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MICROBIOLOGY REVIEW(S)

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Division of Antiviral Drug Products (HFD-530)

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Sponsor: Glaxo Wellcome Inc.
Five Moore Drive
Research Triangle Park, NC 27709

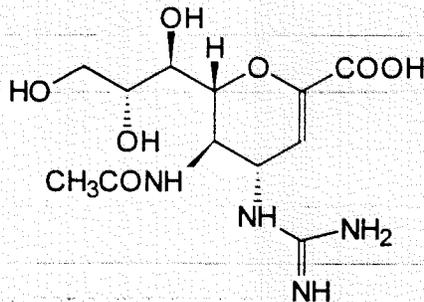
Product Names: Proprietary: Relenza®
Nonproprietary: Zanamivir
Code: GR121167X or GG167

Chemical name: 5-(acetylamino)-4-[(aminoiminomethyl)amino]-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enoic acid.

Empirical formula: C₁₂H₂₀N₄O₇

Molecular Weight: 332.3

Structural Formula:



Dosage form: Inhalation powder (Relenza Rotadisk)

Indication: Treatment of influenza A and B

Related documents: INDs

BACKGROUND

In this NDA Glaxo Wellcome Inc. is requesting approval of Relenza® (inhalation powder) for the treatment of uncomplicated illness due to influenza virus in adults and adolescents who are 12 years old and who have been symptomatic for no more than 2 days. The proposed indication is based on the results obtained from three Phase 3 prospective, double-blind, randomized, placebo-controlled trials. Relenza® treatment effects were based on the assessment of the symptomatic course of influenza illness. The primary endpoint in these studies was the median time to alleviation of clinically significant signs and symptoms of influenza. Secondary endpoints involved multiple criteria including quantitative evaluation of influenza virus titers.

The applicant presented the microbiology portion of the NDA in several sub-sections. They include: the mechanism of action; anti-neuraminidase activity in vitro; antiviral activity in vitro; antiviral activity in animal models; and the development of resistance to zanamivir. Studies on the development of resistance were sub-divided into: resistance to zanamivir in vitro; zanamivir-dependent resistance; resistance due to mutations in NA; resistance due to mutations in NA and HA; drug cross-resistance; and Relenza resistance in clinical studies. The microbiology review that follows addresses the data described and includes a background devoted to the virology of influenza virus (1).

Influenza virology: Three influenza virus types A, B and C are members of a family of related enveloped viruses with a diameter in the range of 80 to 120 nm and of indistinguishable morphology. The division of influenza viruses into types A, B and C is based on the antigenic differences between their nucleoproteins and their matrix protein antigens.

Influenza viruses replicate in the nucleus of the infected cells. Their genome is segmented, single-stranded linear RNA. The viral RNA is 'negative-stranded' (i.e., the virion RNA is complementary to the messenger RNA) and thus the RNA itself cannot serve as messenger RNA for the synthesis of viral proteins. However, in infected cells, the negative-stranded influenza virus RNA is directly transcribed to generate positive-stranded RNA by the combined action of viral and host cellular enzymes. The positive-

stranded RNA then serves both as a template for the synthesis of the viral genomic RNA segments as well as viral proteins.

Influenza viruses A and B contain 8 segments of RNA and code for 10 proteins, which include two envelope proteins. The RNA segments 4 and 6 code for the viral envelope glycoproteins; the former codes for the glycoprotein hemagglutinin (HA), and the latter codes for the glycoprotein neuraminidase (NA). In contrast to influenza virus types A and B, the influenza virus C contains 7 segments and codes for 9 proteins with a single envelope glycoprotein. A single envelope glycoprotein is coded for by the RNA segment 4 of influenza virus C and is referred to as HEF, because it contains the hemagglutinin, esterase and fusion activities. Therefore, sialic analogue inhibitors of NA inhibitors, such as Relenza, the drug of this NDA, as well as other experimental drugs like GS4104, BCX-140 and others undergoing FDA evaluation are expected to inhibit the replication of influenza viruses A and B but not influenza virus C.

The envelope glycoproteins, HA and NA, of influenza virus types A and B play an important role in the initiation of viral infection and pathogenicity. Several studies indicate that the envelope proteins, HA and NA function in a complementary manner in that they mutually compensate the receptor binding function. Influenza virus HA recognizes and binds to the cell receptors i.e., sialic acids (neuraminic acid) and the NA activity releases the virus from receptor sialic acid, thereby promoting the virus release. The molecular target of zanamivir is the viral NA and more specifically the NA enzyme activity portion of the protein without direct effect on the antigenic properties of the NA. Considering the molecular interactions between HA and NA proteins and their complementary role in the viral infection, replication, spread, and pathogenicity, some additional molecular aspects of these two proteins are summarized briefly.

Influenza virus hemagglutinin: HA derives its name from its role in the agglutination of erythrocytes by attachment to specific sialic glycoprotein receptors. The HA is encoded by the 4th largest RNA segment and accounts for 25-27% of the virion protein that is distributed evenly on the surface of the virions. The HA is responsible for the attachment of the virus particle to the cell surface via a sialic acid receptor and also for subsequent fusion of viral and cell membranes which allows the entry of the virus genome into the cell cytoplasm. HA is the major antigen of the virus against which neutralizing antibodies are produced. The variability of HA has been the main factor in

the continuing evolution of influenza epidemics and controlling those epidemics by immunization.

HA is a trimeric envelope glycoprotein. It is initially synthesized as a single 567-amino acid polypeptide that undergoes glycosylation. During infection, the 567 amino acid polypeptide (HA₀) undergoes post-translational cleavage by cellular proteases. The cleavage results in the formation of two subunits, HA₁ (328 amino acids) and HA₂ (221 amino acids) that covalently attach to each other by disulfide bonds. The active HA is a trimer of this disulfide-linked HA₁/HA₂ subunit. Cleavage of HA₀ to form HA₁ and HA₂ polypeptides is required for the viral infectivity because it is the HA₂ fragment that mediates fusion between the viral envelope and the host cell membrane.

