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Short Communication

Oseltamivir–zanamivir combination therapy is not superior to zanamivir monotherapy in mice infected with influenza A(H3N2) and A(H1N1) pdm09 viruses

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ABSTRACT

The efficacy of oseltamivir–zanamivir combination therapy compared to that of monotherapy was evaluated in mice infected with influenza A(H3N2) or A(H1N1)pdm09 viruses. For A(H3N2) virus, zanamivir monotherapy and oseltamivir–zanamivir combination showed significant reduction of mean weight loss compared to oseltamivir. Zanamivir monotherapy also conferred decreased mortality, weight loss and lung viral titers (LVT) compared to oseltamivir for A(H1N1)pdm09 wild-type virus. Intermediate benefits were observed for the oseltamivir–zanamivir combination. For the oseltamivir–resistant A(H1N1)pdm09 H275Y virus, the efficacy of oseltamivir–zanamivir was comparable to that of zanamivir and significantly higher than that of oseltamivir in terms of survival, weight loss and LVT.

Neuraminidase (NA) inhibitors (NAIs) such as oseltamivir and zanamivir represent one of the most valuable options for the control of influenza epidemics and pandemics. Combinations of anti-influenza agents have been thought to provide improved therapeutic potency in addition to reducing the emergence of resistance. At this time, available data on the potential effect of combined NAI therapy is very limited. For instance, combinations of oseltamivir and peramivir demonstrated additive activities *in vitro* and in mice infected with A/NWS/33 (H1N1) virus (Smee et al., 2010). On the other hand, a double-blind, randomized clinical trial carried out in France during the 2008–2009 influenza epidemic found that the oseltamivir–zanamivir combination appeared less efficacious than oseltamivir monotherapy in adults with uncomplicated influenza, mostly A(H3N2) infections (Duval et al., 2010). Nevertheless, a more recent study by the same group has suggested greater effectiveness of combined therapy in the reduction of influenza transmission in household contacts (Carrat et al., 2012). In the A(H1N1)pdm09 background, combinations of oseltamivir and zanamivir showed concentration-related additive to antagonistic antiviral effects *in vitro* (Nguyen et al., 2010), whereas a clinical trial conducted during the 2009–2010 influenza pandemic failed to conclude whether combined therapy improved or reduced the effectiveness of oseltamivir monotherapy in the treatment of A(H1N1)pdm09 virus infection in community patients, due to small sample size (Escuret et al., 2012).

The aim of this study was to evaluate the efficacy of the oseltamivir–zanamivir combination compared to that of monotherapy for the treatment of mice infected with NAI-sensitive A(H3N2) and A(H1N1)pdm09 viruses as well as for A(H1N1)pdm09 viruses harboring the oseltamivir resistance H275Y NA substitution.

A mouse-adapted A/Victoria/3/75 (H3N2) wild-type (WT) virus (a gift from Dr. D. Smee, Utah State University, Logan, UT) was passaged twice in Madin-Darby canine kidney (MDCK) cells. The recombinant influenza A(H1N1)pdm09 WT and its oseltamivir-resistant H275Y NA variant were generated from the first A(H1N1)pdm09 virus isolated in Québec City (A/Québec/144147/09, GenBank accession numbers FN434457-FN434464) using bidirectional pLLBA/G plasmids as previously described (Pizzorno et al., 2011). The recombinant viruses were amplified in ST6GalI-MDCK cells overexpressing the α2,6 sialic acid receptors (kindly provided by Dr. Y. Kawaoka, University of Wisconsin, Madison, WI). All three viruses were sequenced using the ABI 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) and titrated by standard plaque assays.

Susceptibility phenotypes to oseltamivir carboxylate (Hoffmann-La Roche, Basel, Switzerland) and zanamivir

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(GlaxoSmithKline, Stevenage, UK) were determined by NA inhibition assays using the 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA, Sigma, St-Louis, MO, USA) substrate as described elsewhere (Potier et al., 1979), with minor modifications (Pizzorno et al., 2011). Both A/Victoria/3/75 (H3N2) and recombinant A/Québec/144147/09 (H1N1pdm09) WT viruses were susceptible to oseltamivir with 50% inhibitory concentration (IC_{50}) values of 0.26 ± 0.04 nM and 0.46 ± 0.01 nM, respectively. Both viruses were also susceptible to zanamivir, with IC_{50} values of 0.38 ± 0.01 nM and 0.15 ± 0.01 nM, respectively. As expected, the recombinant A/Québec/144147/09 H275Y NA mutant showed highly reduced susceptibility to oseltamivir (IC_{50}: 451.92 ± 26.01 nM), but remained susceptible to zanamivir (IC_{50}: 0.14 ± 0.01 nM).

