Antiviral Research 96 (2012) 130–137

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Oseltamivir–zanamivir bitherapy compared to oseltamivir monotherapy in the treatment of pandemic 2009 influenza A(H1N1) virus infections

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ARTICLE INFO

Article history:
Received 7 February 2012
Revised 2 August 2012
Accepted 4 August 2012
Available online 14 August 2012

Keywords:
Oseltamivir
Zanamivir
Combination therapy
Pandemic
Influenza A(H1N1)pdm09 virus

ABSTRACT

Background: The emergence of oseltamivir resistance in 2007 highlighted the need for alternative strategies against influenza. To limit the putative emergence of resistant viruses this clinical trial aimed to evaluate the antiviral efficacy and tolerability of oseltamivir–zanamivir (O + Z) bitherapy compared to oseltamivir monotherapy (O). This clinical trial was designed in 2008–2009 and was conducted during the A(H1N1) influenza virus pandemic in 2009–2010. The A(H1N1)pdm09 viruses were reported to be sensitive to oseltamivir and zanamivir but resistant to amantadine.

Methods: During the pandemic phase in France, adults with influenza-like illness for less than 42 h and who tested positive to influenza A were randomised into treatment groups: (O + Z) or (O). Patients had a nasal wash at day 0, before the beginning of treatment and daily at days 1 to 4. They also had a nasal swab at days 5 and 7 to check for the negativation of viral excretion. Virological response was assessed using the GAPDH adjusted M gene quantification.

Results: Analysis was possible for 24 patients, 12 in the (O + Z) arm and 12 in the (O) arm. The mean viral load decreased at around 1 $\log_{10}$ cgeq/µl per day regardless of allocated treatment group. We could not detect any significant difference between treatment groups in the duration needed to alleviate symptoms. All treatments were well tolerated. No oseltamivir-resistant H275Y NA mutated virus has been detected in patients of both treatment groups.

Conclusions: The sample size of our study is too limited to be fully informative and we could not detect whether combination therapy (O + Z) improves or reduces the effectiveness of oseltamivir in the treatment of influenza A(H1N1)pdm09 virus infection in community patients. Additional studies are needed to improve the antiviral treatment of patients infected with influenza virus.

1. Introduction

The emergence of oseltamivir-resistance in 2007 and the rapid worldwide spread of the pandemic influenza A(H1N1)pdm09 virus have highlighted the need for effective novel antiviral approaches against influenza.

The neuraminidase inhibitors (NAIs) oseltamivir and zanamivir are the recommended antiviral agents against influenza A and B viruses. Amantadine was one of the first antiviral agents used against influenza A viruses. In the last decade there has been a substantial worldwide increase in amantadine-resistance, starting with the seasonal influenza A(H3N2) viruses (Deyde et al., 2007).

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0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.08.002

When the study was designed, seasonal influenza A(H1N1) viruses were resistant to oseltamivir but mostly sensitive to amantadine. However, as a consequence of the emergence of the A(H1N1)pdm09 virus that carried the S31N mutation in M2 (Dawood et al., 2009), all currently circulating human influenza A viruses are resistant to amantadine (WHO, 2011).

In the winter of 2007–2008, seasonal oseltamivir-resistant A(H1N1) viruses related to the A/Brisbane/59/2007 (H1N1) variant emerged and disseminated in individuals who were not under oseltamivir treatment (Lackenby et al., 2008; Meijer et al., 2009). However, the emergence of these viruses bearing the H275Y NA mutation had a major impact, showing that A(H1N1) viruses may develop the H275Y NA mutation and that the oseltamivir could not be used to treat infected patients. With the displacement of seasonal A(H1N1) by the A(H1N1)pdm09 viruses, all human influenza viruses were sensitive to oseltamivir and zanamivir. However, in order to limit the putative emergence of resistant clones in the context of a A(H1N1) pandemic when the use of NAIs is large, it was important to test possible alternative strategies. Theoretically, antivirals in combination could improve antiviral efficacy, reduce the duration of symptoms, and lower the risk of emergence of antiviral resistance.

The *in vitro* combination of NAIs may be antagonistic because these agents target the same binding pocket in the neuraminidase. However, different routes of administration, orally for oseltamivir and by inhalation for zanamivir, could induce a pharmacologically interesting interaction *in vivo*. During the 2008–2009 season when A(H3N2) virus was predominantly circulating, a clinical trial conducted in France confirmed that the oseltamivir–zanamivir bitherapy was antagonistic (Duval et al., 2010). However, these results may be different for A(H1N1) and A(H3N2) viruses because neuraminidases N1 and N2 show structural differences (Russell et al., 2006). Moreover, in the context of the pandemic it was interesting to study if the results found with the A(H3N2) virus would be confirmed or not with the A(H1N1)pdm09 virus.

The randomised trial was then conducted during the A(H1N1)pdm09 virus pandemic in France, to compare the oseltamivir–zanamivir (O + Z) combination therapy with the oseltamivir monotherapy (O), in terms of antiviral efficacy, resolution of symptoms, tolerability, and the prevention of oseltamivir-resistance emergence.

