluorometric NA inhibition assay (McKimm-Breschkin, 2000). Thus, based on these

in vitro data, we can conclude that no drug-resistant strains due to NA mutations were found in our study, since the range of $IC_{50}$ values for the different viral subtypes was extremely narrow ( < 5-fold changes). Obviously, NA inhibition assays would not detect resistant strains containing hemagglutinin mutations. At the present time, no phenotypic assay would readily detect such mutants, since no tissue culture system adequately reflects the receptor specificity of human cells of the respiratory tract. Thus far, HA mutations have been found very uncommonly by comparing the gene sequence of post- and pre-therapy isolates and such mutants were susceptible to zanamivir in the ferret model (Barnett et al., 2000; Boivin et al., 2000).

Despite the susceptibility of all isolates to the three NA inhibitors tested, we did find some differences in the susceptibility levels related to the type of influenza viruses (A or B) and the type of compounds. First, influenza B viruses were generally less susceptible than A (H3N2) viruses for all three tested drugs (mean differences of 1.5-, 2- and 16-fold for RWJ-270201, zanamivir and oseltamivir carboxylate, respectively). Second, we found that RWJ-270201 was the most potent drug in vitro against influenza A and B viruses including both clinical isolates and vaccine strains (Table 2). For influenza A (H3N2) isolates, oseltamivir carboxylate was found to be more active than zanamivir, whereas the opposite result was obtained for influenza B viruses. Our data, in particular the lower potency of oseltamivir carboxylate against influenza B viruses, are in agreement with previous reports (Gubareva et al., 1999; Bantia et al., 2001). These differences in potency may be explained by the subtle differences in the active site of the NA enzyme from different types (and subtypes) of viruses and by the interaction of these compounds with the active site. For example, the higher potency of RWJ-270201 compared to oseltamivir carboxylate with regard to influenza B viruses may be explained by the additional interaction with the active site of the NA provided by the guanidinium group of RWJ-270201 which may compensate for the unfavorable reorientation of the Glu 276 side chain (Bantia et al., 2001). However, considering the high concentrations of these drugs in the respiratory tract (in the μM range for zanamivir in sputum) (Gubareva et al., 2000) and the low $IC_{50}$ values of the isolates tested in this study (in the nM range), it is unlikely that such in vitro differences would significantly influence the in vivo response in humans. The differences in the chemical structure and in the in vitro potency of these compounds may be more important in the advent of the development of resistance to one of these drugs (Gubareva et al., 2000).

In this study, we reported baseline susceptibility of clinical influenza isolates during the first year in which NA inhibitors were available in Canada. Continuous monitoring of influenza susceptibility is needed as the number of patients receiving these compounds for treatment or prophylaxis will increase. Since resistance to NA inhibitors is expected to be low, surveillance programs should include sampling from treated subjects still excreting viruses on or after day 3 of therapy and from contacts of those being treated.

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