Groups of twelve 6- to 8-week old female C57BL/6 mice (Charles River, ON, Canada) were inoculated intranasally (i.n.) with

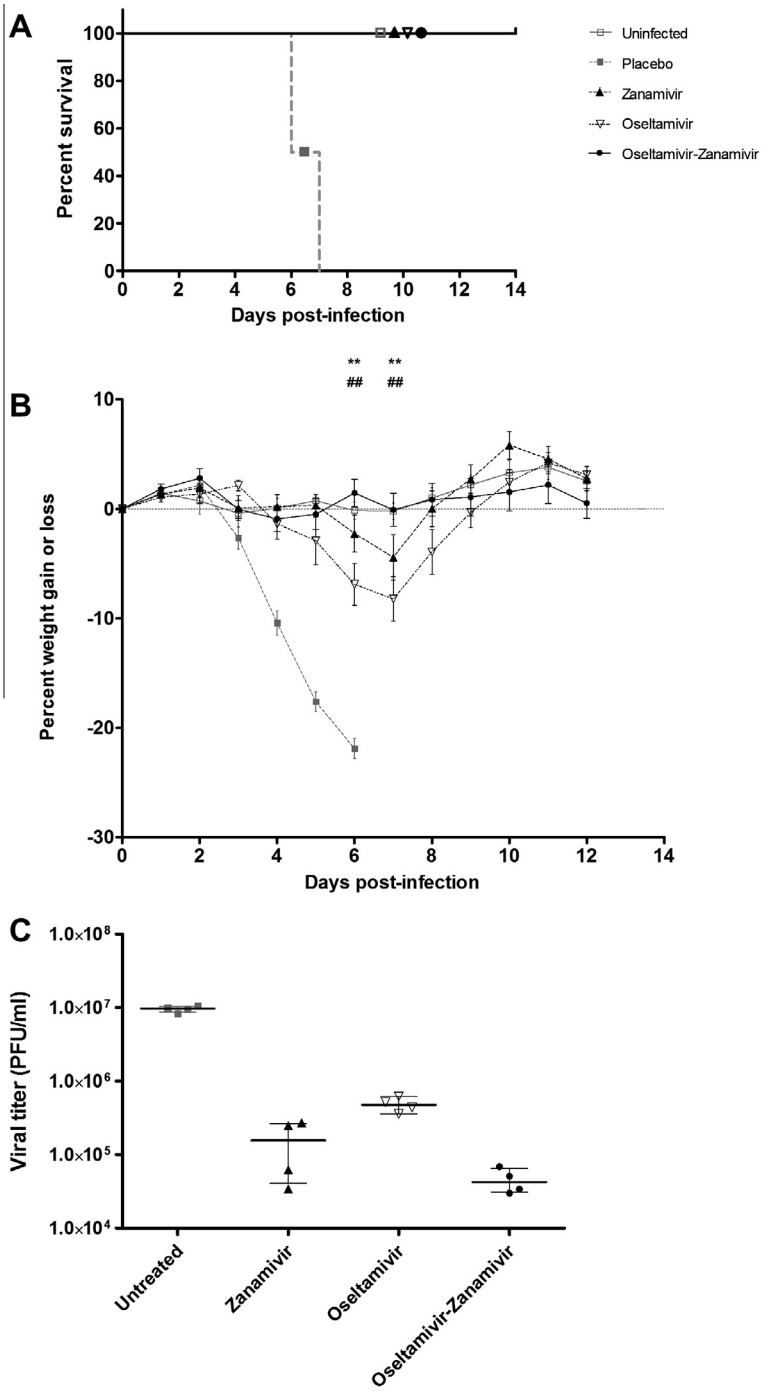

Fig. 1. Mortality (A), weight loss (B) and lung viral titers (LVT) (C) of C57BL/6 mice infected with A/Victoria/3/75 (H3N2) wild-type (WT) virus and treated with neuraminidase inhibitors. Groups of 12 mice were infected with 10^3 PFU of a mouse-adapted strain and treated 48 h post-infection with saline (placebo control), oseltamivir 10 mg/kg by gavage, zanamivir 10 mg/kg intranasally, or oseltamivir–zanamivir combination, twice daily for 5 days. An uninfected group (saline) was added as control. On day 4 p.i., four mice per group were sacrificed and viral titers in lung homogenates were determined by plaque assay in MDCK cells. $^{\#\#**}P < 0.01$ for differences in mean weight loss between mice treated with oseltamivir compared to zanamivir (*) or oseltamivir–zanamivir combination (#), using one-way ANOVA with Tukey's multiple-comparison post-test.

either saline (uninfected control), 10^3 plaque forming units (PFU) of A(H3N2) WT virus, or 10^5 PFU of recombinant A(H1N1)pdm09 WT or H275Y viruses. To mimic clinical conditions, treatment was initiated 48 h post-infection (p.i.) with either saline (placebo control), oseltamivir 10 mg/kg by gavage (plus saline i.n.), zanamivir 10 mg/kg i.n. (plus saline in gavage), or the oseltamivir–zanamivir combination, twice daily for 5 days. Mortality and weight loss were monitored for 14 days. The humane endpoint was determined at 25% weight loss. On day 4 p.i., four mice per group were sacrificed to determine lung viral titers (LVT) by plaque assay in ST6Gall-MDCK cells. Viruses recovered from lungs were sequenced for the presence of unexpected NA mutations. All animal procedures were approved by the Institutional Animal Care Committee of Laval University according to guidelines of the Canadian Council of Animal Care.

As shown in Fig. 1A, i.n. inoculation of 10^3 PFU of A/Victoria/3/75 (H3N2) virus resulted in a 100% mortality rate on day 7 p.i. in untreated mice. On the other hand, treatment with oseltamivir, zanamivir or oseltamivir–zanamivir combination 48 h p.i. resulted in 100% survival. Interestingly, zanamivir monotherapy as well as