Influenza virus neuraminidase: The target of zanamivir is the viral NA. The function of NA is to cleave the sialic acids from the viral HA, viral NA itself, and host cell substrates. This cleavage is believed to prevent virus aggregation, elutes virus from infected cells, and facilitates trafficking of the virions to reach new cells for infection.

Influenza virus NA is a tetrameric envelope glycoprotein of identical subunits. The NA is coded by the 6th largest RNA segment of influenza virus and accounts for about 6% of the virion protein. NA, also known as sialidase or acylneuraminyl hydrolase, catalyzes the cleavage of the α -ketosidic linkage between the terminal sialic acid and an adjacent sugar residue of glycoproteins, glycolipids, and oligosaccharides. Removal of the sialic acids by the NA assists both the entry and release of influenza virus from cells. The NA of human influenza viruses cleaves the NeuAc α 2,6 linkage. The HA of human influenza viruses preferentially recognize NeuAc α 2,6 linkage for binding and initiation of the infection process.

NA is a bifunctional protein in that it acts both as an enzyme, by removing sialic acids from glycoproteins, and as a major antigenic determinant, in that it can undergo antigenic variation. The enzyme plays a role in preventing aggregation of newly synthesized virus particles by the sialidase-catalyzed hydrolysis of acetylneuraminic acid, which is the receptor binding site for the HA of influenza virus. NA may also allow virus to move through the mucous present in the respiratory tract. NA has been crystallized and its three-dimensional structure determined (2). Zanamivir is designed as a substrate analogue of the NA active site, therefore it should bind and inhibit its activity.

Nomenclature of influenza virus subtypes: As stated earlier, influenza viruses are divided into types A, B and C on the basis of antigenic differences of their nucleocapsid and matrix proteins. Influenza A viruses are further subdivided into subtypes on the basis of antigenic differences in the HA and NA. Fourteen HA, and 9 NA subtypes are recognized from all animals. However, to date only 3 HA (H1, H2 and H3) and 2 NA (N1 and N2) subtypes have been recognized in epidemics of human influenza A infection. The most common subtypes of HA and NA combination that have been found to circulate in humans are H1N1, H2N2 and H3N2. The nomenclature system recommended by the World Health Organization include the virus type, geographic origin, isolate number and the year of isolation. The antigenic description of the HA and NA is given in parenthesis, e.g., A/Shanghai/11/87 (H3N2).

SUMMARY

Mechanism of neuraminidase inhibition by zanamivir: Influenza virus NA is a glycohydrolase that cleaves the terminal α -ketosidic linkage between the terminal sialic acid residue and an adjoining sugar residue found on a array of glycoproteins, glycolipids and oligosaccharides. Molecular studies on the x-ray crystal structure of NA defined the active site of the enzyme in 3-dimensions. Mechanistic studies of NA-directed catalysis revealed that the hydrolytic cleavage of the sialic acid proceeds through an oxonium cation transition-state. The dual knowledge of the enzyme active site and the catalytic mechanism through the transition state structure provided opportunities for rational design of NA inhibitors using computational methods. Zanamivir is one of several inhibitors of NA rationally designed by logical substitutions on the sialic acid substrate-based template.

The function of NA in the influenza virus life cycle is not completely understood. The enzyme does not seem to play a direct role in viral entry, replication, assembly, or budding (3). Instead the enzyme binds to the sialic acid containing glycoconjugates and cleaves the terminal sialic acids. Therefore, NA is believed to facilitate the prevention of virus aggregation and promote virus trafficking through the mucus of the respiratory track.

The overall amino acid homology of the ten NAs (nine NA subtypes of influenza virus A and one of B) is low (~30%). However, the 21 amino acids that make up the catalytic site of the enzyme in all of the ten NAs are completely conserved. This highly conserved nature of the enzyme active site predict that rationally designed drugs like zanamivir, oseltamivir and other experimental inhibitors block the activity of the NA of different subtypes. Consistent with this expectation, zanamivir was found to inhibit the activity of all of the NAs and the inhibitory concentrations were within a narrow range (see below).

The applicant selected zanamivir as their choice for the inhibition of NA because of the inhibitor's higher binding affinity ($K_i = \sim 0.38 \cdot 10^{-10}$ M) to the different NAs than the NAs affinity to the substrate ($K_m = \sim 7 \cdot 10^{-6}$ M). This high differential binding property of zanamivir by isosteric conformation permits competitive inhibition of the enzyme activity. Designers of sialic acid-based inhibitors (sialomimetics) also pointed out (2) that mutants of the highly adaptable influenza virus could emerge by reversal in the differential binding properties of the substrate and inhibitor, i.e., preferential binding of the substrate over the inhibitor. Indeed NA mutants of influenza virus have been isolated from both in vitro and in clinical settings after exposure of the virus to zanamivir (see below).

Inhibition of NA activity by Zanamivir: The applicant determined the 50% inhibitory concentration (IC_{50}) of zanamivir for NA activity from a range of influenza A and B viruses, which included both laboratory strains and clinical isolates. In parallel experiments, the inhibitory activity of zanamivir was also compared with other NA inhibitors. In most of the activity studies a simple synthetic substrate, 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUN) was used as a substrate. Influenza virus NA cleaves the α -glycosidic linkage releasing the fluorescent compound 4-methylumbelliferone which is measured fluorimetrically. This synthetic substrate used in many of these studies provides the minimal requirements for cleavage by the enzyme neuraminidase. Thus, the artificial substrates that can be used for determining NA activity in vitro need to bear little homology to the natural substrates and therefore, the inhibitory concentrations reported with this substrate have limited value for extrapolation to the in vivo situation (see below).

