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Anti-influenza virus activity of peramivir in mice with single intramuscular injection

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Abstract

In the event of an influenza outbreak, antivirals including the neuraminidase (NA) inhibitors, peramivir, oseltamivir, and zanamivir may provide valuable benefit when vaccine production is delayed, limited, or cannot be used. Here we demonstrate the efficacy of a single intramuscular injection of peramivir in the mouse influenza model. Peramivir potently inhibits the neuraminidase enzyme N9 from H1N9 virus in vitro with a 50% inhibitory concentration (IC_{50}) of 1.3 ± 0.4 nM. On-site dissociation studies indicate that peramivir remains tightly bound to N9 NA ($t_{1/2} > 24$ h), whereas, zanamivir and oseltamivir carboxylate dissociated rapidly from the enzyme ($t_{1/2} = 1.25$ h). A single intramuscular injection of peramivir (10 mg/kg) significantly reduces weight loss and mortality in mice infected with influenza A/H1N1, while oseltamivir demonstrates no efficacy by the same treatment regimen. This may be due to tight binding of peramivir to the N1 NA enzymes similar to that observed for N9 enzyme. Additional efficacy studies indicate that a single injection of peramivir (2–20 mg/kg) was comparable to a q.d. × 5 day course of orally administered oseltamivir (2–20 mg/kg/day) in preventing lethality in H3N2 and H1N1 influenza models. A single intramuscular injection of peramivir may successfully treat influenza infections and provide an alternate option to oseltamivir during an influenza outbreak.

Keywords: Peramivir; Influenza; Mouse model; Intramuscular

1. Introduction

Influenza is a respiratory infection associated with significant morbidity in the general population, mortality in elderly and high-risk patients and presents the ongoing threat of a new pandemic (CDC, 2000; Hien et al., 2004a). While immunization remains the primary means of prevention, antiviral drugs are an important adjunct. This is particularly true in situations in which vaccines are unavailable or ineffective due to viral antigenic changes or poor host immune response. Although the M2 channel inhibitors, amantadine and rimantadine, are effective against influenza A strains, their use is limited because of central nervous system and gastrointestinal side effects, emergence of viral resistance and lack of effectiveness against influenza B (Hayden et al., 2004). Of note, recent human clinical isolates of the avian H5N1 virus have shown resistance to this class of drugs (Hien et al., 2004b).

Because of the importance of influenza NA in viral replication and pathogenesis, interest has focused on the development of selective inhibitors of this enzyme. The neuraminidase (NA) inhibitors are an important advancement in the management of influenza. Inhaled zanamivir and oral oseltamivir are effective in both prophylaxis and treatment of influenza A and B viruses (Hayden et al., 1999a, 1999b; Kaiser et al., 2003). The need for an inhaler device and the risk of bronchospasms limits the use of zanamivir. Oseltamivir is being used although the gastrointestinal effects and emergence of resistant variants in some treated populations has limited the use of this drug (Kiso et al., 2004). Oseltamivir is currently stockpiled by many countries in the event of a major influenza outbreak or pandemic. The availability of another anti-influenza agent as an alternative to oseltamivir would be valuable. Using structure-based drug design, we have previously synthesized peramivir, a potent inhibitor of influenza NA (Babu et al., 2000).

Peramivir (BCX-1812, RWJ-270201) is a cyclopentane analogue and is a potent and selective inhibitor of influenza NA

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and has demonstrated activity against various influenza A and B viruses, including highly pathogenic H5N1 viruses, in vitro and in vivo (Babu et al., 2000; Bantia et al., 2001; Drusano et al., 2001; Govorkova et al., 2001; Sidwell et al., 2001; Smee et al., 2001). Peramivir was evaluated in a number of toxicology studies following oral, intravenous and intramuscular (IM) administration in mice, rats, primates, and dogs. Peramivir was well tolerated and demonstrated a good safety profile in these animal studies. Additionally, peramivir has demonstrated efficacy in humans with experimentally induced influenza A and B infections (unpublished data). Although peramivir showed significant antiviral activity against influenza A and B infections in phase 2 and phase 3 clinical trials, the primary endpoint of time to relief of symptoms did not reach statistical significance (www.shareholder.com/biocryst/news/20020625-83347.cfm?ReleaseID=83347). The lack of significant clinical effects may be due to the relatively low blood levels which were obtained following oral administration, considering the low oral bioavailability of peramivir (≤3%) (www.biocryst.com/pdf/peramivirfacts.pdf). Parenteral formulations of peramivir should allow the compound to achieve blood levels that would greatly enhance clinical activity of this neuraminidase inhibitor.