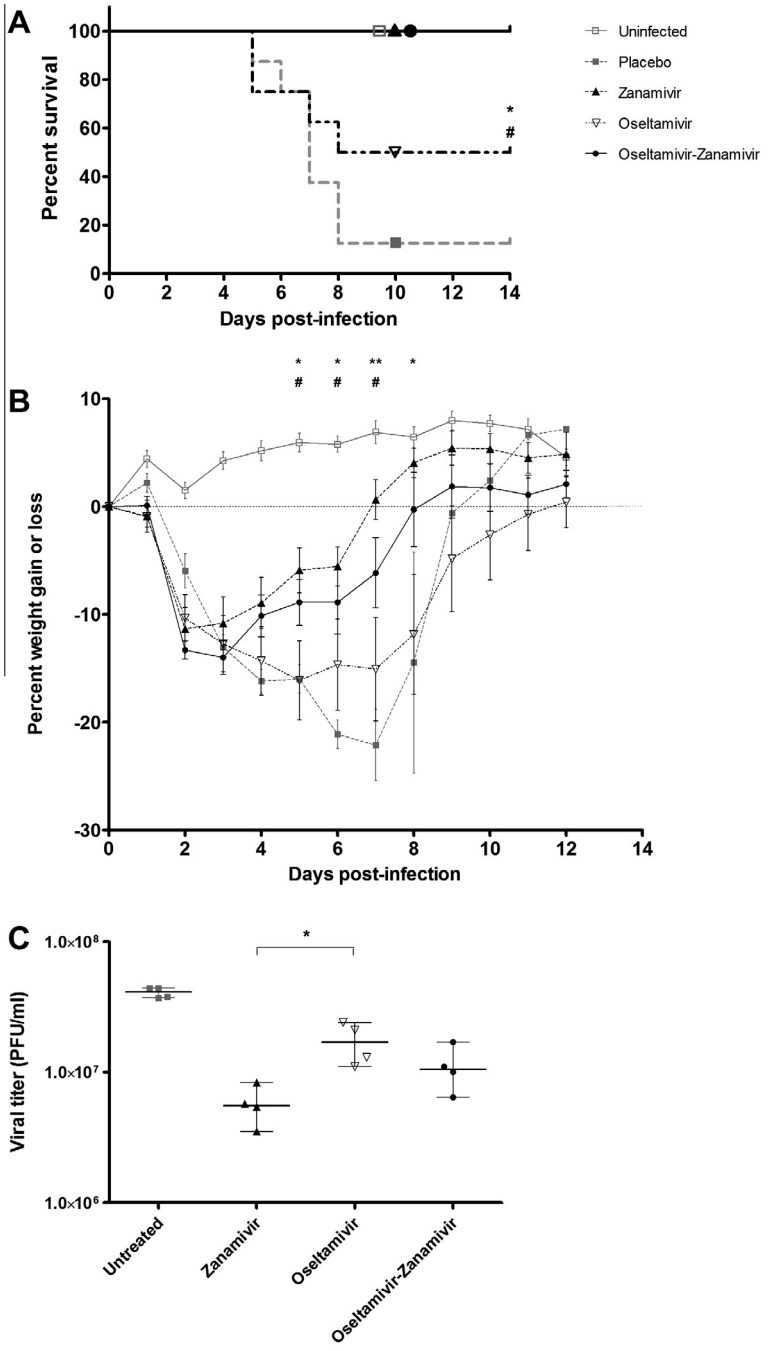

Fig. 2. Mortality (A), weight loss (B) and lung viral titers (LVT) (C) of C57BL/6 mice infected with recombinant A/Québec/144147/09 (H1N1pdm09) wild-type (WT) virus and treated with neuraminidase inhibitors. Groups of 12 mice were infected with 10^5 PFU of the recombinant WT strain and treated 48 h post-infection with saline (placebo control), oseltamivir 10 mg/kg by gavage, zanamivir 10 mg/kg intranasally, or oseltamivir–zanamivir combination, twice daily for 5 days. An uninfected group (saline) was added as control. On day 4 p.i., four mice per group were sacrificed and viral titers in lung homogenates were determined by plaque assay in MDCK cells. $^{\#*}P < 0.05$, $^{\#\#**}P < 0.01$ for differences between mice treated with oseltamivir compared to zanamivir (*) or oseltamivir–zanamivir combination ($^\#$), using Log-Rank (Mantel-Cox) test for comparing Kaplan–Meier survival plots, and one-way ANOVA with Tukey's multiple-comparison post-test for comparing mean weight loss and LVT.

