RWJ-270201 (BCX-1812): a novel neuraminidase inhibitor for influenza

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The influenza virus neuraminidase (NA) is important in the pathogenesis of infection and, thus, is an attractive target for agents used in the treatment and prophylaxis of influenza. This article describes preclinical and early clinical data related to RWJ-270201 (BCX-1812), a novel, orally active NA inhibitor that was rationally designed for having potent and selective activity against influenza A and B viruses. RWJ-270201 is a unique NA inhibitor with a cyclopentane ring structure and high selectivity for the influenza NA. RWJ-270201 has efficacy comparable to or better than earlier NA inhibitors against a wide range of influenza A and B isolates, including recently emerged and avian strains, both *in vitro* and in a lethal murine model of influenza. Based on the high selectivity and efficacy of RWJ-270201 against both type A and B influenza strains in preclinical studies as well as murine pharmacodynamic studies supporting the potential for once-daily administration, clinical trials were initiated in order to determine the tolerability and antiviral activity of RWJ-270201 in humans. To date, clinical studies have indicated that RWJ-270201 is well tolerated and has antiviral activity in human experimental influenza models when administered orally once daily.

Keywords: influenza; neuraminidase inhibitor; RWJ-270201

1. INTRODUCTION

Influenza is a global public health concern and is responsible for significant morbidity and mortality due to both annual epidemics and unpredictable pandemics. Despite the devastation of this respiratory disease, only limited options for control are available.

The primary method of defence against influenza is the prevention of viral infection through vaccination. Immunization is highly effective when administered annually in response to the seasonal antigenic variation of the virus, which results from rapid mutation of the viral surface proteins, in particular haemagglutinin (HA) and neuraminidase (NA), due to selective pressure.

Antiviral agents have been developed for supplementing vaccination in the prevention of infections, as well as for treating influenza infections. Supplementation of vaccine prophylaxis may be necessary if an unpredicted influenza strain that is not countered by the vaccine circulates widely, as evidenced in the 1994–1995 influenza season (Wood 1998). Vaccine shortages may also occur, as experienced during the 2000–2001 influenza season due to unforeseen vaccine manufacturing and production problems (Centers for Disease Control and Prevention 2000). Antiviral agents may also supplement vaccination by treating infected individuals who were either not vaccinated or were infected despite vaccination. Vaccinated individuals occasionally become infected even when the vaccine closely matches the circulating viral strains and certain populations, including the elderly and those who are immunocompromised (e.g. patients with

acquired immune deficiency syndrome (AIDS) or cancer or transplant recipients) are more likely to have incomplete immune responses to the vaccine (Nichol 1998).

Amantadine and rimantadine were the first anti-influenza compounds developed, but they were not completely satisfactory since they were only effective against the influenza A strain and were associated with the rapid development of viral resistance. The most recently developed anti-influenza compounds are NA inhibitors. NA is thought to play an important role in the pathogenesis of influenza by cleaving the terminal sialic acid from carbohydrates on the cell surface and viral envelope in order to release viral progeny from infected cells (Liu *et al.* 1995; Calfee & Hayden 1998). Electron microscopy has shown that NA-deficient viruses are infectious, but aggregate on the host cell surface rather than being completely released, thus inhibiting the spread of infection (Liu *et al.* 1995). It has also been suggested that NA facilitates viral infection by impairing the ability of respiratory mucus for inactivating the virus (Burnet 1948; Colman 1994).

NA is a good therapeutic target because its active site is highly conserved among the many characterized influenza A and influenza B viral strains and subtypes (Colman *et al.* 1983; Burmeister *et al.* 1992; Varghese *et al.* 1992). This conservation suggests that NA inhibitors could protect against newly emerged, potentially pandemic influenza strains. X-ray crystallography has been used for determining the structure of NA complexed with sialic acid (Burmeister *et al.* 1992; Varghese *et al.* 1992), which is its natural substrate or with 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA), which is a transition state analogue of sialic acid with non-selective NA inhibitory

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activity (Bossart-Whitaker *et al.* 1993). This work has led to rational drug design of NA inhibitors that are highly specific to the influenza virus (Von Itzstein *et al.* 1993; Kim *et al.* 1997; Babu *et al.* 2000).

Two NA inhibitors, namely zanamivir (2,4-dideoxy-2,3-didehydro-4-guanidinosalic acid) and oseltamivir (ethyl (3R,4R,5S)-4-acetoamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate) (Hayden *et al.* 1997; Treanor *et al.* 2000), have recently proven to be clinically effective. Zanamivir, which is the first NA inhibitor approved for the treatment of influenza, is delivered by inhalation, whereas oseltamivir is an orally administered prodrug that must be metabolized to its active form, oseltamivir carboxylate.