This paper compares the efficacy of a single IM injection of peramivir to 5 days of oral treatment (q.d.) of oseltamivir or peramivir in the mouse influenza A model. Efficacy of single IM injections of peramivir was compared to single IM injections of oseltamivir (both drug and prodrug forms) in the same model. Furthermore, this paper describes the tight binding nature of peramivir to the NA enzyme which makes it an effective anti-influenza agent with just a single IM injection.

2. Materials and methods

2.1. Viruses

The influenza A viruses used in this study were obtained from American Type Culture Collection, Manassas, VA, USA (A/NWS/33;H1N1) and Dr. Robert Sidwell, Utah State University, Logan, UT, USA (A/Victoria/3/75;H3N2) and were mouse adapted. Purified N9 crystals from A/H1N9 (NWS/G70) avian virus were obtained from Dr. Graeme Laver, Australian National University, Canberra, Australia.

2.2. Mice

Specific pathogen-free female BALB/c mice (10–19 g) were obtained from Charles Rivers Laboratories (Raleigh, NC, USA). They were quarantined for 24 h prior to infection and maintained on rodent diet from Harlan Teklad and tap water from the laboratory animal research center of BioCryst Pharmaceuticals, Inc.

2.3. Compounds and reagents

Peramivir, oseltamivir, oseltamivir carboxylate and zanamivir were synthesized by BioCryst Pharmaceuticals, Inc. (Birmingham, AL, USA). Each compound was prepared in sterile 0.9% sodium chloride for in vivo experiments. A mixture of 5% isoflurane/95% oxygen was administered as anesthesia.

2.4. NA assay

A standard fluorimetric assay was used to measure influenza virus NA activity (Potier et al., 1979). The substrate (2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid, MuNANA) is cleaved by NA to yield a fluorescent product that can be quantified. The assay mixture contained inhibitor at various concentrations and NA enzyme in 32.5 mM MES (2-(*N*-morpholino)-ethanesulfonic acid) buffer, 4 mM calcium chloride at pH 6.5 and incubated for 10–30 min. The reaction was started by the addition of the substrate. After incubation for 30–120 min fluorescence was recorded (excitation: 360 nm and emission: 450 nm) and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting percent inhibition of NA activity versus the inhibitor concentration. The results are reported as the average of three experiments.

2.5. On-site dissociation determination

To determine the rate of on-site dissociation of peramivir and oseltamivir carboxylate, the complex forms of NA-inhibitor were first prepared by incubating purified N9 NA enzyme with the inhibitors (at a concentration of 100 nM) for 1 h at 37 °C. The free compound was removed from the complex by a Bio-Spin column (P6, Bio-Rad). The on-site dissociation rates of the NA-inhibitor complex were measured by mixing the complex with substrate (75 μM of MuNANA) and recording the fluorescence (excitation: 360 nm and emission: 450 nm).

2.6. General procedure for in vivo antiviral experiments

Mice were anesthetized with isoflurane and exposed to 100 μL of virus by intranasal instillation. In the prophylaxis model, drug was administered 1 or 4 h before viral infection; in the treatment model, drug was given at times indicated after the viral infection. Each infected, drug and saline-treated group contained 5–10 mice. All mice were observed daily for changes in weight and for any deaths. Parameters for evaluation of antiviral activity included weight loss, reduction in mortality and/or increase in mean day to death (MDD) determined through 16 or 21 days.