2. Patients, material and methods

This phase II clinical trial was a multicentered, randomised, and unblinded study of two parallel groups.

2.1. Study population

The study targeted adult out-patients of both sexes, aged 18 to 64 years, with influenza-like illness (ILI) for less than 42 h, not vaccinated against influenza in the year of the study and who tested positive for influenza A (QuickVue® Influenza A+B test). Patients also had to be free from chronic diseases, have medical insurance, and give informed written consent. Exclusion criteria were pregnancy (a pregnancy test was performed before inclusion), lactation, lack of effective contraceptive methods, ongoing chronic obstructive pulmonary disease (COPD), asthma, renal failure, epilepsy, confusional state, hallucinations, severe uncontrolled psychotic or neurotic state, depression with antidepressant treatment, congestive cardiac insufficiency, peripheral oedema, orthostatic hypotension and a hypersensitivity to one component of the study drugs. Use of drugs like nasal topics, corticosteroids, immunosuppressive drugs, neuroleptics or antiemetics was not permitted during the study.

Patients were recruited by general practitioners in the community (in Lyon and Paris, France) during the peak circulation of the influenza A(H1N1)pdm09 virus.

The study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Lyon Ethics Committee (*Comité de Protection des Personnes Lyon B*) on 9 September 2009.

2.2. Study protocol

Randomisation was performed after checking eligibility, obtaining patient consent, and collecting baseline data. A permuted-block algorithm was used for randomisation. Concealed allocation was performed by calling the coordination centre. Patients were randomised into two groups according to the antiviral treatment they received: the oseltamivir–zanamivir (O + Z) bitherapy or the oseltamivir (O) monotherapy.

Oseltamivir (Tamiflu®) was administered orally at the recommended dosage of 75 mg, two times a day for 5 days (150 mg per day). Zanamivir (Relenza®) was administered by inhalation with the Diskhaler system, at the recommended dosage of two inhalations of 5 mg, two times a day for five days (20 mg per day). For the combination therapy both drugs were given concomitantly: there was no more than a few minutes of delay between the oral administration of oseltamivir and the inhalation of zanamivir. Oseltamivir was provided for free by Roche SA. Zanamivir was purchased from GlaxoSmithKline. Drugs were packaged by Creapharm SA.

Specimens were collected at patients' homes by a study nurse. Specimens consisted of a nasal wash within two hours after the first visit (H0), and every 24 h until 96 h after treatment start. The nasal washes were obtained by instilling 2.5 ml of physiological saline solution in each nostril and then aspirating the nasal secretions with a silicone pipe connected to a vacuum pump. To ensure good viral conservation, the viral transport medium (Sigma Virocult®, Medical Wire & Equipment Co.) was aspirated and mixed with the nasal wash. Then, the mixture was kept at 4 °C during transport to the virological laboratory. Subsequent specimens consisted of nasal swabs (Virocult®, Medical Wire & Equipment Co) performed on days 5 and 7. We changed from nasal washes to nasal swabs to alleviate the sampling performed in patients; we first assumed that the viral load would be near zero on these days and nasal swabs were done to check for the negativation of viral load.

Baseline data included the patient's medical history and influenza symptoms. Follow-up data included the evolution of symptoms, and compliance to treatment (assessed by pill count by number of study days). A follow-up visit was performed on day 6 to assess a potential carry-over effect.

Data were collected on Case Report Forms by the investigators, and entered into a database using Clininfo SA software (Clininfo SA, 99 rue de Gerland, 69007 Lyon, France).

2.3. Laboratory procedures: virological analysis

Nasal washes and nasal swabs were added to a viral medium culture for a final volume of at least 1.5 ml. Then the samples were divided into aliquots and frozen at −80 °C. All the samples for an individual were then tested in a same assay run for quantification.

RNA was extracted from 200 μl of nasal wash without antibiotics using the automated NucliSens easyMAG system (Biomerieux). Elution of the extracted nucleic acids was performed in 70 μl.

Influenza A virus was detected and quantified using a real time reverse transcription quantitative polymerase chain reaction (rt RTqPCR) on the influenza A M gene as described previously (Duchamp et al., 2010). The results were expressed in $\log_{10}$ copies of RNA genome equivalent/μl of nasal wash or nasal swab (abbreviated

$\log_{10}$ cgeq/μl). In order to control sample quality (presence of cells in the sample), a rt RTqPCR was performed on the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) intracellular gene (Wong et al., 2005; Duval et al., 2010) and an adjustment was made for the amount of GAPDH in the sample and the mean GAPDH for all samples. The GAPDH quantification gave comparable cell numbers results between groups with a mean Ct of 25.07 ± 3.01 (5.06 $\log_{10}$ cgeq/μl) and 25.78 ± 2.61 (4.69 $\log_{10}$ cgeq/μl) for (O) and (O + Z) groups respectively. The GAPDH adjusted M gene values were obtained by dividing the M gene value by the ratio (GAPDH value/GAPDH mean for all values). The results were expressed in $\log_{10}$ cgeq/μl. The final statistical analysis was performed considering the initial and the GAPDH adjusted M gene quantification values (Duval et al., 2010). As the results were similar and in order to avoid any confusion, we will present only the viral load corresponding to the GAPDH adjusted M gene quantification values.