oseltamivir–zanamivir combination showed a moderate but significant reduced maximum mean weight loss compared to oseltamivir monotherapy (4.4%, 0.1% and 8.2%, respectively, $P < 0.01$), on day 7 p.i. (Fig. 1B). However, no significant differences in mean LVT on day 4 p.i. were observed among the groups treated with either oseltamivir ($4.9 \pm 1.1 \times 10^5$ PFU/ml), zanamivir ($1.5 \pm 0.6 \times 10^5$ PFU/ml), or oseltamivir–zanamivir combination ($4.6 \pm 1.8 \times 10^4$ PFU/ml) (Fig. 1C).

In mice infected with 10^5 PFU of recombinant A/Québec/144147/09 (H1N1pdm09) WT virus, treatment with zanamivir or oseltamivir-zanamivir resulted in 100% survival, compared to 50% ($P < 0.05$) for the oseltamivir group and 12.5% ($P < 0.05$) for the saline group (Fig. 2A). Zanamivir monotherapy also led to a greater reduction in mean weight loss compared to oseltamivir as well as lower mean LVT on day 4 p.i. ($5.7 \pm 1.9 \times 10^6$ vs $1.7 \pm 0.6 \times 10^7$ PFU/ml, respectively, $P < 0.05$). Intermediate values were observed for the oseltamivir–zanamivir combination group (Fig. 2B,C). For the A/Québec/144147/09 H275Y infection (Fig. 3A–C), the efficacy of oseltamivir–zanamivir combination was comparable to that of zanamivir monotherapy and