2.7. In vivo anti-influenza efficacy of a single IM injection of peramivir and 5-day oral treatment with oseltamivir or peramivir

Mice were infected intranasally with an approximately 70–90% lethal dose of the A/NWS/33(H1N1) or A/Victoria/3/75 (H3N2) influenza virus. Oral treatment with peramivir or oseltamivir (prepared in injection-grade saline) began 1 or 4 h before virus exposure (prophylaxis model) and continued once daily for 5 days unless indicated. A single IM treatment was administered 1 or 4 h before virus exposure or at times indicated (treatment model). Normal and saline-treated control mice

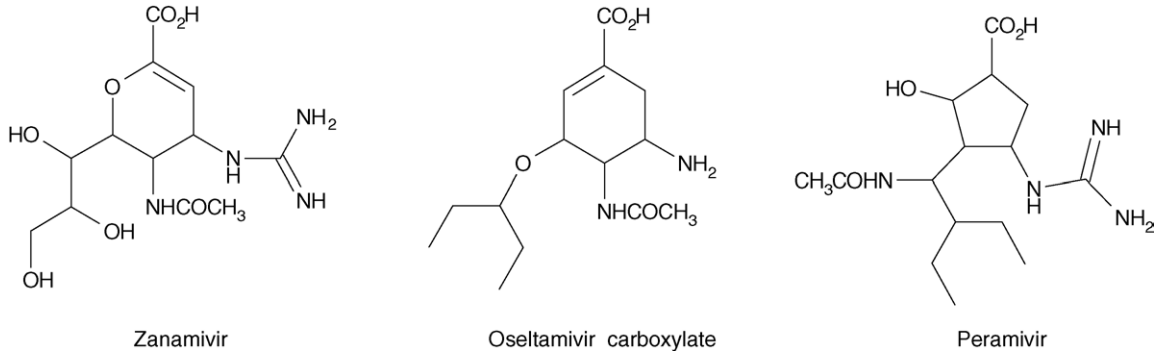

Fig. 1. Structures of compounds under investigation.

were included in the same treatment schedule. Parameters studied were reduction in mortality and/or increase in mean day to death.

2.8. Statistical analysis

The data was analyzed by Sigma Plot (Windows Version 4.01, SPSS, Chicago, IL, USA) and Sigma Stat (Windows Version 2.0, Jandel Corporation, San Rafael, CA, USA). The *t*-test was used to evaluate differences in mean day to death. One-way analysis of variance (ANOVA) was performed using the Holm–Sidak test for pairwise multiple comparisons to evaluate differences in weight loss. Kaplan–Meier survival analysis (log rank or Gehan–Breslow tests) were applied to survival number differences.

3. Results

3.1. In vitro NA inhibition

The ability of peramivir to inhibit the NA activity of N9 (from the H1N9 virus) was tested and compared to zanamivir and oseltamivir carboxylate. The structures of these compounds are shown in Fig. 1. The IC_{50} for peramivir, oseltamivir carboxylate, and zanamivir against N9 enzyme were not significantly different and ranged from 1.3 to 2.1 nM (Table 1). The IC_{90} values for peramivir, oseltamivir carboxylate, and zanamivir are also listed in Table 1.

3.2. On-site dissociation of inhibitors from NA enzyme

To understand the difference between the binding affinities of the various NA inhibitors to the enzyme, on-site dissociation studies were performed. In this experiment, the NA-inhibitor complex was prepared and the unbound inhibitor was removed by passing through a biospin column. Different amounts of NA/inhibitor complex (10 and 50 μL) were added into the reaction mixture containing 75 μM substrate in order to initiate the reaction. Fig. 2 shows one set of experimental data using 10 μL of NA/inhibitors complex and NA control. As shown in the figure, NA alone reached a plateau (23,000 fluorescence units) at about 3.5 h, but the complex of NA/oseltamivir carboxylate and NA/zanamivir reached a plateau at about 4 h. However, the complex of NA and peramivir never reached the plateau of 23,000 fluorescence units up to 24 h (data is displayed only for 5 h). The $t_{1/2}$ (time for half the substrate to be converted to the product) is 0.75 h for free NA and 1.25 h for the NA/zanamivir and NA/oseltamivir carboxylate complex, whereas the $t_{1/2}$ for the NA/peramivir complex is >24 h. This result indicates that peramivir is tightly bound and has a slow off-rate from the N9-inhibitor complex compared to oseltamivir carboxylate or zanamivir. The amino acid residues which define the active site of neuraminidase are highly conserved from strain to strain, thus suggesting peramivir may also bind tightly to different subtypes of neuraminidases, including N1 and N2.