The influenza A virus subtyping was performed on the first sample (nasal wash) received for each patient using a RT-PCR specifically targeting the N1 segment of A(H1N1)pdm09 virus (primers and probes are available upon request at grippe@pasteur.fr).

The one-step allelic discrimination real-time RT-PCR used on A/Brisbane/59/2007 (H1N1) – related viruses (Carr et al., 2008) was adapted and carried out on A(H1N1)pdm09 viruses. This RT-PCR uses two probes, which specifically detect the presence of a His (H) or Tyr (Y) in position 275 of N1 for A(H1N1)pdm09 viruses. The oligonucleotide primers (900 nM final concentration, Eurogentec) were as follows: N1SWFor (sense): 5′-CAG GCC TCA TAC AAG ATC TTC AGA-3′ and N1SWRev (antisense): 5′-CAC TAG AAT CAG GAT AAC AGG AGC-3′. The two allele specific probes (200 nM final concentration, Applied) with 5′dyes and 3′ minor groove binding non fluorescent quenchers (MGBNFQ) were as follows: the oseltamivir sensitive probe N1SWH275 VIC – 5′-CTC ATA GTG ATA ATT A-MGBNFQ and the oseltamivir resistant probe N1SWH275 FAM – 5′-CCT CAT AGT AAT AAT TA-MGBNFQ. We used 5 μl of RNA eluate in a 25 μl reaction volume with the SuperScriptIII Platinum One-Step qRT-PCR system (Invitrogen) on Applied Biosystems 7500 with the following cycling parameters: 50 °C for 15 min, 95 °C for 15 s, and 60 °C for 40 s for 45 cycles. The capacity of this RT-PCR to detect H275Y NA mutated viruses was confirmed using two oseltamivir-resistant viruses isolated by cell culture from patients admitted in intensive care unit and treated by oseltamivir. The IC_{50} for oseltamivir of these isolates, determined by fluorometric NA inhibition assay using the MUNANA substrate (Sigma–Aldrich) as described previously (Ferraris et al., 2005), were 232 nM and 178 nM compared to a mean of 0.46 ± 0.23 nM for 192 sensitive A(H1N1)pdm09 influenza viruses isolated in 2009–2010 (Escuret et al., 2011). Sequencing of these isolates confirmed that their oseltamivir-resistance was due to the H275Y NA mutation. As in previous studies, we amplified a 112-bp cDNA fragment from isolates with a sequence confirmed for H275 or Y275 with the Qiagen one-step RT-PCR kit. These amplicons were purified using a PCR purification kit (Qiagen) and quantified on a Nanodrop (ND-1000 spectrophotometer). We generated standard curves with serial dilutions of quantified amplicons. Assays were performed in triplicate for each target. Linear curves were obtained over the range of 10^1 to 10^8 copies, the slopes were −3.35 and −3.33 for H275 and Y275 respectively with correlation coefficients >0.99 for both targets. The sensitivity of this RT-PCR was 10 copies for the detection of the H275Y NA mutation (20/20 reactions detected) and was between 10 and 100 copies for the detection of the wild type virus (17/20 reactions detected for 10 copies). Mixed populations of wild-type and H275Y NA mutated influenza A(H1N1)pdm09 viruses could be detected using this allelic discrimination RT-PCR. At least 10% of H275Y NA mutated virus could be detected in a mixed viral population.

Frozen aliquots of nasal samples that were detected positive for influenza by the M gene rt RTqPCR were inoculated in MDCK (Madin Darby Canine Kidney) cells (from ATCC, product N° CCL-34). Cells were cultured in a growth medium: Eagle's Minimum essential Medium (EMEM, Biowhittaker 12–125F) with L-Glutamine 1% (200 mM; Biowhittaker 17–605E), Penicillin–Streptomycin 2% (25000 U/ml and 25000 μg/ml; Biowhittaker 17–719R) and SVF 2%. For the virus culture the medium was EMEM with L-Glutamine 1%, Penicillin–Streptomycin 2% and trypsin TPCK 2 μg/ml (Boehringer Mannheim 109819).

The presence of virus in cell culture was assessed by the M gene rt RTqPCR performed on dilutions (1/1000) of supernatant collected at day 4 after inoculation. However when the Ct was high (Ct ⩾ 38) and could correspond to a dilution of the inoculum we did a second passage in cell culture. The virus presence was tested by fluorometric NA activity assay with MUNANA substrate in the 2nd passage supernatant as previously described (Ferraris et al., 2005).

2.4. Sample size

The sample size was calculated using the one-step Fleming method (Fleming, 1982).