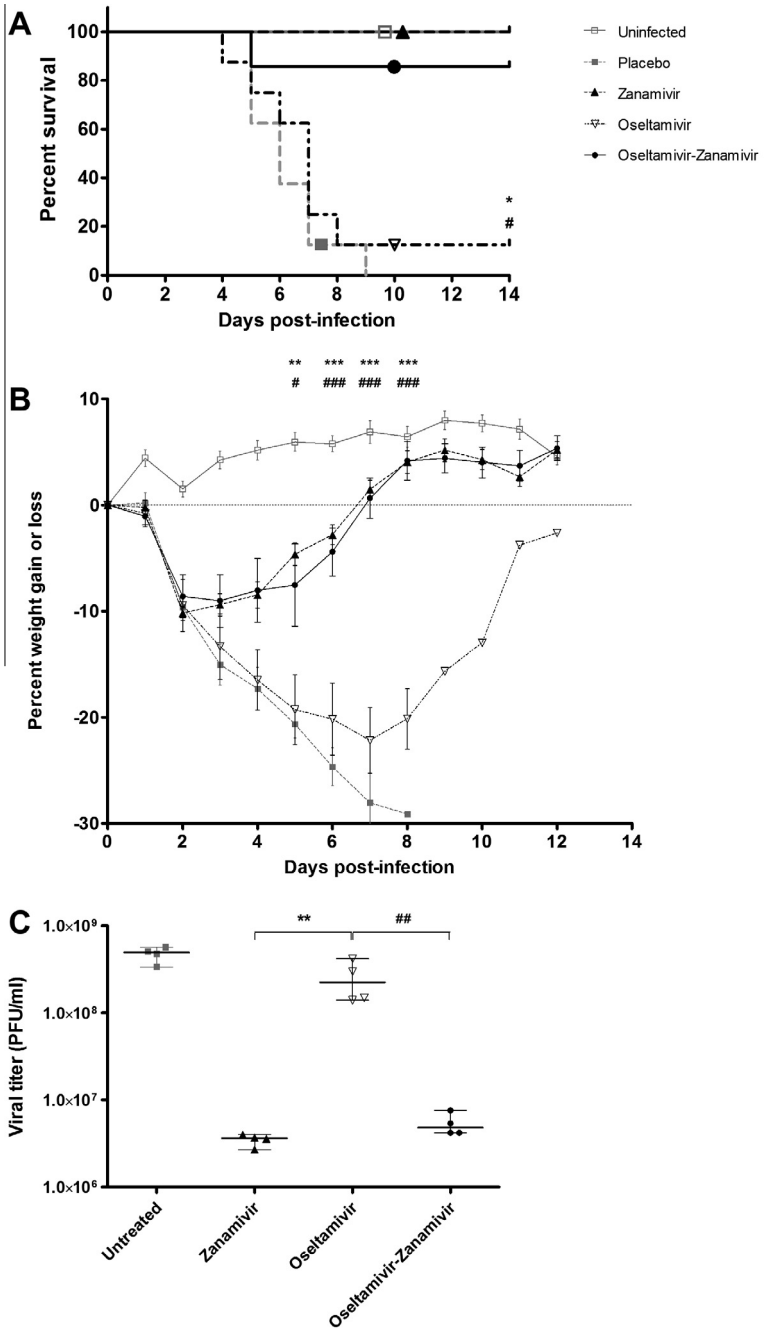

Fig. 3. Mortality (A), weight loss (B) and lung viral titers (LVT) (C) of C57BL/6 mice infected with recombinant A/Québec/144147/09 (H1N1pdm09) H275Y mutant virus and treated with neuraminidase inhibitors. Groups of 12 mice were infected with 10^5 PFU of the recombinant H275Y strain and treated 48 h post-infection with saline (placebo control), oseltamivir 10 mg/kg by gavage, zanamivir 10 mg/kg intranasally, or oseltamivir–zanamivir combination, twice daily for 5 days. An uninfected group (saline) was added as control. On day 4 p.i., four mice per group were sacrificed and viral titers in lung homogenates were determined by plaque assay in MDCK cells. $^{\#**}P < 0.05$, $^{\#\#**}P < 0.01$, $^{\#\#\#***}P < 0.001$ for differences between mice treated with oseltamivir compared to zanamivir (*) or oseltamivir–zanamivir combination (#), using Log-Rank (Mantel-Cox) test for comparing Kaplan–Meier survival plots, and one-way ANOVA with Tukey's multiple-comparison post-test for comparing mean weight loss and LVT.

significantly higher than that of oseltamivir in terms of survival (85.7% and 100% vs 12.5%, respectively, $P < 0.05$), weight loss and mean LVT on day 4 p.i. ($5.4 \pm 1.6 \times 10^6$ and $3.5 \pm 0.6 \times 10^6$ vs $2.5 \pm 1.3 \times 10^8$ PFU/ml, respectively, $P < 0.01$). In all cases, comparison of NA viral sequences collected from lung homogenates with those of inoculated viruses confirmed the absence of unexpected mutations or mixed viral populations.