Table 1
Inhibition of N9 (H1N9) neuraminidase activity[a]

NA inhibitors	IC_{50} (nM)	IC_{90} (nM)
Peramivir	1.3 ± 0.4	5.0 ± 1.1
Oseltamivir carboxylate	2.1 ± 0.4	10.4 ± 0.7
Zanamivir	1.6 ± 0.3	10.0 ± 1.2

[a] Fluorimetric assay used to measure influenza NA activity. Values are expressed as mean of three experiments ± S.E.

3.3. Influenza A mouse model: treatment and prophylaxis

In the mouse influenza model, viral infection leads to loss of body weight and high mortality, and this decrease in body weight

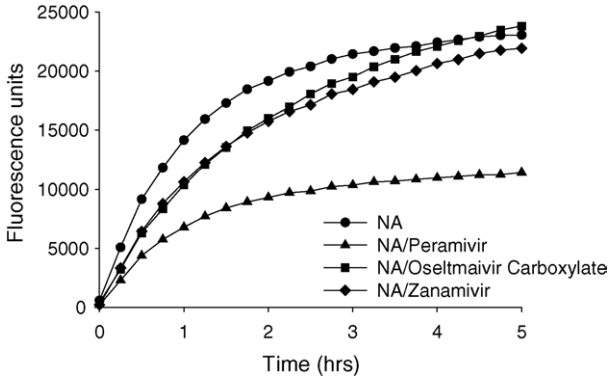

Fig. 2. On-site dissociation of neuraminidase-inhibitor complex. The onsite dissociation rates of the NA-inhibitor complex were measured by mixing the complex with substrate (75 μM MuNANA) and recording the fluorescence (excitation: 360 nm and emission: 450 nm).

correlates with pulmonary viral titer and pulmonary lesion score (Johansson et al., 1993). Therefore, the efficacy of orally and IM administered peramivir, oseltamivir and zanamivir was evaluated on the basis of the weight loss, mean day to death and survival rate, measured for 16 or 21 days post-infection for treated, infected animals relative to untreated, infected (control) animals.

In the prophylaxis model, a single IM injection of peramivir, given 4 h before viral challenge with H1N1 virus, was compared to an oral treatment of peramivir once daily for 5 days at doses of 1 and 10 mg/kg/day. Complete protection against lethality was observed in the mice treated at 10 mg/kg with both treatment regimens. However, at the 1 mg/kg dose, 60% of the mice survived in the oral treatment group versus 40% survival in the IM treatment group (Table 2, Experiment 1). Mean weight loss data for day 5 is presented in Table 2 to show a comparison between all experimental groups before deaths occurred. Mice treated with a single IM injection of peramivir at 10 mg/kg demonstrated no weight loss by day 5, whereas peramivir given orally for 5 days at the same dose lost 0.22 g.

Peramivir was also administered at 2, 10, 20 mg/kg as a single IM injection 4 h before the viral infection with the same virus from the previous experiment. Complete protection against lethality was observed at all doses, however, none of the five saline-treated control mice survived (Table 2, Experiment 2). By comparison, complete protection against lethality was also observed in the mice treated orally with oseltamivir at both 2 and 10 mg/kg/day (q.d. × 5 days). No signs of drug-related toxicity were observed when peramivir was administered IM at the highest dose (20 mg/kg). Peramivir and oseltamivir showed a dose response relationship when the weight loss of infected mice over time was followed. At day 8, the maximum mean weight loss in the 2, 10, and 20 mg/kg peramivir-treated groups were 3.3, 0.98, and 0 g, respectively. Additionally, oseltamivir provided a similar effect with the greatest mean weight loss of 1.34 and 0 g occurring at day 8 for the 2 and 10 mg/kg groups, respectively. Day 5 weight loss shows a similar trend (Table 2). In general, a lower dose resulted in greater weight loss when compared with a higher dose.