The effect of zanamivir on the activity of NA from all of the nine subtypes of avian influenza virus in vitro is presented in Table 1. This appears to be the only study in the

NDA in which feutin (one of the many natural substrates) was used as a substrate for enzyme activity determinations. The results suggest that zanamivir inhibited the NA activity of all nine NA subtypes of influenza A virus as expected based on the conserved nature of the catalytic site of the enzyme. However, the IC_{50} of zanamivir against different NAs was variable (range of 5.5 to 95.2 nM) and fell into two distinct groups. Group one is comprised of viruses of the N1, N2, N3, N4 and N8 subtypes which are inhibited at drug concentrations similar to the human strain H2N2, and group two is comprised of N5, N6, N7 and N9 subtypes. The sensitivity of group two viruses to the inhibitor is 4-16 times lower than the responses of subtypes in group one.

Table1. In vitro inhibition of all nine neuraminidase subtypes by zanamivir.

Neuraminidase Subtype	Virus	IC_{50} (nM)
N1	Duck/Alberta/37/76	7.6
N2	Mallard/New York/6750/78	6.7
N3	Duck/Alberta/48/76	5.5
N4	Turkey/Ontario/6118/68	8.1
N5	Shearwater/Australia/73	35.1
N6	Duck/England/56	37.3
N7	Chick/Germany/49	71.5
N8	Duck/Memphis/928/74	9.5
N9	Duck/GDR/72	95.2
N2	Singapore/1/57 ^a	8.8

a = Human strain used for comparison with avian viruses.

In other studies using MUN as a synthetic minimal substrate (data not shown), the applicant reported that IC_{50} values for inhibition of NA activity by zanamivir was in a narrower range. In studies with minimal substrate the mean IC_{50} values for the inhibition of influenza A virus NAs was in the range of 0.64 to 5.6 nM and for the Influenza B virus NAs the IC_{50} was in the range of 1.9 to 7.5 nM (data not shown).

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Selectivity of sialidase inhibition by zanamivir: In studies to evaluate the inhibition specificity of zanamivir, the applicant determined the IC_{50} of zanamivir against one purified human NA (lysosomal sialidase from placenta) using a simple synthetic substrate. The results suggest that zanamivir is a weak inhibitor of the human lysosomal NA. The IC_{50} of zanamivir required for the inhibition of human NA was 10^5 times higher than the IC_{50} required for inhibition of influenza virus NAs. In a comparative analysis for evaluation of zanamivir selectivity, the applicant reported the inhibitory activity of zanamivir against NAs from parainfluenza virus type-2, *V.cholera*, *A. urefaciens*, *C. perfringens* and sheep liver. In these studies, zanamivir showed substantially weaker inhibitory activity compared to the inhibitory activity on influenza virus NAs (data not shown).

These limited data on evaluation of specificity of NA are insufficient to draw conclusions on the selective inhibition of zanamivir for influenza virus NA. A single human NA was tested when humans have many endogenous NAs participating in a range of metabolic activities. In contrast to the synthetic substrate, MUN, used in these studies the natural substrates for NA are numerous and of multiple molecular forms (glycoproteins, glycolipids and oligosaccharides) and of diverse conformations. The sialic acid in the natural substrates may be in conformations with limited accessibility to the enzyme compared to that of the simplistic substrate. Furthermore, in these studies the purity of the enzymes, the metal ion requirements for optimal activity of the enzymes, the substrates and a variety of other factors were not optimized. In brief, the studies reported on the selectivity of zanamivir for inhibition of influenza NA are inadequate to draw any conclusions.

In Vitro anti-influenza virus activity of zanamivir: The applicant carried out several studies to determine the anti-influenza virus activity of zanamivir. In these studies, both laboratory adapted and clinical isolates of influenza A and B viruses were used. In parallel experiments the antiviral activity of zanamivir was compared with other anti-influenza virus agents; amantadine, rimantadine, ribavirin and Neu5AC2en. Several assay systems were used in these studies to evaluate the effect of zanamivir on influenza virus. They include: (a) influenza virus induced plaque formation on MDCK indicator cells, (b) influenza virus growth measured by the yield of virus into the culture medium of influenza virus infected MDCK cells, (c) reduction in the cytopathic effect (cpe) of influenza virus due to treatment of cells with zanamivir, and (d) the growth of influenza

virus in human respiratory epithelium explants (a cell type representative of the natural site of infection in man).

In the antiviral activity assays, confluent monolayers of recipient cells were infected with influenza virus strains at a multiplicity of infection of 0.01 plaque forming units per cell and the test compounds were added at different concentrations. In plaque reduction assays, 3 days after infection the virus induced plaques were counted and the percentage of plaque reduction at each compound concentration was calculated. In the inhibition of virus yield assays, the viral titers were determined from infected MDCK cells either by plaque assays or by hemagglutinin assay, and in human respiratory epithelium explants, zanamivir effect was determined by inhibition of virus yield.

In all of these assays, the inhibitory effect of zanamivir and the comparator compounds was expressed as IC_{50} and IC_{90} values (the compound concentrations required to reduce the plaque numbers by 50% and 90%, respectively). The values were determined by taking the mean percentage inhibition from three experiments and plotting the values against the compound concentration. The IC_{50} and IC_{90} values were then determined by linear interpolation.

Combined results from the antiviral activity studies indicate that zanamivir was active against a range of influenza viruses with a broad range of sensitivity against different isolates. The IC_{50} values for zanamivir ranged from 0.005 to 16.0 μ M and the IC_{90} values ranged from 0.05 to >100 μ M. In general, the comparator compounds, amantadine, rimantadine, ribavirin and Neu5AC2en, required much higher concentrations than zanamivir, indicating that under these conditions zanamivir is a more potent inhibitor of influenza NA than the comparator anti-influenza agents.