The development of additional antiviral agents, including NA inhibitors, remains an important goal in the control of influenza. This ongoing search is driven by the desire for agents with improved safety, efficacy and convenience. The availability of an arsenal of effective agents is being encouraged by ongoing pandemic planning efforts (World Health Organization 1999) as well as by the burden that annual epidemics place on health care systems worldwide.

This article describes preclinical and early clinical data for RWJ-270201 (BCX-1812), which is a novel NA inhibitor with a unique cyclopentane ring structure. This compound is highly selective for the influenza NA and is active against a range of influenza A and B isolates, including recent clinical isolates and avian strains with the potential for direct to human transmission, both *in vitro* and in murine models. Based on the high selectivity and broad efficacy of RWJ-270201 in preclinical studies as well as murine pharmacodynamic studies supporting further investigation of once-daily administration, clinical trials were initiated in order to determine the tolerability and antiviral activity of RWJ-270201. To date, clinical studies have indicated that RWJ-270201 is well tolerated and has antiviral activity in human experimental influenza models when administered orally once daily.

2. THE STRUCTURAL DEVELOPMENT OF A NEW NA INHIBITOR

RWJ-270201 was developed by a rational, structure-based approach using the crystallographic structure of the highly conserved NA active site and its substrate interactions. The crystal structure of the influenza NA complexed with sialic acid (figure 1a) or with DANA (figure 1b), which inhibits NA activity (Meindl *et al.* 1974), has revealed that the NA active site is composed of four binding pockets where the major functional groups of sialic acid and DANA are positioned: (i) an acidic pocket in which three arginine residues form hydrogen bonds with the carboxylic acid side chain of DANA, (ii) a hydrophobic pocket bound by the glycerol side chain of DANA, (iii) a hydrophobic pocket occupied by the acetamido group of DANA, and (iv) a negatively charged pocket occupied by the C4-hydroxyl group of DANA. An ordered water molecule mediates the negative charges in the fourth pocket.

A six-membered ring structure serves as the scaffold for the functional groups of sialic acid and its analogues DANA, zanamivir and oseltamivir carboxylate.

However, five-membered ring structures were used as the novel starting scaffold for RWJ-270201 development, since α/β -6-acetyl-amino-3,6-dideoxy-D-glycero-altro-2-nonulofuranosonic acid (figure 1c) was reported to have influenza NA inhibitory activity comparable to that of DANA (Yamamoto *et al.* 1992). The crystal structure of this compound bound to NA revealed that, although the ring structure is positioned differently from that of DANA, the four key functional groups have similar active site interactions (Babu *et al.* 2000). Therefore, the positioning of the functional groups rather than of the scaffold structure is an important indicator of the potency of NA inhibition.

NA inhibitors of increasing potency were synthesized based on the structure of the compound in figure 1c (Babu *et al.* 2000). The cyclopentane derivative (figure 1d) was designed with a guanidino group (Babu *et al.* 2000) replacing the C4-hydroxyl group of DANA in the negatively charged binding pocket of NA, as with zanamivir (Von Itzstein *et al.* 1993). In addition, replacement of the polar glycerol side chain was attempted in order to optimize its pharmacokinetic properties. The crystal structure of this compound bound to NA verified that the guanidino group bound the negatively charged residues lining the fourth binding pocket in a manner similar to that of zanamivir.

Based on the structure and activity of the compound shown in figure 1d, the cyclopentane derivative, which is also shown in figure 1e, was designed with the addition of an *n*-butyl side chain in order to fit the hydrophobic region of the pocket bound by the glycerol side chain of DANA (Babu *et al.* 2000). This compound was synthesized as a racemic mixture and the most active isomer was selected by soaking an influenza A NA crystal in the mixture and then determining the structure of the isomer bound to the NA active site. Interestingly, the conformation of the *n*-butyl group of the compound in figure 1e was quite different when bound to influenza A from its conformation when bound to influenza B (Babu *et al.* 2000). Although the active site of NA is highly conserved even between influenza A and B viruses, this altered conformation is probably due to a small sequence difference that is responsible for lesser flexibility within the influenza B NA active site.