Single IM injections of peramivir or oseltamivir at 10 mg/kg dose were evaluated when administered 4 h prior to inoculation with H1N1 virus (Table 3). Oseltamivir provided only 30% protection which is not significantly different from the control group in which 90% of the mice died. However, in the peramivir-treated group, complete protection against lethality was observed. The peramivir group did not show any substantial weight loss (∼1.7% of initial weight). On the other hand, the oseltamivir group lost significant weight, about 4 g (25% of initial weight), and only 3 out of 10 mice survived (Fig. 3). In the same model, a comparison of single oral treatments of a 10 mg/kg dose of either peramivir or oseltamivir was assessed. Peramivir provided better protection orally with a survival rate of 50%, whereas, only 10% of mice survived in the oseltamivir group (Table 3).

To determine if similar protective effects are observed using different viruses, the efficacy of a single IM injection of peramivir was compared to the oral treatment of oseltamivir

Table 2
Intramuscular treatment of peramivir compared to oral treatment with oseltamivir or peramivir in mouse influenza model

Experiment number	Viral strain	Compound	Dose (mg/kg/day)	Dosing schedule	Treatment time (h)	Route of administration	Survival	Mean day to death ± S.E.	Mean weight change (g) (day 5)
1	A/NWS/33 (H1N1)	Peramivir	1	q.d.	−4	IM	2/5	14.7 ± 0.33**	−1.04*
			10				5/5*	>16.0 ± 0.0*	+0.14*
		Saline	–				0/5	10.6 ± 1.0	−2.8
		Peramivir	1	q.d. × 5 days	−4	Oral	3/5	10.0 ± 1.0	−2.3**
			10				5/5*	>16.0 ± 0.0*	−0.22*
		Saline	–				0/5	9.8 ± 0.49	−3.1
2	A/NWS/33 (H1N1)	Peramivir	2	q.d.	−4	IM	5/5*	>21.0 ± 0.0*	−0.22*
			10				5/5*	>21.0 ± 0.0*	+0.24*
			20				5/5*	>21.0 ± 0.0*	+0.48*
		Oseltamivir	2	q.d. × 5 days	−4	Oral	5/5*	>21.0 ± 0.0*	+0.14*
			10				5/5*	>21.0 ± 0.0*	+0.20*
		Saline	–	q.d.	−4	IM	0/5	8.8 ± 0.37	−2.4
3	A/Victoria/3/75 (H3N2)	Peramivir	20	q.d.	−1	IM	9/10*	9.0 ± 0.0*	−2.8*
		Oseltamivir	20	q.d. × 5 days	−1	Oral	9/10*	10.0 ± 0.0*	−2.8*
		Saline	–	q.d.	−1	IM	1/11	7.1 ± 0.43	−4.6

* $P < 0.003$; ** $P < 0.02$ vs. saline, infected (Oral, IM).

Table 3
Single oral and intramuscular treatment of peramivir and oseltamivir in mouse influenza model

Viral strain	Compound	Dose (mg/kg/day)	Dosing schedule	Treatment time (h)	Route of administration	Survival	Mean day to death ± S.E.
A/NWS/33 (H1N1)	Peramivir	10	q.d.	−4	IM	10/10*	>21 ± 0.0*
	Oseltamivir	10	q.d.	−4	IM	3/10	9.6 ± 0.53
	Peramivir	10	q.d.	−4	Oral	5/10	9.4 ± 0.51
	Oseltamivir	10	q.d.	−4	Oral	1/10	8.3 ± 0.37
	Saline	–	q.d.	−4	Oral	1/10	9.7 ± 0.58

* $P < 0.001$ vs. saline-treated control.