Selectivity of zanamivir: To distinguish cytotoxic effect from antiviral effect of zanamivir, the applicant investigated the in vitro cytotoxicity of the drug against four established cell lines to determine the specificity of zanamivir. In these studies the test cells were exposed to different concentrations of the drug for 5 days with appropriate controls in parallel and determined the viability and division of cells exposed to zanamivir. The cell lines used in these studies were: MRC-5, a cell line derived from human lung tissue; PANC-1, a cell line derived from human pancreatic tissue; 161-BR, a cell line derived from human epithelial tissue; and MDCK, a canine cell line derived

from kidney tissue. The cytotoxicity in all of the four cell lines was determined by measurement of the uptake of a vital stain neutral red, and by the uptake of [³H]-thymidine.

Results of the selectivity studies (data not shown) of zanamivir indicate that the compound had no effect on cell viability or cell division at a concentration as high as 10 mM. The therapeutic index, (i.e., the ratio of zanamivir concentration that caused cytotoxic effect to the concentration of zanamivir that exerted antiviral activity, as measured by plaque assay) of zanamivir in MDCK cells calculated was in the range of >62.5 to >250,000 and was dependent on the virus subtype used. At zanamivir concentrations of <10 mM cytotoxicity was not detectable on MRC-5, PANC-1 or 161-BR cell lines. The data on the cytotoxicity of zanamivir indicates a favorable therapeutic index.

Efficacy of zanamivir in animal models of influenza virus infection: Three animal models of influenza virus infection, mouse, ferret and chicken, are conventionally used to evaluate the anti-influenza activity of test compounds. In these animal models, the influenza infection is predominantly confined to the respiratory tract as is the case with the majority of influenza infections. The ferret model more closely matches the clinical course of human infection in that the infected ferrets develop an illness of limited duration with symptoms similar to those seen in humans. In the mouse model, infected animals develop pneumonia and show high mortality rate. Chickens infected with highly pathogenic avian influenza virus strains develop disseminated infection with high mortality rate.

The applicant investigated the prophylactic and therapeutic anti-influenza virus A and B efficacy of zanamivir in the mouse and the ferret animal models. The present NDA is for a treatment indication of influenza infection and therefore, the review mainly deals with the therapeutic effect of zanamivir in these animal models.

Efficacy of zanamivir in the mouse model: Ether anaesthetized mice were infected by intranasal inoculation with a 50µl suspension (approximately 25µl into each nostril) of influenza virus type A/singapore/1/57 containing 1.5×10^2 TCID₅₀ (50% tissue culture infectious dose) units of influenza virus. Treatment with zanamivir or placebo (phosphate buffered saline) was initiated 3 hours post-infection and continued twice

daily on days 1 to 3. The lung influenza virus was titered in MDCK cells, and the results expressed as the area under the virus titer/time curve (AUC) for the test and control groups. The effect of therapy was determined by calculating the extent to which the AUC for the test group is reduced relative to the phosphate buffered saline controls.

Table 2. Effect of delayed treatment on the efficacy of zanamivir in mice

Delay element in treatment protocol	Virus Titer (%AUC)*	
	0.39mg/kg	12.5 mg/kg
(1) No delay in treatment- 2 prophylactic doses given at 18- and 3-hr post infection ⁺	0.068	≤0.0036
(2) Delayed treatment – one prophylactic dose at 3-hr before infection ⁺	0.68	0.0047
(3) Delayed treatment-first dose at 3-hr post infection ⁺	5.74	0.0065

* %AUC values calculated as mean AUC for compound divided by the mean AUC for the relevant control and multiplied by 100.

⁺ Subsequent doses given twice daily on days 1-3.

Results presented in table 2 show that in influenza A-infected mice, zanamivir is less effective (10 to 100-fold) when administered post-infection than prophylactically. Three hours post-infection, the efficacy of zanamivir at doses of 0.39 and 12.5 mg/kg decreased the AUC of virus titer to 5.74% and 0.0065% of the control, respectively. The dose requirements of zanamivir needed for efficacy at later times (after >3-hour post infection) are unknown, and it is expected that even higher concentrations of zanamivir may be needed to achieve similar levels of reductions in virus load. The high therapeutic dose of zanamivir required to reduce viral load in the lungs of this model is consistent with the observation that high concentrations of the drug are required to inhibit influenza virus replication in cell cultures in vitro.

Efficacy of zanamivir in the ferret model: In the evaluation of the therapeutic efficacy of zanamivir in the ferret model, isoflurane anaesthetized female ferrets (weight range of 700-1200g) were challenged with 10^4 TCID₅₀ of influenza virus A/Mississippi/1/85 by intranasal instillation of 0.25 ml of diluted stock. Treatment with

zanamivir was initiated at 5 hours after infection with one group and 22 hours after infection with a second group. Treatment of both ferret groups was continued twice daily on days 1 to 6 with a single dose on day 7.

Table 3. Effect of delayed treatment on the efficacy of zanamivir in ferrets

Delay element in treatment protocol	Nasal wash titer (%AUC)*		
	0.05 mg/kg	0.3 mg/kg	1.5 mg/kg
(1) No delay in treatment-prophylactic dose given at 26 and 2-hr before infection	11.4	1.38	0.8
(2) Delayed treatment-first dose 5-hr post-infection	13.98	1.47	0.95
(3) Delayed treatment-first dose 22-hr post-infection	31.82	2.88	1.10

* % AUC values calculated as mean AUC for compound divided by the mean AUC for the relevant control and multiplied by 100

Data presented in table 3 show the efficacy of zanamivir at dose levels of 0.05, 0.3 and 1.5 mg/kg body weight in a dose volume of 0.25 mg/kg was determined from the nasal wash samples. Virus titer in the case of ferrets was determined by [redacted] and not by infectious virus assay as was the case in the mouse model. Each nasal wash sample was assayed in triplicate at eight dilution levels (ranging from $1:10^7$) along with virus and cell controls. The titer was expressed in virus equivalents as TCID₅₀. The AUC for nasal wash virus titer on days 1-9 for each dose was calculated and the geometric mean of the AUC value for the group of ferrets determined from the ELISA TCID₅₀. The AUC value was then converted into a log₁₀ value.