The compound in figure 1f, which is known as RWJ-270201, is a modified version of figure 1e that is designed for occupying both hydrophobic pockets of the NA active site (Babu *et al.* 2000). The active conformation, which is selected by soaking the NA crystal with a racemic mixture, was the same as that of figure 1e. However, RWJ-270201 has greater NA inhibition activity than figure 1e.

The positioning of the guanidino group in compounds *e* and *f* in figure 1 was altered in a manner that changes their active site interactions compared with figure 1d and zanamivir (Babu *et al.* 2000). Since this region of the active site is mutated in some zanamivir-resistant strains, the altered positioning of the compound in figure 1e was expected to impact on cross-reactivity with these strains. Indeed, the zanamivir-resistant NA variants with glycine or alanine replacing Glu119 retained susceptibility to RWJ-270201 (Gubareva *et al.* 2000).

Therefore, a novel and potent NA inhibitor with a cyclopentane ring structure was developed by structure-based

Figure 1. The chemical structures (a) of sialic acid, (b) DANA and (c–f) various cyclopentane compounds examined during the development of RWJ-270201 (f) (adapted from Babu *et al.* 2000).

drug design. The functional groups of RWJ-270201 and their NA active site interactions suggested that this NA inhibitor would be orally available, selective and potent. These characteristics have been verified in preclinical and early clinical studies, as outlined in §§ 3 and 4.

3. PRECLINICAL STUDIES WITH RWJ-270201

Preclinical studies have demonstrated that RWJ-270201 is a potent and selective inhibitor of influenza NA. Studies comparing the efficacy of RWJ-270201 to other antiviral agents against a wide range of influenza A and B strains were performed *in vitro* and in a lethal murine model of influenza. These strains included recent clinical isolates and avian strains with potential pandemic significance. The preclinical studies described in this section have also explored the emergence of resistance in response to RWJ-270201 exposure, the efficacy of RWJ-270201 in an immunocompromised host and the pharmacodynamic parameters of RWJ-270201.

RWJ-270201 was determined as being highly selective for influenza NA inhibition as compared with NAs from non-influenza sources. Using a fluorometric assay for measuring NA inhibition *in vitro*, its IC_{50} values against bacterial, mammalian and other viral NAs were more than 300 000 nM, as compared with IC_{50} values of 0.09–11 nM against influenza A and B NAs (Bantia *et al.* 2001).

The potency of RWJ-270201 was tested against a wide range of clinically significant influenza A and B isolates both *in vitro* and *in vivo*. The activity of RWJ-270201 against a number of influenza A and B strains *in vitro* is shown in table 1. RWJ-270201 was generally as potent as oseltamivir carboxylate against the tested influenza A NA subtypes in the study by Bantia *et al.* (2001), except for the NA from the H1N1 subtype, which was more sensitive to RWJ-270201. RWJ-270201 was more potent than zanamivir against the H2N2 and H3N2 subtypes based on their IC_{50} values, but was equivalent in activity against the NA activity from the H1N1 and H6N2 influenza A subtypes. RWJ-270201 had lower IC_{50} values in general. However, the inhibitory activity of RWJ-270201 was similar to that of zanamivir and greater than that of oseltamivir carboxylate for the eight influenza B NAs tested (Bantia *et al.* 2001).

The inhibitory activity of RWJ-270201 was measured in tissue culture assays against a series of clinically significant isolates in a second *in vitro* study (Smee *et al.* 2001): 5 A/H1N1 strains, 12 A/H3N2 strains, 2 A/H5N1 strains and 5 B strains. Inhibition of the virus-associated cytopathic effect (CPE) in infected Madin–Darby canine kidney (MDCK) cells, which was measured as EC_{50} values, was evaluated by visual examination for CPE (table 1) and by measurement of increases in the cellular uptake of neutral red dye. In general, RWJ-270201 was

Table 1. Inhibitory activity of RWJ-270201, zanamivir and oseltamivir carboxylate against clinical isolates.

(The values for the Bantia *et al.* (2001) study are IC₅₀ ranges (nM), which are the concentrations of drug required for inhibiting 50% of NA activity as determined by a fluorometric assay measuring NA activity. The values for the (Smee *et al.* 2001) study are EC₅₀ ranges (µM), which are 50% effective (virus inhibitory) concentrations as determined visually by the CPE inhibition assay in MDCK cells.)