In this study, we used a controlled animal model to evaluate the clinical and virological responses to the treatment of severe influenza infections with NAI-sensitive and NAI-resistant influenza viruses. With the focus of improving oseltamivir therapy, we hypothesized that the combination of anti-influenza agents having different interactions with the NA substrate, namely oseltamivir and zanamivir, could potentially improve antiviral effectiveness while reducing the emergence of drug-resistant variants. Our results showed that, in mice infected with A(H1N1)pdm09, the efficacy of the oseltamivir–zanamivir regimen was comparable to that of zanamivir alone but greater than that of oseltamivir, when treatment was initiated at 48 h p.i. This trend was also seen in the A(H3N2) infection model, but with lesser differences among groups.

Interestingly, our findings are not in line with what has been reported in the randomized placebo-controlled trial by Duval and colleagues (Duval et al., 2010), in which oseltamivir showed greater clinical and virological efficacy as compared to either zanamivir or oseltamivir–zanamivir combination in 541 patients with uncomplicated influenza. Nonetheless, this discrepancy could be attributed to many factors. Firstly, in the mentioned clinical trial, almost all influenza viruses detected were of the A(H3N2) subtype, whereas the most important differences in treatment outcomes observed in our study were found for the A(H1N1)pdm09 virus, for which the only clinical trial reported to date failed to be fully informative (Escuret et al., 2012). Secondly, patients enrolled in the study by Duval and colleagues had uncomplicated influenza, whereas we studied the effects of single or combined NAI therapy in a model of severe infection. Finally, the French group pointed out the possibility of a slight underestimation of performance in both zanamivir and oseltamivir–zanamivir arms of their study. Actually, in a subset analysis of the same cohort, treatment of index patients with oseltamivir–zanamivir combination within 24 h of onset of symptoms proved to be more effective than monotherapy for reducing household transmission (Carrat et al., 2012).

The fact that the influenza A(H3N2) strain used in our study is a mouse-adapted strain may represent a limitation of our model. Also, even if clinical trials have not shown any significant pharmacokinetic differences between inhaled and intranasal zanamivir in humans (Cass et al., 1999), no such data are available in mice. Finally, only one dose of each NAI was tested in this study. Although the 10 mg/kg dose of oseltamivir given to mice is considered a good estimate of the normal dose given to humans (75 mg), a similar correlation has not yet been defined in the case of zanamivir. Based on a previous report using the same route of zanamivir administration in mice (Kubo et al., 2010), we arbitrarily used the same concentration of the two drugs; therefore the results presented here should be interpreted with caution.

Although most of the oseltamivir-resistant viruses remain susceptible to zanamivir, the unpredictable concentrations of zanamivir in peripheral lungs after inhalation as well as the potential induction of bronchospasm, especially in young children (Moscona, 2005), constitute an important limitation for such treatment. However, the advent of intravenous zanamivir, currently in phase III clinical trials, may represent a promising alternative. In that regard, a crossover study carried out on healthy Thai adults found no clinically significant pharmacokinetic interactions between oseltamivir and zanamivir (Pukrittayakamee et al., 2011).

The main conclusion of our study is that zanamivir was superior to oseltamivir therapy in A(H3N2) and particularly A(H1N1)pdm09 infections in our mouse model. Moreover, there was no additional benefit of combination therapy over zanamivir alone. Such differential effects of the two NAIs could not be explained by their *in vitro* activity but are probably related to their different pharmacokinetic properties. Additional controlled studies are needed to determine the best therapeutic regimens in case of severe influenza infections, while minimizing the emergence of drug resistance.

Transparency declarations

G.B. has received grant support from GlaxoSmithKline Canada. All other authors: none to declare.

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