Our hypotheses were based on the results of previous studies (Hayden et al., 1999; Treanor et al., 2000). The first controlled trial on oseltamivir efficacy was conducted in 69 healthy adult volunteers inoculated intranasally with influenza A/Texas/36/1991 (H1N1) virus. There were 4 treatment groups with oseltamivir: 20 (n = 15), 100 (n = 14), 200 mg twice daily (n = 14), 200 mg once daily (n = 13) and 1 placebo group (n = 13). Administration began at 28 h after inoculation and continued for 5 days. The median duration of viral excretion estimated in cell culture was reduced from 107 h in the placebo group (13 patients) to 58 h in the combined oseltamivir treated patients (56 patients) (Hayden et al., 1999). In the study conducted in naturally influenza-infected patients, the patients were treated by oseltamivir 75 mg twice daily (n = 124), or oseltamivir 150 mg twice daily (n = 121) or placebo (n = 129). The median duration of illness before study was similar in all 3 treatment groups (26, 24 and 27 h in the placebo, oseltamivir 75 mg and 150 mg groups, respectively). The proportion of patients shedding virus at each time point were similar in all 3 groups: 15% and 72% of patients had a negative viral excretion estimated in cell culture after 24 h and 72 h respectively, whether they were treated by oseltamivir or placebo (Treanor et al., 2000). We then hypothesised that 50% and 80% of the patients would have a negative nasal wash in cell culture at 48 and 72 h respectively with an oseltamivir monotherapy according to the results of these previous studies (Hayden et al., 1999; Treanor et al., 2000).

Comparisons between cell culture and the rt RT-qPCR on the M gene showed that samples with less than 3 $\log_{10}$ cgeq/μl generally do not grow in cell culture. This threshold was defined after analysis of 405 nasal specimens collected between the 1-09-2009 and the 31-12-2009 for routine surveillance in south of France in the frame of the GROG (*groupes régionaux d'observation de la grippe*) network, detected positive by rt RT-qPCR for influenza A M gene and A(H1N1)pdm09 and inoculated for viral cell culture. Briefly, on a total of 405 samples, 268 and 137 samples had a positive and negative viral isolation in cell culture respectively. We compared the results given by different thresholds (between 5.23 and 0.85 $\log_{10}$ cgeq/μl) with the observed results of viral cell culture. We could then define proportions of true positive (when viral load was > threshold with viral isolation), false positive (when viral load was > threshold with no viral isolation), false negative (when viral load was < threshold with viral isolation) and true negative (when viral load was < threshold with no viral isolation). After analysis of the results, the threshold of 3 $\log_{10}$ cgeq/μl gave the best compro-

mise with a sensitivity of 0.95 (which means that 95% of infectious samples have a viral load >3 $\log_{10}$ cgeq/μl); a positive predictive value of 0.76 (which means that there is a probability of 76% that the sample is infectious if its viral load is superior to 3 $\log_{10}$ cgeq/μl) and a negative predictive value of 0.80 (which means that there is a probability of 80% that the sample is not infectious if its viral load is inferior to 3 $\log_{10}$ cgeq/μl).

The objective was to demonstrate that the combinations of antiviral drugs would have a 48-h efficacy compared with the 72-h efficacy of oseltamivir alone. Therefore, bitherapies would be considered effective if the percentage of patients treated by bitherapies with an influenza A viral load under 3 $\log_{10}$ cgeq/μl (the M gene) is 80% at 48 h.

With $\alpha = 5\%$, power = 80%, with P0 = maximum probability of inefficacy fixed at 50%, Pa, the minimum probability of inefficacy fixed at 80%. The following formulae give the number of patients needed per group S:

$$N = ([z1 - \beta(pa(1-pa))1/2 + z1 - \alpha(p0(1-p0))1/2]/(pa-p0))^2$$
$$S \geqslant [Np0 + (z1 - \alpha(Np0(1-p0))1/2]^* + 1$$

The minimum required sample size was 15 people per arm, but we aimed at recruiting 20 per arm. We assumed that 25% of patients would not meet inclusion criteria. Patients with missing samples were not replaced.

2.5. Efficacy end points

The primary criterion was the percentage of patients with a viral load under 3 $\log_{10}$ cgeq/μl. This was quantified 48 h after treatment start using a rt RTqPCR on the influenza A M gene, adjusted to the GAPDH gene.

Other criteria included the evolution profile of the influenza viral load, the duration of influenza symptoms, the safety of the bitherapies, and the occurrence of oseltamivir-resistance.

2.6. Statistical analysis

The statistical analysis was performed according to the intention to treat principle, i.e. all patients were analysed in the treatment group they were allocated in. However, missing data were not replaced.

The percentages of patients with an influenza A viral load under 3 $\log_{10}$ cgeq/μl at 48 h were calculated and compared between oseltamivir–zanamivir bitherapy and the oseltamivir monotherapy using a chi^2 test. They were then compared to the 80% theoretical threshold using an exact test based on the binomial distribution. The viral load decrease was estimated and compared between treatment groups using a multilevel linear regression model accounting for repeated measurements over time.

The analysis was performed considering the time from the beginning of treatment or time from the beginning of symptoms (data not shown) and conducted to similar results.

The median delay for alleviating all symptoms was defined as the time between drug initiation and symptom alleviation. Symptom alleviation was defined as the first day during which all the studied symptoms (chills, aches, headache, fatigue, cough, pharyngitis, sore throat or nasal congestion, expectoration, otitis, digestive disturbance) were absent or mild. It was compared between groups using a Kruskal–Wallis test. For patients with no symptom alleviation at the last visit, the delay was considered to be seven days (corresponding to the last visit).