		<i>in vitro</i> inhibitory activity		
	number of strains tested	RWJ-270201	zanamivir	oseltamivir carboxylate
Bantia <i>et al.</i> (2001)				
H1N1	6	0.09–0.81	0.30–0.80	0.69–2.20
H2N2	4	0.17–1.40	0.76–1.80	0.01–1.50
H3N2	4	0.14–0.83	0.68–2.30	0.21–0.56
H6N2	1	1.1	1.1	0.84
B	8	0.60–11.0	1.50–17.0	5.00–24.0
Smee <i>et al.</i> (2001)				
H1N1	5	0.09–21.0	0.22–100.0 +	0.17–100.0 +
H3N2	12	< 0.01–0.19	< 0.01–0.65	< 0.01–0.50
H5N1	2	0.01–0.02	0.20–0.22	0.22–0.26
B	5	0.06–3.20	0.03–1.30	0.11–3.00

more potent than zanamivir and oseltamivir carboxylate against H1N1 and H5N1 viruses and had similar antiviral activity against H3N2 and B viruses (table 1).

The efficacy of RWJ-270201 against multiple influenza A and B strains was tested *in vivo* using a lethal murine model of influenza based on its strong antiviral effects and selectivity *in vitro* (Sidwell *et al.* 2001). Mice (8 to 10 per drug dose) were intranasally infected with A/Shangdong/09/93 (H3N2), A/Victoria/3/75 (H3N2), A/NWS/33 (H1N1) or B/Hong Kong/05/72 virus. RWJ-270201, oseltamivir, ribavirin or a saline solution were administered by oral gavage twice daily for 5 days beginning 4 h prior to virus exposure. Body weight, toxicity, arterial oxygen saturation (S_aO_2) decline, lung consolidation, lung viral titres and the mean day to death were determined.

RWJ-270201 treatment resulted in significantly increased numbers of survivors and higher mean day to death values for all four viral strains tested as compared with saline, as shown in table 2 (Sidwell *et al.* 2001). RWJ-270201 doses as low as 1 mg kg⁻¹ d⁻¹ were effective against some strains. In comparison, the same doses of oseltamivir were generally less effective than RWJ-270201. All uninfected mice monitored for drug toxicity survived and both RWJ-270201 and oseltamivir were well tolerated. Decreases in S_aO_2 were also inhibited in infected mice treated with RWJ-270201 (Sidwell *et al.* 2001). The mean S_aO_2 values were significantly greater at day 11 in mice treated with RWJ-270201 than in saline-treated mice.

In addition to being active against a wide range of influenza A and B strains, RWJ-270201 was also shown to have low IC₅₀ values for NAs from more recent North American clinical isolates. RWJ-270201 was tested against viral isolates collected from Canadian influenza patients during the 1999–2000 influenza season after the introduction of NA inhibitor treatment in Canada (Boivin & Goyette 2000). Fluorometric and chemiluminescent NA assays were used for determining the inhibitory activity of RWJ-270201 *in vitro* against 65 viral isolates (38 A/H3N2, 4 A/H1N1 and 23 B isolates) from untreated patients, as well as isolates from four zanamivir-treated patients. All

viral isolates were highly sensitive to NA inhibition by RWJ-270201. When NA inhibitor activity against pre-treatment isolates was compared to activity against isolates obtained after 3 days of zanamivir treatment, the mean IC₅₀ values for RWJ-270201 did not change significantly, indicating that post-treatment isolates from patients receiving zanamivir maintained susceptibility to RWJ-270201.

Other recent clinical isolates against which RWJ-270201 is active include those implicated in two disease outbreaks in Hong Kong that were controlled by region-wide poultry extermination programmes, namely a 1997 outbreak caused by a highly pathogenic H5N1 strain and a 1999 outbreak caused by an H9N2 strain (Subbarao *et al.* 1998; Peiris *et al.* 1999). Inhibition of the NA activity of two H5N1 strains was evaluated in infected MDCK cells by a visual CPE inhibition assay (Smee *et al.* 2001). RWJ-270201 was very potent against both A/duck/MN/1525/81 and A/gull/PA/4175/83 (mean EC₅₀ values of 0.01 and 0.02 µM, respectively). As shown in table 1, this potency was 10-fold greater than that of zanamivir and oseltamivir (0.20–0.26 µM) (Smee *et al.* 2001).