At 5 hours post-infection, nasal wash virus titer in the ferrets exposed to 0.05, 0.3 and 1.5 mg/kg showed percent AUC reductions to 13.98, 1.47 and 0.95, of the control, respectively. At 22 hours post-infection, the nasal wash virus titers were 31.82, 2.88 and 1.10, percent of the control, respectively. Thus, each treatment protocol showed a dose related reduction in nasal wash titer. In addition, temperature measurements suggested no marked pyrexia in ferrets dosed with 1.5 or 0.3 mg/kg (data not shown).

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The results show that administration of zanamivir at 5 and 22 hours post infection was effective in the ferret model as determined by reduction of viral titers or by pyrexia. Comparative efficacy evaluation could not be made because in the mouse model of influenza virus infection the duration of treatment was shorter (3days) compared to the ferrets where the treatment duration was prolonged to 7 days. Furthermore, determination of viral titer in ferrets was carried out by an immunochemical method and in the case of mice it was by infectious viral titer. The high therapeutic dose of zanamivir required to reduce viral load in the lungs of ferrets is consistent with the observations that high concentrations of the drug is required to inhibit influenza virus replication in vitro and in vivo.

Influenza virus challenge studies in human volunteers: The applicant conducted several double-blind placebo-controlled Phase 1 studies to determine the antiviral activity of zanamivir on human challenge strains of influenza virus A and B. In these studies, healthy human volunteers were experimentally inoculated intranasally with influenza A/Texas/91(H1N1) virus or influenza B/Yamagata/16/88 virus, and the prophylactic and therapeutic effects of intranasal zanamivir administered at different doses and regimens was determined. Zanamivir effects were evaluated by determination of virus isolation, hemagglutinin inhibition titer, influenza-like illness, and non-febrile respiratory illness (see medical officer's review for details).

Summary results of the combined studies provide evidence of antiviral activity of zanamivir against the human challenge strains of influenza virus A and B used. Zanamivir reduced the viral shedding, with prophylactic (treatment before virus inoculation) administration being more effective than therapeutic (treatment after virus inoculation) administration. The antiviral effect of zanamivir on influenza virus A infection was more convincing than on influenza virus B infection. However, it is to be noted that in these studies, the route of administration of the drug, the timing of treatment, viral strains and sites of infection may be different from the clinical situations (see medical officer's review).

Influenza vaccine interaction study: The applicant conducted a double-blind, randomized, placebo-controlled study to evaluate the effects of inhaled zanamivir (10 mg once daily) on anti-hemagglutinin antibody production following administration of trivalent influenza vaccine. In this vaccine interaction study, healthy subjects (n = 138)

who received a single dose of trivalent inactivated influenza vaccine, were randomized to receive the active drug or placebo for 28 days. Serum hemagglutination inhibition antibody titers at 2 and 4 weeks after vaccine administration in zanamivir and placebo recipients was determined (see medical officer's review for details).

Table 4: Geometric mean increases in hemagglutination inhibition titer after vaccination.

Week	Influenza Type	Vaccine + Relenza (n = 70)	Vaccine + Placebo (n = 68)
2	B	4.8	5.0
	A (H1N1)	18.6	11.7
	A (H3N2)	15.5	9.8
4	B	4.4	4.6
	A (H1N1)	16.0	11.5
	A (H3N2)	13.9	8.7

Results presented in table 4, indicate that there was no clear difference in hemagglutinin inhibition titer at 2 and 4 weeks after vaccine administration between zanamivir and placebo recipients. Therefore, the data is consistent with the interpretation that zanamivir when given for 28 days does not impair the protective immune response as determined by hemagglutinin antibody titer.

Variation in influenza virus and mechanisms of emergence of drug resistance: As an RNA virus, the influenza viruses inherently exhibit high mutation rates that are primarily attributed to the lack of proofreading during RNA replication. The estimated mutation rate of 1.5×10^{-5} mutations per nucleotide per infectious cycle of influenza viruses is similar to other RNA viruses (1). This high rate of mutation itself provides an ample source of selection of naturally occurring variants. In addition, the rate of mutation for some of the component genes of influenza virus varies. The mutation rate for HA (a neutralizing antigen) is estimated at 6.7×10^{-3} , suggesting that the accelerated rate of mutation in this gene may be selected by immune pressure (1). The combined effects of the accumulation of point mutations due to replication errors and the immune pressure accelerate the genetic drift.

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In addition to genetic drift other events such as RNA recombination, genetic reassortment among and between human and animal strains due to the segmented nature of the viral genome and other genetic effects may further enhance the rate of variation contributing to genetic shifts, drug resistance, and potential pathological consequences.

Available evidence on the inhibition of anti-influenza virus activity by the use of sialomimetic drugs designed to block NA activity appear to induce yet another type of variation in influenza virus. Previous studies with NA inhibitors indicate those genetic changes in HA alone without changes in the activity or sensitivity of NA, appear to be another mechanism of escape from NA inhibition. Thus, multiple mechanisms (inherent, induced and those due to molecular interactions) come into play to overcome drug pressure and allow the virus to rapidly adapt to new drug challenges.

In vitro studies on the emergence of zanamivir resistance: The applicant investigated the potential of zanamivir to induce changes in susceptibility of influenza viruses as a consequence of exposure to the compound. In the susceptibility studies recipient MDCK cells infected with different strains of influenza viruses were passaged in vitro in the presence of increasing concentrations of the compound. The resulting virus isolates were sequentially analyzed for alterations in their phenotype and genotype. Results of these studies indicate that replication of influenza viruses in MDCK cells in the presence of zanamivir led to the selection of virus variants which were resistant to the compound. Interestingly, the variant viruses were found to possess a number of different phenotypes, although all of the viruses have, in common, a reduced sensitivity to zanamivir. Biological studies and nucleic acid sequence analysis of the variant viruses showed that resistance to zanamivir can result from amino acid sequence changes either to the viral HA, or the viral NA, or both. Results of some of these studies are summarized below.