3. Results

3.1. Patients

Between 1 October 2009 and 18 January 2010, 25 out-patients were recruited, 20 in the Lyon area and 5 in the Paris area. Out of these, 12 and 13 patients were randomised into the (O + Z) and (O) groups respectively. The virus was not amplified with the specific M gene RTqPCR in one patient's nasal sample, even though he had first a positive diagnosis for influenza A by rapid testing. A total of 24 patients were analysed, 12 in each group of treatment. All the patients had a proven infection with the A(H1N1)pdm09 influenza virus as the N1 gene specific of A(H1N1)pdm09 virus was detected positive on the first nasal wash of each patient. We could not detect any significant differences in the demographic and clinical characteristics of the patients between treatment groups before treatment (Table 1). Most patients presented general constitutional symptoms such as chills, aches, fatigue, headache and cough. Other symptoms (pharyngitis, sore throat or nasal congestion, expectoration, otitis, or digestive disturbance) were less frequent.

3.2. Primary criterion

At inclusion, all patients had more than 3 $\log_{10}$ cgeq/μl in their nasal wash. After two days of treatment, only 1 (8.3%) patient in each group had a viral load under 3 $\log_{10}$ cgeq/μl (Table 2). The percentage of patients under the threshold of 3 $\log_{10}$ cgeq/μl was far below the 80% rate expected in the (O + Z) group. After using logistic regression to analyze the proportion of patients under the threshold, we did not identify any significant differences between treatment groups. We obtained interpretable cell culture results for 9 (75%) and 7 (58.3%) patients, in the (O + Z) and (O) treatment groups, respectively. After two days of treatment 5 (55.6%) and 2 (28.6%) patients had a negative influenza virus cell culture in the (O + Z) and (O) groups respectively (Table 3). We could not detect any significant difference between the treatment groups but 8 (88.9%) and 3 (42.9%) patients had a negative influenza virus in cell culture after 3 days of treatment in the (O + Z) and (O) groups respectively (Table 3).

Table 1
Demographic and clinical characteristics of the patients at inclusion before treatment.

	Oseltamivir + Zanamivir	Oseltamivir
Demographic characteristics		
N	12	12
Male sex ratio (n, %)	4 (33.3)	6 (50.0)
Age (years)		
Mean (SD)	35.9 (10.6)	32.8 (12.5)
(min–max)	(21.9–55.2)	(19.1–51.7)
Delay from the beginning of symptoms (hours)		
Mean (SD)	25.0 (13.5)	23.9 (9.4)
Moderate or severe clinical symptoms		
Highest temperature (°C)		
Mean (SD)	38.9 (0.4)	39.2 (0.4)
Constitutional symptoms		
Chills and/or sweats (n, %)	10 (83.3)	9 (75.0)
Aches (n, %)	11 (91.7)	10 (83.3)
Fatigue (n, %)	11 (91.7)	11 (91.7)
Headache (n, %)	12 (100.0)	8 (66.7)
Respiratory symptoms		
Cough (n, %)	9 (75.0)	12 (100.0)
Pharyngitis (n, %)	4 (33.3)	5 (41.7)
Sore throat or nasal congestion (n, %)	7 (58.3)	6 (50.0)
Other symptoms		
Expectoration (n, %)	3 (25.0)	1 (9.1)
Otitis (n, %)	1 (8.3)	0 (0.0)
Digestive disturbance (n, %)	1 (8.3)	3 (25.0)

Table 2
Number of patients with a viral load above 3 $\log_{10}$ cgeq/μl according to treatment group and time of sampling.

Days of treatment	Treatment		p Value[a]
	(O + Z) (n = 12)	(O) (n = 12)	(O + Z) versus (O)
Number of patients with viral load above the 3 $\log_{10}$ cgeq/μl threshold (%)			
Day 0	12 (100.0%)	12 (100.0%)	
Day 1	11 (91.7%)	11 (91.7%)	
Day 2	11 (91.7%)	11 (91.7%)	1.00
Day 3	10 (83.3%)	8 (66.7%)	
Day 4	6 (50.0%)	4 (33.3%)	
Day 5	0/11[b] (0.0%)	4/11[b] (36.4%)	
Day 7	0/11[b] (0.0%)	2/11[b] (18.2%)	

(O + Z) means oseltamivir–zanamivir bitherapy, (O) means oseltamivir monotherapy.
At days 0–4 specimens consisted in nasal washes. At days 5 and 7 specimens were nasal swabs performed in order to control the negativation of viral excretion.
[a] Statistical analysis was performed considering the number of patients with viral load under the 3 $\log_{10}$ cgeq/μl threshold.
[b] Samples were missing for some patients at days 5 and 7 and percentages were calculated out of 11 patients.

3.3. Mean viral load decrease based on treatment group

At day 0, the mean viral load was between 6.4 and 6.6 $\log_{10}$ cgeq/μl according to the group. These mean viral loads decreased regularly in the two groups to reach between 2.6 and 2.8 $\log_{10}$ cgeq/μl after four days of treatment. The mean viral load decreased regularly and similarly between the treatment groups. The mean viral load decreased 0.96 $\log_{10}$ per day and 0.94 $\log_{10}$ per day in the (O + Z) and (O) groups respectively. There was no significant difference between the two groups (Table 4).