The efficacy of RWJ-270201 against H5N1 and H9N2 viral strains was also tested in mice (Govorkova *et al.* 2001). The animals were treated with 0.01, 0.1, 1.0 or 10 mg kg⁻¹ d⁻¹ of RWJ-270201 or oseltamivir for 5 days beginning 4 h before infection with A/HK/156/97 (H5N1) or mouse-adapted A/quail/HK/G1/97 (H9N2). All RWJ-270201 doses tested provided significant protection against the H5N1 strain, with complete protection observed at a dose of 10 mg kg⁻¹ d⁻¹. Complete protection for the H9N2 strain was achieved at a dose of 1.0 mg kg⁻¹ d⁻¹. Statistical analyses indicated that oseltamivir and RWJ-270201 provide comparable protection. Uninfected mice treated with 100 mg kg⁻¹ d⁻¹ of either drug showed no signs of toxicity.

These two recent outbreaks in Hong Kong were caused by direct transmission of avian influenza viruses to humans. This suggests that the many viral subtypes not currently identified as human pathogens are potential future threats, possibly leading to pandemics. Since

Table 2. Efficacy of RWJ-270201, oseltamivir and ribavirin in protecting mice against lethal influenza virus infections (adapted from Sidwell *et al.* 2001).(The mean days to death are prior to day 21. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus saline-treated mice.)

virus	compound	dose (mg kg ⁻¹ d ⁻¹)	survivors/total	mean $\pm$ s.d. days to death
A/Shangdong/09/93 (H3N2)	RWJ-270201	100	10/10***	> 21.0 $\pm$ 0.0***
		10	9/10***	8.0 $\pm$ 0.0
		1	5/10***	11.0 $\pm$ 2.7**
	oseltamivir	10	5/10***	13.0 $\pm$ 1.9***
		1	0/10	11.0 $\pm$ 1.9**
	ribavirin	75	10/10***	> 21.0 $\pm$ 0.0***
A/Victoria/3/75 (H3N2)	RWJ-270201	100	10/10***	> 21.0 $\pm$ 0.0***
		10	7/10**	13.0 $\pm$ 2.1
		1	0/10	11.0 $\pm$ 0.7
	oseltamivir	10	1/9	12.0 $\pm$ 1.6
	saline	—	2/16	11.0 $\pm$ 2.1
	RWJ-270201	10	10/10***	> 21.0 $\pm$ 0.0
A/NWS/22 (H1N1)	RWJ-270201	10	10/10***	> 21.0 $\pm$ 0.0
		1	10/10***	> 21.0 $\pm$ 0.0
		10	10/10***	> 21.0 $\pm$ 0.0
	oseltamivir	10	10/10***	> 21.0 $\pm$ 0.0
	saline	—	3/20	11.0 $\pm$ 3.1
	RWJ-270201	100	10/10***	> 21.0 $\pm$ 0.0***
B/Hong Kong/05/72	RWJ-270201	100	10/10***	> 21.0 $\pm$ 0.0***
		10	6/10***	11.0 $\pm$ 3.4**
		1	4/10**	7.5 $\pm$ 2.0
	oseltamivir	10	1/10	9.3 $\pm$ 1.7**
	saline	—	1/10	8.6 $\pm$ 1.7*
	ribavirin	75	9/10***	5.0 $\pm$ 0.0
	saline	—	0/19	7.1 $\pm$ 1.6

vaccine production typically takes at least six months (Couch *et al.* 1996), antivirals may play a critical role in the early response to future pandemics. Therefore, the potency of RWJ-270201 against avian strains of all nine NA subtypes (two strains per subtype) was measured and compared to that of zanamivir and oseltamivir carboxylate (Govorkova *et al.* 2001). RWJ-270201 effectively inhibited the enzymatic activity of all nine NA subtypes (mean IC₅₀ values of 0.9–4.3 nM) *in vitro*, as measured by a fluorometric NA inhibition assay. In comparison, the mean IC₅₀ values were generally higher for zanamivir (2.2–3.0 μ M) and oseltamivir carboxylate (1.9–69 μ M). Unlike RWJ-270201, the efficacy of zanamivir and oseltamivir carboxylate was more variable according to subtype (table 3). In addition to inhibiting NA activity, RWJ-270201 inhibited viral replication of strains producing all nine NA subtypes in MDCK cells (EC₅₀ range of 0.5–12 μ M) (Govorkova *et al.* 2001).

Several preclinical studies have investigated the potential for the emergence of drug-resistant strains in response to RWJ-270201. Resistance development, which is a general concern for all antimicrobial agents, has been an issue for the anti-influenza drugs amantadine and rimantadine. Drug-resistant viruses have been isolated from *ca.* 30% of patients treated with these compounds, sometimes within 2–3 days after treatment initiation (Hayden & Hay 1992). According to several studies of oseltamivir and zanamivir (Gubareva *et al.* 1998, 2001; McKimm-Breschkin 2000), drug resistance to NA inhibitors appears to emerge less rapidly than resistance to amantadine or rimantadine and has not been problematic in the clinical

Table 3. RWJ-270201, oseltamivir carboxylate and zanamivir inhibition of the NA activity of avian influenza viruses (adapted from Govorkova *et al.* 2001).