One of the viruses selected for zanamivir susceptibility studies was influenza virus strain A/NWS/G70C (H1N9), which contained the N9 from the avian influenza virus and the remainder from the human influenza virus NWS (H1N1). This strain of influenza was selected because the crystal structure of the NA of this virus was known (zanamivir was rationally designed inhibitor of NA based on the 3-dimensional structure of the N9 enzyme) and any changes in the NA could be related to its 3-dimensional structure. The

H1N9 virus was passaged in MDCK cells in the presence of increasing concentrations of zanamivir or its close analogue 4-amino-Neu5Ac2en. Virus replication was evaluated by the viral cytopathic effect on MDCK cells, and virus growth in cell supernatant was determined in a slot blot immunoassay using a monoclonal antibody for NA protein.

Zanamivir-dependent influenza variants: Influenza virus variants emerged as early as 1 passage and up to 6 passages. Variants that emerged at 5-6 passages were selected for further characterization (4). Phenotypic analysis of the variants isolated showed that 90% had some level of drug dependence based on the following properties:

- (a) titers of virus produced in the presence of the compound were four-to-eight fold higher when grown in the presence of compound than in the absence of compound.
- (b) Some variants gave larger and greater numbers of plaques in presence of the drug.
- (c) The variants were up to 1,000-fold less sensitive to the selecting compound in the plaque assays.
- (d) Hemagglutination elution assays showed that the variants eluted much more rapidly than the parental virus (1 hour vs. overnight).
- (e) The concentration of the compound required to inhibit hemagglutination elution was considerably more for variants than for the parental virus.

The significance of the emergence of the in vitro drug-dependent variants that acquired a variety of phenotypic properties is unknown. If this were to happen in the clinic, these in vitro observations might predict the emergence of drug-dependent variants with robust growth in the presence of the drug and consequent lack of prophylactic and/or therapeutic effect of the drug. These in vitro observations on the emergence of drug-dependent variants calls for investigations on the emergence of drug-dependent variants in clinical settings.

Zanamivir resistance due to mutations in hemagglutinin: Three variants; 1, 5, and 7, from the A/NWS/G70C (H1N9) study were selected for further characterization of the resistance properties. Variant 1 was passaged exclusively in the comparator (4-amino-Neu5Ac2en); variant 5 was passaged originally in comparator and subsequently in zanamivir and variant 7 was passaged in zanamivir throughout. The following observations show that the phenotypic differences in these viral variants were not due to changes in the NA:

- (a) Inhibition of NA activity studies with the variants showed no changes in enzyme activity; that is the resistant variants retained sensitivity to inhibition by zanamivir as parental virus.
- (b) The kinetics of binding studies showed that there were no significant differences in the kinetic or equilibrium parameters.
- (c) Addition of exogenous NA to the culture medium did not increase the number of plaques indicating the phenotypic change is not due to loss of NA activity, and
- (d) Sequence analysis of the NA gene showed no changes in residues involved in the binding of the substrate or inhibitor.

The HA and NA genes of each virus were then sequenced after reverse transcription of the virion RNA and amplification of the resulting DNA by [] Genotypic analysis of these variants showed that all of the 3 variants contained mutations in the HA sequences. The mutations in the HA were associated with the sialic acid binding site at positions Thr-155-Ala, Val-233-Ile and Arg-229-Ile or Arg-229-Ser. The amino acids mutated in these resistant viruses were in the active site of the sialic acid binding site as observed in the X-ray crystal structure of the HA. Two of the 3 variants showed a single mutation in the NA gene at positions involved neither in substrate or inhibitor binding and thus probably not involved in altering the affinity of the enzyme or the substrate or the inhibitor.

Zanamivir resistance due to mutations in neuraminidase: The target of zanamivir is the viral NA and the sub-site of interference is the enzyme active site of influenza NA. Continuous exposure to zanamivir is predicted to induce mutations in the active site of the enzyme which may reduce the affinity and thus confer resistance to the compound. To explore the possibility of resistance due to reduction in NA activity the applicant passaged influenza A virus NWS/G70C (H1N9) in the presence of increasing concentrations of zanamivir as described above and characterized the viral variants for potential alterations in their phenotypic and genotypic properties.

Phenotypic analysis of the serially passaged viruses at passage 7 (5, and report SR1997/00038/00) generated a variant virus with reduced sensitivity to zanamivir as determined by both plaque assay as well as NA inhibition assay. Analysis of the kinetics of the binding of zanamivir to the variants isolated from passage 7 or subsequent passage NA showed that the enzyme had lower affinity to the compound. Genotypic analysis of the

variant showed a mutation in a highly conserved region of the viral NA at position Glu-119-Gly. This amino acid Glu-119 is one of the amino acids in the active site of the enzyme that interacts with the guanidino group of zanamivir. Electron microscopy of the purified NA showed that whereas clear tetramers were seen with the wild type NA, the mutant NA structure was diffuse and appeared to be disrupted. X-ray analysis of zanamivir complexed with both parental and mutant NA showed that in the mutant the Glu-119 interactions with the inhibitor are replaced by interactions with a water molecule, thus providing unequivocal evidence for the structural consequences of this mutation. The mutation resulted in the loss of stabilizing interactions between the guanidino moiety of zanamivir and the carboxylate at amino acid residue 119.

Similar studies designed to evaluate changes in susceptibility of NA using other strains of influenza A and B showed amino acid changes in the carboxylate binding region of the enzyme active site resulting in loss of the catalytic activity of the enzyme. The other critical amino acids changes occurred at amino acid positions 292 and 229 of NA which were also the active site amino acids. It is unknown what effect the loss of NA activity on the pathogenicity of the virus.