3.4. Resolution of symptoms of influenza-like illness during treatment

The median duration of illness before inclusion was of 24 h whatever the group of treatment. There was no significant difference in the median delay for symptom alleviation between groups, with 4 days in the (O + Z) group versus 5.5 days in the (O) group.

3.5. Adverse events

Treatment-related adverse events were reported for 3 and 1 patients in the (O + Z) and (O) groups, respectively. These adverse events were headaches, nausea, vomiting, and gastralgia in the (O + Z) group and nightmares in the (O) group.

Two severe adverse events were notified during the study but only one was judged to be oseltamivir related. It was a post prandial gastralgia successfully treated by lanzoprazole.

Table 3
Number of patients with a positive influenza virus culture according to treatment group and time of sampling.

Days of treatment	Treatment	
	(O + Z) (n = 9[a])	(O) (n = 7[a])
Number of patients with a positive influenza virus in cell culture (%)		
Day 0	9 (100.0%)	7 (100.0%)
Day 1	7 (77.8%)	5 (71.4%)
Day 2	4 (44.4%)	5 (71.4%)
Day 3	1 (11.1%)	4 (57.1%)
Day 4	0 (0.0%)	1 (14.3%)
Day 5	0 (0.0%)	0/6[b] (0.0%)
Day 7	0/8[b] (0.0%)	0/6[b] (0.0%)

(O + Z) means oseltamivir–zanamivir bitherapy, (O) means oseltamivir monotherapy.
[a] These results are for a subset of patients with a day 0 sample positive for influenza and with cell culture results available for all the samples received.
[b] Samples at days 5 and 7 were missing for some patients. We could suppose that these missing samples would have been negative as the viral excretion was already negative for these patients at least one day before.

3.6. Emergence of resistance to oseltamivir

We tested the presence of the H275Y NA mutation responsible for oseltamivir-resistance with an allele-specific rt RT-PCR in order to detect a possible emergence of oseltamivir-resistance. This test was performed on the nasal wash after 3 days of treatment (or 2 days of treatment when the detection of the M gene was negative at day 3) for all patients and on all samples for patients presenting a low viral load decrease. The mean Ct detecting the presence of an H in position 275 of the NA was 32.4 ± 5.44 and 34.6 ± 4.07 for samples after 3 days of treatment (or 2 days of treatment in three cases) from patients treated by (O) monotherapy or (O + Z) combination therapy respectively. With this approach we did not detect any H275Y NA mutation in the patients treated by (O + Z) combination therapy or (O) monotherapy.

4. Discussion

This clinical trial aimed to study the antiviral efficacy and tolerability of oseltamivir–zanamivir (O + Z) combination therapy compared to oseltamivir monotherapy (O) and was conducted during the A(H1N1)pdm09 virus pandemic in 2009–2010. To our knowledge, our study is the first one to evaluate the antiviral efficacy of this combination therapy *in vivo* on the A(H1N1)pdm09 virus.

We hypothesised that a combination of antiviral agents may lead to more rapid viral clearance and to reduction in the emergence of resistant viral strains. Our hypotheses were the extrapolations of previous studies' results (Hayden et al., 1999; Treanor et al., 2000) that are briefly described in material and methods section. We hypothesised from these studies that around 15%, 50% and 80% of patients should have a negative viral excretion after 24, 48 and 72 h of treatment by oseltamivir monotherapy. We then expected that 50% and 80% of the patients would be detected negative by influenza virus cell culture and would have a viral load under the threshold of 3 $\log_{10}$ cgeq/μl after 48 h of oseltamivir monotherapy and oseltamivir–zanamivir bitherapy respectively. The results we obtained after viral cell culture for a few samples are similar to those of previous studies albeit the low number of included patients prevents extensive interpretation. However, it is only after 3 days of treatment, and not 2 days as expected, that nearly 43% and 89% of patients had a negative viral excretion with oseltamivir monotherapy or oseltamivir–zanamivir bitherapy respectively.

If we consider the results of the M gene amplication, after 48 h of treatment, patients with a viral load under 3 $\log_{10}$ cgeq/μl were only 8.3% in both the (O + Z) and (O) groups respectively. In our study, the detection of viral excretion was performed by real-time RTqPCR. When samples were collected in patients presenting less than three days after symptom onset, viral culture and RT-PCR gave comparable results for the detection of A(H1N1)pdm09 virus (Cheng et al., 2010). In our hands, there was a probability of 80% that the A(H1N1)pdm09 viral culture was negative when the viral load estimated by M gene quantification was under the threshold of 3 $\log_{10}$ cgeq/μl. Previous studies estimated viral load by tissue culture-infective doses and discrepancies between tissue culture-infective doses and real time RTqPCR could explain differences between the hypotheses and the observed results. There is a large difference between the expected and observed percentages of patients with a viral load under the threshold of 3 $\log_{10}$ cgeq/μl.

Table 4
Mean viral load decrease from day 0 and day after day according to treatment group.