(Two HA subtypes were tested for each NA subtype. The IC₅₀ values are the concentrations of drug necessary for reducing the NA activity to 50% of the activity of controls as determined by a fluorometric assay.)

NA subtype	range of mean IC ₅₀ values in nM		
	RWJ-270201	zanamivir	oseltamivir carboxylate
N1	2.6–3.4	5.8–20	36.0–53.0
N2	2.0–2.7	4.7–7.4	1.9–2.7
N3	3.1–3.8	4.9–5.9	2.9–3.3
N4	2.3–3.2	2.2–4.5	5.5–47
N5	1.2–2.7	2.7–14	33.0–37.0
N6	1.3–3.1	3.4–7.2	3.2–4.9
N7	1.5–4.3	5.9–7.2	4.4–8.2
N8	1.1–1.2	5.3–30	30.0–69.0
N9	0.9–1.6	10.0–12.0	9.5–18.0

setting to date (Jackson *et al.* 2000). However, the impact of resistance to NA inhibitors cannot be fully determined until these agents have been used more extensively.

In order to investigate the potential for cross-resistance to RWJ-270201, previously isolated oseltamivir carboxylate- and zanamivir-resistant NA variants were tested *in vitro* for cross-resistance to RWJ-270201 (Gubareva *et al.* 2000). According to the NA inhibition assay, RWJ-270201

retained inhibitory activity against some of the variants. NA variants with mutations in residues that play structural roles in the active site (e.g. position 119) were sensitive to inhibition by at least one of the NA inhibitors. This was most probably related to differences in the interactions and positioning of the three NA inhibitors within the active site. However, residues that play essential catalytic roles in the active site by interacting with the sialic acid substrate (e.g. position 152) were resistant to all three NA inhibitors in some cases (Gubareva *et al.* 2000).

Several studies have attempted to isolate RWJ-270201-resistant variants by serially passaging virus in the presence of RWJ-270201 (Bantia *et al.* 2000; Smee *et al.* 2000). A resistant strain was first isolated at passage 15 when increasing RWJ-270201 concentrations (0.3 nM–1 mM) were added to cultured MDCK cells infected with A/Singapore/1/57 (Bantia *et al.* 2000); this resistant strain was *ca.* 10-fold less susceptible to RWJ-270201 and zanamivir than wild-type virus, but was *ca.* 3000-fold less susceptible to oseltamivir carboxylate. The passage 15 virus contained a Gly130Asp substitution in HA and an Arg292Lys substitution in the NA active site. These mutations have been reported previously in response to zanamivir and oseltamivir treatment and were shown to be less infective in mice (Gubareva *et al.* 1997; Tai *et al.* 1998).

Mouse-adapted A/Shangdong/09/93 (H3N2) was passaged in the presence of increasing concentrations of RWJ-270201 (110 µM) in another study (Smee *et al.* 2000). A significantly resistant strain that exhibited reduced sensitivity to RWJ-270201 (EC_{50} of 23 µM versus 0.007 µM for the wild-type parental strain by plaque assay) and to zanamivir and oseltamivir carboxylate was identified. This variant, which contains a Lys189Glu substitution in HA, was less virulent in mice than its parental strain (Smee *et al.* 2000).

Unlike the *in vitro* studies described above, no resistance emerged during a study that was designed for selecting RWJ-270201-resistant variants in mice (Sidwell *et al.* 2000a). Mice infected with A/Shangdong09/93 were treated with placebo or RWJ-270201 (1, 10 or 100 mg kg⁻¹ d⁻¹) twice daily for 5 days beginning 4 h before infection. Viral isolates collected from the lungs on day 5 retained sensitivity to RWJ-270201, as determined by an *in vitro* antiviral assay, even after the recovered virus was passaged again in mice. To date, therefore, similar to the other NA inhibitors, resistance development does not appear to be as prevalent for RWJ-270201 as for amantadine and rimantadine, but the limited amount of available data precludes any firm conclusions from being drawn.