Zanamivir resistance due to mutations in both hemagglutinin and neuraminidase: In studies on the evaluation of the emergence of resistance to zanamivir the majority of resistant viruses were found to contain mutations in the amino acid sequences of both the viral HA and NA (6,7). Results of in vitro sensitivity studies showed that Glu-119-Gly NA mutation in an otherwise wild type background or Arg-292-Lys mutation in NA, conferred approximately 10-fold resistance to zanamivir in plaque reduction assays. Similarly viruses containing Asn-199-Ser mutation or Ser-186-Phe HA mutation in an otherwise wild type background conferred a 10-fold resistance in plaque reduction assays. However, viruses containing the Glu-119-Gly mutation in NA and the Ser-186-Phe mutation in HA showed a 100-fold resistance in plaque reduction assays. This observation indicates that multiple mutations can act synergistically to increase resistance in vitro. It was also observed that the extent of resistance was dependent on the choice of parameters in plaque assays such as size, diffuse nature of the plaque or drug dependence of the plaque.

Cross-resistance: Resistance to sialomimetic drugs occur by more than one mechanism; due to mutations in the viral NA, or viral HA or both. Mutations in the HA

that reduce the affinity for receptor binding appear to circumvent the need for significant NA activity for virus release. Such HA mutants that alter the host cell-virus interactions confer cross-resistance among all NA inhibitors (6,8). Thus, zanamivir resistance due to mutations in HA is expected to be cross-resistant to the investigational drugs GS4071 and BCX-140 and to all other sialomimetics and vice versa. Zanamivir resistance due to mutations in the HA was observed in both in vitro and clinical isolates (9). The effect of zanamivir-associated mutations for cross-resistance to other NA inhibitors was not been tested directly but is predicted to impart cross-resistant to other NA inhibitors.

In studies on the evaluation of changes in influenza virus susceptibility to zanamivir the applicant exposed the virus in parallel experiments to zanamivir structural analogues as comparators to evaluate the emergence of resistance and cross-resistance to these NA active site inhibitors. When zanamivir or its analogue 4-amino-Neu5Ac2en, were used in resistance evaluation studies, variants emerged which demonstrated resistance to each of the tested compounds. In addition, cross-resistance between zanamivir or its analogue 4-amino-Neu5Ac2en was observed regardless of the initial compound used in resistance studies. Most significantly, equivalent levels of cross-resistance to the compounds (zanamivir or its analogue 4-amino-Neu5Ac2en) was observed regardless of the drug used in the initial resistance selection (5).

In drug susceptibility studies, a zanamivir-resistant mutant virus with a single mutation at the catalytic site in NA at Arg-292-Lys emerged and this isolate was further analyzed in cross-resistance studies. Arg 292 plays an important role in substrate binding and is conserved in all viral and bacterial NAs. This substitution at the catalytic site raised the question of cross-resistance to other NA inhibitors whose designs are based on NA active site structure. In vitro analysis of this mutant showed that this virus was resistant to zanamivir but more importantly, it was highly cross-resistant to NA inhibitor GS4071 (6,10), an investigational drug currently being developed as an anti-influenza virus agent. These results suggest that cross-resistance among NA inhibitors specifically designed to inhibit the activity of the enzyme by interfering with the active site of NA is possible.

Viral resistance to zanamivir in clinical samples: To determine whether shifts in susceptibility to zanamivir occurred during therapy the applicant evaluated a subset of pre- and post-treatment matched clinical viral isolates derived from subjects in phase 2 and 3 clinical trials. In some samples, viral susceptibility was monitored phenotypically

using plaque reduction assays in MDCK cells and enzyme assays for NA activity. In other cases, genotypic assays were also carried out to determine the molecular basis of resistance by sequencing the HA and NA genes. In one instance, susceptibility was monitored in ferrets. In all of the susceptibility studies no assay was used consistently across the clinical trial isolates which made it difficult to draw conclusions on shifts in susceptibility.

In the phase 2 European study NAIB2005, 15 pre- and post-treatment matched viral isolates (11 influenza B strains and 4 influenza A) and 5 placebo matched pairs were evaluated. In plaque reduction assays one of the post-treatment isolates showed a 1000-fold reduction in susceptibility to zanamivir relative to the pre-treatment isolate. However, both viruses in the matched pair appeared to show no changes in the NA enzyme activity assays. Sequencing the HA gene also showed no changes in the sequence between the matched pairs. Furthermore, the virus was fully susceptible to zanamivir in the ferret.

In the phase 2 U.S. study NAIA2005, a total of 17 matched pairs (12 treated pairs + 5 placebo pairs) were analyzed for susceptibility to zanamivir using the plaque reduction assay and plaque size assay. Three of the isolates showed some shifts in susceptibility but all were found to be susceptible to zanamivir in the NA enzyme activity assay.

In the phase 2 European study NAIB2008, a total of 21 matched pairs (14 treated pairs + 7 placebo pairs) were evaluated. Interestingly, 4 isolates did not have sufficient NA activity for susceptibility assays which indicated the requirement of this enzyme for viral replication was minimal. The remaining isolates were found to be sensitive in NA enzyme activity assays. Plaque reduction assays showed variable results. Sequencing of the NA gene showed mutations that result in changes in amino acids (not in the active site domain) at different residues (Ile-29-Pro or Ile-20-Met). The applicant considered these mutations not related to susceptibility of zanamivir.

In the phase 3 study NAIA/B3002, 12 matched isolates were evaluated for NA enzyme activity and the isolates were not tested in any biological assays such as the plaque reduction assay. In the NA enzyme assay the applicant stated that none of the isolates showed shifts in susceptibility to zanamivir.

In a summary statement the applicant claimed that in acute infection with influenza virus A or B no evidence of zanamivir resistance was detected from any individual. However, analysis of certain clinical isolates suggest that shifts in susceptibility did occur.