		Treatment		p Value
		(O + Z) (n = 12)	(O) (n = 12)	(O + Z) versus (O)
Mean viral load decrease (SD)				
Decrease from day 0[a]	Day 0–1	1.30 (0.98)	1.05 (0.70)	
	Day 0–2	2.58 (1.05)	2.16 (1.55)	
	Day 0–3	3.18 (1.23)	2.73 (2.12)	
	Day 0–4	3.79 (1.56)	3.76 (1.89)	
Decrease day after day[b]	Day 0–1	1.30 (0.98)	1.05 (0.70)	
	Day 1–2	1.28 (0.78)	1.11 (1.20)	
	Day 2–3	0.68[c] (0.51)	0.58 (1.03)	
	Day 3–4	0.59[c] (1.09)	1.02 (1.65)	
	Estimated slope	0.96 (0.12)	0.94 (0.12)	0.90

(O + Z) means oseltamivir–zanamivir bitherapy, (O) means oseltamivir monotherapy.
Viral load is expressed in (M gene adjusted to GAPDH) $\log_{10}$ cgeq/μl of nasal wash.
[a] Mean viral load decrease from day 0 was calculated by subtracting the viral load at day 1, 2, 3 or 4 to the viral load at day 0 for each patient in each group. Then a mean and a standard deviation (SD) was calculated for each day (1 to 4) and for each patient group.
[b] Mean viral load decrease day after day was calculated by subtracting the viral load at day 1, 2, 3, or 4 to the viral load of the day before. Then a mean and a SD was calculated for each day (day 0 to 1; day 1 to 2, etc.) and for each patient group.
[c] For one patient in the (O + Z) group the nasal wash received at day 3 had no GADPH gene detected and could not be interpreted. The calculation of mean viral load decrease between days 2 and 3 and between days 3 and 4 was then obtained considering 11 instead of 12 patients.

Because of this we think the evolution profile of influenza viral load and the occurrence of oseltamivir-resistance are better criteria for evaluating antiviral efficacy.

We could not detect any significant difference between treatment groups in the mean viral loads, the evolution of the viral loads, the delay for symptom alleviation and the occurrence of adverse events. The included population corresponds to the targeted population (age, sex, free of concomitant disease) as well as the symptom duration before treatment. However, the absence of significant difference could be due to the low number of patients included in our study that represents only 80% of the minimum expected number of patients (a minimum of 15 evaluable patients per group were expected).

Our study was conducted in healthy adults infected with influenza A(H1N1)pdm09 virus and we could not find any difference in treatment efficacy between the oseltamivir–zanamivir combination and oseltamivir alone. In the randomised placebo-controlled trial conducted in adults with 2008–2009 seasonal influenza A(H3N2) virus infection, the oseltamivir–zanamivir combination appeared less effective than oseltamivir monotherapy (Duval et al., 2010). In a study conducted *in vitro* on the A(H1N1)pdm09 virus, the combination of zanamivir and OC or peramivir showed additivity to moderate antagonism against influenza A(H1N1)pdm09 virus (Nguyen et al., 2010). Due to the low number of patients we were probably not able to perform analyses of viral load profiles powerful enough to detect small differences between treatment groups.

Our study was performed in healthy adults. However, the optimization of the antiviral treatment is important for critically ill patients. A study was conducted in Denmark in 21 patients admitted in intensive care unit (ICU), infected with influenza A(H1N1)pdm09 virus and treated with oseltamivir (150 mg twice a day) via a nasogastric tube and zanamivir (50 mg twice a day) administered in a soluble form for intravenous administration by a nebulizer in the inspiratory ventilator tube. The treatment was started more than 48 h after the first symptoms appeared and could explain a low efficiency of the antiviral treatment for these patients: 76% and 47,6% had a positive RT-PCR for A(H1N1)pdm09 at 7 and 10 days after the treatment start respectively. The mortality in these patients was high despite the combined antiviral treatment which could not prevent the development of acute lung injury (Petersen et al., 2011).

One major strength of our study is the close patient monitoring and virological analysis with nasal washes or swabs performed nearly every day for seven days and RTqPCR which allowed for a good estimation of viral decrease under treatment. We observed a regular decrease around 1.0 $\log_{10}$ cgeq/μl every day in both treatment group. When comparing our results with those of the previous trial conducted on A(H3N2), we observed a similar decrease of 2 $\log_{10}$ cgeq/μl in the viral load after two days of treatment despite some differences between trials, based on the type of neuraminidase and the duration of viral load follow-up (Duval et al., 2010).

We did not detect any H275Y NA mutation responsible for oseltamivir-resistance in both groups of treatment. The risk of oseltamivir-resistance emergence was low. This may be due to the fact that our population was adult and that influenza symptoms were mild. Oseltamivir-resistance emergence is preferentially observed in the context of prolonged viral excretion often associated with a paediatric population (Whitley et al., 2001; Kiso et al., 2004; Tamura et al., 2011), immunosuppression (Ison et al., 2006; Tramontana et al., 2010; Hill-Cawthorne et al., 2010), or severe cases (WHO, 2010; Petersen et al., 2011).