Since NA inhibitors inhibit viral replication but do not destroy virus that is already present, a functional immune system is necessary for clearing virus from an infected host. Therefore, it was important to determine the effect of RWJ-270201 on the immune response. An immunosuppressive antiviral might impact negatively on the effectiveness of post-treatment vaccination or might permit reinfection after treatment. Murine studies indicated that RWJ-270201 is not immunosuppressive (Sidwell *et al.* 2000b). RWJ-270201 at a dose of 100 mg kg⁻¹ d⁻¹ did not negatively impact on immune cell responses or populations in mice infected with the influenza virus. Surviving mice developed humoral

immunity, as indicated by high titres of neutralizing antibodies and resistance to reinfection with the same viral strain (Sidwell *et al.* 2000b).

The ability to treat influenza infections in immunocompromised hosts effectively is an important clinical issue for a number of special populations, including the elderly, transplant recipients and patients with AIDS. In order to determine the efficacy of RWJ-270201 in immunosuppressed mice, BALB/c mice were treated with 100 mg kg⁻¹ cyclophosphamide administered intraperitoneally (Sidwell *et al.* 2000b). Immunosuppressed mice were treated by oral gavage with doses of 1, 10 or 100 mg kg⁻¹ d⁻¹ RWJ-270201 twice daily for 5 days beginning 24, 42 or 60 h following infection. Treatment with all RWJ-270201 doses and at all initiation times resulted in significant increases in the number of survivors.

Pharmacodynamic studies were performed in mice in an effort to predict an effective clinical regimen for RWJ-270201. In the murine efficacy studies described above, RWJ-270201 was administered according to the same schedule used for oseltamivir, namely twice-daily dosing. However, the pharmacodynamic evaluation of RWJ-270201 in the murine influenza model predicted the effectiveness of once-daily dosing (Drusano *et al.* 2001). In order to understand the pharmacodynamic relationships between the dose and schedule of administration in mice better, BALB/c mice were challenged with influenza A/Shangdong/09/93 (H3N2) and then were treated with five doses of placebo or RWJ-270201 (1–10 mg kg⁻¹ d⁻¹) administered every 8, 12 or 24 h. Treated animals were monitored for day of death compared with untreated, infected mice. The RWJ-270201 total dose rather than the schedule of administration was the most significant determinant of RWJ-270201 effectiveness when the effects of the various doses and schedules were evaluated by Kaplan–Meier and Cox proportional hazards analyses. It was further determined that the area under the drug concentration–time curve had the strongest impact on outcome by Cox proportional hazards analyses and was the pharmacodynamically linked variable. The final model, which included the area under the drug concentration–time curve, was not impacted on further by inclusion of the schedule of administration, indicating that this variable is not important for determining the time to death. These data suggested that it is appropriate to investigate once-daily administration of RWJ-270201 in clinical studies.

4. RWJ-270201 CLINICAL DATA

Given the promising preclinical results reported for RWJ-270201, clinical studies were initiated. The tolerability, antiviral activity and pharmacodynamic characteristics of RWJ-270201 have recently been examined in phase I and phase II clinical trials. These early studies, which are described below, indicated that RWJ-270201 is well tolerated at multiple doses and schedules and is effective in an experimental model of human influenza infections in otherwise healthy adults.

The phase I studies evaluated the safety and pharmacokinetics of RWJ-270201 (the data are on file at The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ, USA). The orally administered compound

was absorbed rapidly, with maximal plasma concentrations achieved in 2–4 h after dosing in most subjects. The plasma elimination half-life was 12–25 h and absorbed drug was eliminated solely by renal excretion of unchanged drug. The mean trough plasma concentrations during a 400 mg once-daily dosing regimen surpassed the IC_{90} values for the least sensitive of all tested strains of influenza A and B (11 ng ml^{-1}). Assessment of the safety profile of RWJ-270201 after single or multiple doses ranging from 0.5 mg kg^{-1} to $800 \text{ mg kg}^{-1} \text{ d}^{-1}$ for 10 days indicated that RWJ-270201 is well tolerated. A summary of the incidence of frequently reported adverse events ($\geq 2\%$) from 13 phase I studies is shown in table 4. Adverse events were reported with similar frequencies for placebo (37%) and RWJ-270201 (39%).

The phase II challenge studies were designed for determining the safety, tolerability and efficacy of RWJ-270201 in healthy, susceptible adult volunteers that were experimentally inoculated with influenza A or B (Hayden *et al.* 2000). The influenza challenge model has previously proven to be useful for predicting the value of antivirals against naturally occurring influenza infections (Treanor & Hayden 1998). The randomized, double-blind, placebo-controlled trials of RWJ-270201 were also used for predicting the clinically effective dose and schedule of RWJ-270201 for influenza A and B viruses.