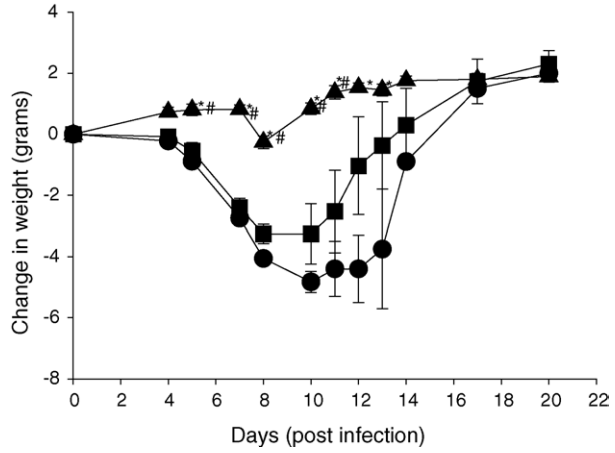

Fig. 3. Effects of single intramuscular treatment of peramivir and oseltamivir, at a dose of 10 mg/kg on weight loss in influenza A (H1N1) infected mice. Number of mice in each group was 10. (●) vehicle group (1/10 survived), (▲) peramivir treatment group (10/10 survived), and (■) oseltamivir treatment group (3/10 survived). Weight loss is statistically significant at $^{*}P < 0.05$ vs. control; Weight loss is statistically significant at $^{\#}P < 0.05$ vs. oseltamivir.

(q.d. × 5 days) in mice infected with the H3N2 virus (Table 2, Experiment 3). In this study the drug was administered 1 h prior to viral inoculation. The single IM treatment of peramivir at 20 mg/kg dose provided almost complete protection against lethality (9/10 survived). Oseltamivir also demonstrated similar protective effects (9/10 survived). The mean weight loss was almost identical in both treatment groups in that the oseltamivir-treated mice lost 28% of their weight (∼5.1 g) compared to 25% (4.5 g) weight loss in the peramivir group by day 8.

In the delayed treatment model, IM administration of a 10 mg/kg single dose of peramivir 24 h or 48 h post-infection gave complete protection against lethality, whereas, in the saline-treated group, 70% lethality was observed. There was no significant weight loss by day 5 in both 24 and 48 h peramivir-treated groups given both orally (q.d. × 5 days) and by single IM injection, whereas, the saline-treated group lost 2.1 g (Table 4).

4. Discussion

Peramivir is a specific and potent inhibitor of influenza NA and has demonstrated in vitro activity that is comparable or better than oseltamivir carboxylate and zanamivir. Oral administration of peramivir also demonstrated in vivo activity both in mice and ferrets (Bantia et al., 2001; Sidwell et al., 2001; Sweet et al., 2002). However, in human clinical trials, in spite of significant reduction in viral titer, peramivir did not demonstrate a statistically significant decrease in time to relief of symptoms and this may be because of low oral bioavailability of the drug in humans (www.biocryst.com/pdf/peramivirfacts.pdf; www.shareholder.com/biocryst/news/20020625-83347.cfm?ReleaseID=83347).

To circumvent the problem of low oral bioavailability, peramivir was tested as a single IM injection in the mouse influenza model. Peramivir is active when administered IM in both prophylaxis and treatment mouse influenza virus infection models. Initial studies were performed with smaller number of animals, whereas, the number of mice was expanded to more than eight mice per group in latter studies. In three different studies (prophylaxis model) using two different strains (H1N1 and H3N2) of influenza A virus, efficacy of a single IM injection of peramivir was compared to oral treatment (q.d. × 5 days) of either oseltamivir or peramivir (Table 2, Experiments 1–3). Although, 5 days (b.i.d.) of oseltamivir is normally used in the clinic for treatment of influenza, our studies have also shown that once daily dosing for 5 days is also effective (Table 2). In