There are some limitations to our study. First, the low number of enrolled patients prevented us from detecting small differences between treatment groups. We decided to monitor patients closely for seven days to detect any possible oseltamivir-resistant influenza viruses. It was very difficult to include more patients with such a close follow-up. However, if combination therapy had a marked benefit in term of antiviral efficacy as expected in our first hypothesis, this could have been shown even with the few enrolled patients. We decided not to continue the study for a second flu season because we could not detect any significant difference and previous studies and *in vitro* data were unable to detect any advantage with the combinations therapies tested either. Secondly, our trial was conducted in adult outpatients that are at a low risk for oseltamivir-resistance. Close patient monitoring allowed us to detect possible oseltamivir-resistant viruses that occured late in the course of treatment. However, it was difficult to implement our clinical trial in children, immunocompromised patients or those with a severe presentation making them at a higher potential risk of developing oseltamivir-resistant variants.

Our study could not detect whether the combination therapy (O + Z) improves or reduces the effectiveness of oseltamivir in

clinical practice for the treatment of influenza A(H1N1)pdm09 virus infection in an out-patient setting.

Alternative antiviral treatments to oseltamivir monotherapy are still needed against influenza infections mostly in severely ill and/or immunocompromised patients (Petersen et al., 2011). The triple combination of oseltamivir carboxylate, amantadine, and ribavirin was shown to be synergistic *in vitro* for amantadine- or oseltamivir-resistant strains (Nguyen et al., 2010), was superior to double combinations and single agents at suppressing resistance emergence *in vitro* (Hoopes et al., 2011), and was synergistic for A(H5N1) and A(H1N1)pdm09 virus infection in mice (Nguyen et al., 2012). Clinical studies are in progress to identify the potential benefits of this triple combination in humans (Nguyen et al., 2012). New antiviral agents, like favipiravir or DAS181 are promising as new influenza antiviral agents with novel mechanisms of action (Boltz et al., 2010).

5. Conclusions

Due to the limited sample size we could not detect any therapeutic benefit in using oseltamivir–zanamivir bitherapy compared to oseltamivir monotherapy for ambulatory patients infected with influenza A(H1N1)pdm09 virus. Other studies are necessary to confirm this result and to improve the antiviral treatment of patients infected with influenza, especially for those who are immunocompromised and severely ill.

Role of the funding source

The funder had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report and in the decision to where to submit the paper for publication.

Disclosure statement

V.E. received a travel grant from Roche Pharmaceuticals to attend the Fourth European Scientific Working Group on Influenza (ESWI) Influenza Conference.

A.M. has membership in the ministry of health advisory board on influenza, was involved in epidemiological studies partially or fully granted by Roche and GSK and declares travel grants from Roche for participation in scientific meetings.

X.D. had a conference invitation from GSK and lecture fees from Roche and Gilead.

S.V.D.W. had a conference invitation and travel grants from GSK, research grant and joined patent from institution on unrelated subjects from GSK, member of the advisory committee on influenza of the French ministry of health, is a member of ESWI, is a member of the GEIG (Groupe d'Expertise et d'Information sur la Grippe) and is vice-president of the GROG network.

B.L. declares potential conflicts of interests with Roche, GSK, BMS, and BioCryst. B.L is a member of the scientific board of the GEIG and of the ESWI Board of Directors.

C.C., F.B., M.B.D., S.G., T.B., C.L., F.G. do not have any conflicts of interest to declare.

Acknowledgements

The authors thank all of the study participants and especially, the patients who contributed to the study.

Coordination centre: We are indebted to Frédéric Pugeat and Sandrine Thérond at the Clinical Investigation Centre of Lyon for study coordination. We thank Kent Neal (supported by the French Cochrane Center) for proofreading the manuscript.

Centre of virology: We are very grateful to Audrey Bonnetin and Reda Ihsani for their excellent technical assistance. We thank Martine Valette, Gwendolyne Burfin, Sylvaine Faure, and Rémi Laganier for their advice and technical assistance.

Clinical investigators: We thank the doctors who included patients (number of patients enrolled) in Lyon, France: Martine Kessous (9), Patrick Terrasse (7), Luc Hillion (6), Christophe Lablanche (4), Roland Cezanne Bert (3), Martine Chapdaniel (1), Delphine Alibert (1); Anthony, France: Philippe Berthier (3), Mathieu Tiphine (3), Jacques Bigotte (1), and Philippe Bossi (1).

Study nurses (in charge of nasal washes in patients' homes and taking samples to the virological laboratory): We thank Marion Caratini, Sabrina Soufi, Danièle Burlat, Joelle Levie, Christope Lenne, Marie-Laure Bourdier, and Ladame Frédéric.

Study sponsor: We are grateful to Valérie Plattner and Irène Zublena for clinical research management, Johanna Boulant and Amélie Amiot for study monitorying, and Delphine Bertram for pharmacovigilance.

Statistical analysis: We thank Alvine Bissery, René Ecochard, and Florent Boutitie at the *Service de Biostatistiques, Hospices Civils de Lyon et Laboratoire de Biostatistiques-Santé UMR 5558 CNRS Université de Lyon*.

Financial support: The study was funded by Roche (Michèle Pecking and Sylvie Dantin).

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