Subjects in both the influenza A and influenza B challenge studies were inoculated intranasally with virus 24 h prior to treatment initiation and then treated for 5 days (Hayden *et al.* 2000). The primary endpoint was the reduction in viral shedding, as measured by the area under the mean viral titre–time curve. Secondary endpoints included composite symptom evaluation scores.

A total of 90 subjects in the influenza A challenge study were randomly assigned to one of five treatment groups: oral tablets of placebo or oral tablets of RWJ-270201 in one of four regimens (100 mg once daily, 200 mg once daily, 200 mg twice daily or 400 mg once daily). After randomization, the subjects were inoculated with A/Texas/36/91 (H1N1). In this study, the area under the mean viral titre–time curve of the mean viral titre over time, the primary efficacy endpoint, was significantly reduced for the 400 mg RWJ-270201 once-daily regimen (73% reduction versus placebo) ($p < 0.005$). The composite symptom scores of the group treated with RWJ-270201 were lower than those of the group treated with placebo.

Subjects in the influenza B challenge study were inoculated with B/Yamagata/16/88 and then treated with (i) placebo, (ii) 800 mg RWJ-270201 once daily, or (iii) an 800 mg loading dose of RWJ-270201 on day 1 followed by 400 mg doses once daily on days 2–5. Viral replication, which was measured as the area under the mean viral titre–time curve, was significantly reduced by both RWJ-270201 regimens (loading dose 49% and 800 mg once daily 61% versus placebo) (both $p < 0.05$) in this study.

RWJ-270201 was well tolerated in both studies. Adverse events occurred at similar incidences in the placebo and RWJ-270201 groups. Twenty-eight per cent of the patients in the influenza A study treated with RWJ-270201 reported adverse events, as compared with 44% of those treated with placebo. The rates of adverse events in the influenza B study were 33% in the RWJ-270201 group

Table 4. Treatment-emergent adverse events with a 2% or over reported incidence in phase I studies.

(The values in parentheses are percentages. All doses were combined for RWJ-270201. Twenty-seven out of the 31 pharyngitis events and all 14 out of the 14 coughing events were reported post-bronchoscopy in studies without placebo-treated subjects. The data are on file at The R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ, USA.)

adverse event	incidence	
	RWJ-270201 (<i>n</i> = 378)	placebo (<i>n</i> = 59)
any adverse event	146 (39)	22 (37)
headache	58 (15)	11 (19)
dizziness	10 (3)	3 (5)
pharyngitis	31 (8)	0
coughing	14 (4)	0
upper respiratory tract infection	10 (3)	0
nausea	15 (4)	3 (5)
diarrhoea	10 (3)	3 (5)
fatigue	12 (3)	3 (5)
somnolence	6 (2)	3 (5)
micturition frequency	2 (1)	2 (3)

versus 37% in the placebo group. These experimental human challenge studies indicate that RWJ-270201 has antiviral activity when administered once daily and is well tolerated at the doses used in the studies.

Pharmacodynamic analysis of the phase II studies confirmed that, as shown in the murine studies, the RWJ-270201 schedule of administration (once versus twice daily) has no impact on its effectiveness, which was measured as the time required for viral clearance (Drusano *et al.* 2000). Therefore, once-daily dosing of RWJ-270201 is supported by efficacy, tolerability and pharmacodynamic analyses.

5. CONCLUSIONS

RWJ-270201 is a novel NA inhibitor that was designed by a rational, structure-based approach with the objectives of achieving high selectivity for influenza NA inhibition and broad-ranging activity against influenza A and B strains with an orally active compound. Preclinical studies have demonstrated the selectivity and potency of RWJ-270201 against an array of clinically significant influenza strains, including recent clinical isolates and avian strains with potential pandemic implications. In general, the activity of RWJ-270201 *in vitro* and in mice was comparable to or better than that of zanamivir and oseltamivir.

As a result of the encouraging preclinical data, clinical trials were initiated in order to determine the tolerability and efficacy of RWJ-270201. Phase II clinical studies in which healthy adults were challenged experimentally with influenza A or B strains have provided further evidence that RWJ-270201 is well tolerated and has antiviral activity when administered orally and once daily. These studies support the continued development of RWJ-270201 in phase III trials in individuals with naturally acquired influenza infections; such trials are currently underway. A favourable outcome of these trials could lead

to the availability of a new weapon for combating the significant morbidity and mortality of annual epidemics and unpredictable pandemics of influenza.

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