Table 4
Comparison of oral and intramuscular treatment of peramivir in mouse influenza model (delayed treatment)

Viral strain	Compound	Dose (mg/kg/day)	Dosing schedule	Treatment time (h)	Route of administration	Survival	Mean day to death ± S.E.	Mean weight change (g) (day 5)
A/NWS/33(H1N1)	Peramivir	10	q.d.	+24	IM	8/8*	>21 ± 0.0*	+0.31*
		10	q.d. × 5 days	+24	Oral	8/8*		+0.16*
	Peramivir	10	q.d.	+48	IM	8/8*	>21 ± 0.0*	+0.16*
		10	q.d. × 5 days	+48	Oral	8/8*		+0.30*
	Saline	–	q.d.	+24	IM	3/10	9.0 ± 0.49	−2.1

* $P < 0.002$ vs. saline-treated control.

all three studies the efficacy of a single IM injection of peramivir at doses of 10 or 20 mg/kg was comparable to the oral treatment (q.d. × 5 days) of oseltamivir or peramivir at the same dose in terms of survival, mean day to death, and weight loss. At doses of 2 mg/kg, as a single IM injection, peramivir demonstrated comparable efficacy in terms of survival (Table 2, Experiment 2). Nevertheless, the maximum weight loss was greater in the single IM peramivir-treated group versus the oral (q.d. × 5 days) oseltamivir-treated group. Although the maximum weight loss for the treatment groups was observed around days 8–10, weight loss data for day 5 is presented to show a comparison between all experimental groups before deaths occurred. It should also be noted that the lowest dose of peramivir (1 mg/kg) was not effective in terms of survival, yet, there was a significant increase in the mean day to death (Table 1, Experiment 1). In another experiment, we were able to show that peramivir was highly effective in mice with a viral challenge that caused 70% lethality when treatment was initiated as late as 48 h post-infection (Table 4).

Single IM injections of either peramivir or oseltamivir were also compared in the H1N1 mouse influenza model. The survival data (Table 3) indicate that a single IM injection of peramivir is efficacious and provides complete protection against lethality. Conversely, a single IM injection of oseltamivir provides no significant protection against lethality. The weight loss data is consistent with the survival data and indicates that a single IM injection of peramivir is effective in preventing weight loss in infected mice, unlike the oseltamivir group (Fig. 3). These studies indicate that peramivir is efficacious when given as a single IM injection whereas oseltamivir is not effective by the same route of administration in the mouse influenza model. A single IM injection of oseltamivir carboxylate in mice showed a similar effect as the single IM injection of oseltamivir (data not shown).

The IC_{50}s of peramivir and oseltamivir carboxylate are subnanomolar against H1N1 at 0.11 and 0.69 nM (Bantia et al., 2001) and H3N2 at 0.59 and 0.55 nM, respectively (unpublished data). In spite of similar potency against neuraminidase enzymes, a single IM injection of oseltamivir (carboxylate) is not effective. However, peramivir as a single IM injection is superior to oseltamivir (carboxylate) given as single IM injections and the reason for this may be the slow off rate of peramivir from the NA enzyme compared to zanamivir or oseltamivir carboxylate (Fig. 2). Although the slow off-rate of peramivir was demonstrated with the N9 neuraminidase, one would expect peramivir to bind tightly to both N1 and N2 neuraminidases since the amino acid residues in the active site are highly conserved among different NA subtypes. This data is consistent with efficacy data from the single oral administration of peramivir or oseltamivir studies (Table 3). Furthermore, this data also provides an explanation for the superior activity of peramivir compared to oseltamivir carboxylate and zanamivir in the intranasal treatment of influenza A infected mice (Bantia et al., 2001).

In summary, peramivir is a potent inhibitor of NA activity and because of the tight binding and slow off-rate of peramivir from the NA enzyme, prophylactic and delayed single IM administrations were effective in preventing lethality and weight loss in the mouse influenza model. In view of the in vivo and in vitro data, we conclude that peramivir is effective as a single IM injection and could be used in the treatment of human influenza virus infections.

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