Resistance to zanamivir has also been observed in an immunocompromised patient infected with influenza virus B who was treated for 2 weeks with nebulized zanamivir. Analysis of sequential isolates obtained from this patient indicate the emergence of resistance, as determined by different susceptibility assays. The post treatment clinical samples contained mutations in both the HA and NA. One HA mutation at The-198-Glu, was first identified in the day 8 treatment isolate. This critical mutation abolishes the glycosylation site at Asn-196 and also altered the antigenic properties of the mutant virus as determined by a HA receptor-binding assay. The NA mutation, Arg-152-Lys, first identified in the day 12 treatment isolate, occurred in the enzyme's active site. Parallel in vitro measurements of NA activity in the parent and the mutant virus showed that the mutant virus retained only 3-5% of the enzyme activity of the pre-treatment virus. In addition, the NA of the 12-day isolate was about 1000-fold less sensitive to zanamivir than the parent's enzyme. However, in MDCK cell culture the mutant was as sensitive as the parent. This result is consistent with the emergence of resistance in clinical settings.

Combined data on the emergence of zanamivir resistance in influenza virus indicate that resistance does occur albeit with low frequency. Support for the emergence of resistance came from both the in vitro experiments and from analysis of clinical samples. The extent of information on the emergence of resistance in clinical samples provided by the applicant is insufficient to characterize the risk of emergence of resistance in clinical use. The sponsor should be urged to carryout additional surveillance studies.

CONCLUSIONS

In vitro anti-NA activity studies demonstrate that zanamivir is a strong, competitive inhibitor of the NA of influenza virus A and B. In vitro antiviral activity studies show that zanamivir inhibits the replication of laboratory strains and clinical isolates of influenza virus. The in vitro anti-NA activity and antiviral activity data are consistent with the observed antiviral efficacy in animal models and in experimental infection of human volunteers.

The applicant stated that zanamivir is specific to the influenza virus NA by comparing the drug's effect on a single human enzyme. The extent to which zanamivir compromises the endogenous activities of multiple human NAs was not adequately evaluated, and therefore, the conclusion that zanamivir is specific for viral NA could not be supported. However, long term exposure of animals to zanamivir appear to show no significant toxicity (see Pharmacology review).

The IC_{50} for natural substrates is likely to be higher than that with the low molecular weight, minimal synthetic substrate used in the determination of anti-NA activity of zanamivir. The natural substrates for NA are numerous and are high molecular weight glycoconjugates with multiple conformations. Furthermore, in vivo desialation of mucopolysaccharides is the net result of two competing enzyme systems (equilibrium); the multiple cellular sialyl transferases which add sialic acids to a multitude of cellular substrates and a single influenza NA that can remove the sialic acid residues. Extrapolation of this information suggests the need for higher concentrations of the drug for antiviral activity in vivo than that projected from the anti-NA activity studies.

The role of NA in influenza virus replication is not well understood. NA may be a suboptimal target for efficient inhibition of influenza virus replication because it does not appear to play a role in viral entry, replication, assembly or budding (11), mutants (in vitro or clinical isolates) with NA activity of <5% of the parent enzyme replicate well in cell culture and in ferrets (9). However, if the viral titer is directly related to influenza pathology, NA enzyme inhibitors could potentially lessen the severity of infection.

The remarkable adaptability of influenza virus including the error-prone nature of its replication endows the virus with the possible pre-existence of any conceivable mutant providing opportunities for selection and selective amplification of the mutants under drug pressure. Zanamivir susceptibility studies showed the emergence of drug resistance within 10 passages of the virus in vitro and within 25 replication cycles in vivo (1). Additionally, the drug has a low genetic barrier for resistance, i.e., a single mutation is sufficient to overcome the drug pressure. However, the emergence of resistance to zanamivir appears to be slower with lower frequency than the approved anti-influenza drugs, amantadine and rimantadine.

The molecular basis for the emergence of resistance and cross-resistance to sialomimetic drugs is due to changes in the viral NA and HA. These viral envelope glycoproteins are the major antigenic determinants and the drug-selected mutations in these genes can result in antigenic variation beyond the inherent variation. The HA in particular is

immunologically very important in that it is this antigen against which protective antibodies develop and its variability has been the main factor in the continuing evolution of influenza epidemics. Therefore, the potential of the sialomimetic drugs like zanamivir or oseltamivir to select for variants in these antigenic molecules, makes it crucial to characterize these mutants for changes in transmissibility and virulence.

Draft microbiology label: Attached is the microbiology label as of 7-26-99. There may be revisions to portions of the label.

MICROBIOLOGY:

DRAFT

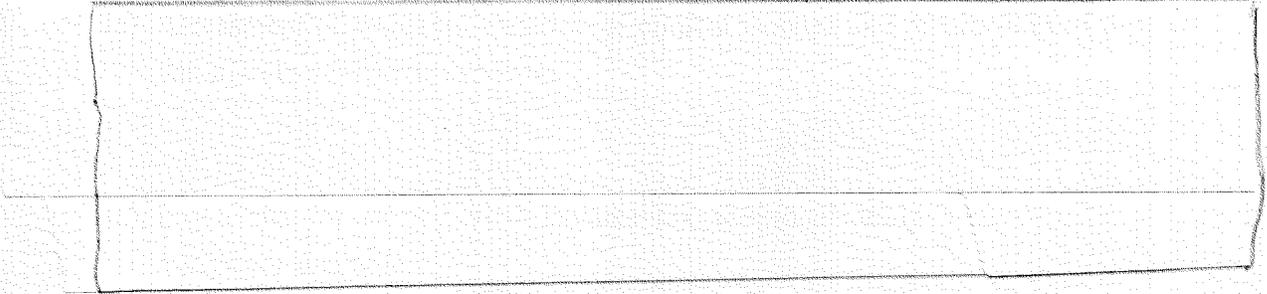
LABELLINE

DRAFT LABELING

RECOMMENDATION: With respect to microbiology, this application is recommended for approval.

Phase 4 considerations: The following is a draft list of microbiology issues for consideration as Phase 4 commitments.

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/S/

Narayana Battula

Concurrence:

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