Oseltamivir, an Anti-influenza Virus Drug, Produces Hypothermia in Mice: Comparison Among Oseltamivir, Zanamivir and Diclofenac

Hideki ONO,* Yuko NAGANO, Noriaki MATSUNAMI, Shinichi SUGIYAMA, Shohei YAMAMOTO, and Mitsuo TANABE

Laboratory of CNS Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan.

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Oseltamivir phosphate (Tamiflu), an anti-influenza virus drug, is hydrolyzed by carboxylesterase to an active metabolite. The metabolite inhibits the influenza virus-specific neuraminidase. In this study, the effects of oseltamivir on normal core body temperature were studied in mice. Oseltamivir (30–300 mg/kg, intraperitoneally (i.p.) and 100–1000 mg/kg, orally (p.o.)) dose-dependently lowered the body temperature. The effects of oseltamivir (p.o.) continued longer than those of oseltamivir (i.p.), and approximately triple doses of oral oseltamivir were needed to produce the same peak effects as intraperitoneal oseltamivir. The non-steroidal anti-inflammatory drug diclofenac (1–30 mg/kg, i.p.) did not affect body temperature, and (at 30 and 60 mg/kg, s.c.) did not interact with the hypothermic effects of oseltamivir (100 mg/kg, i.p.). Zanamivir, which also inhibits neuraminidase, did not produce hypothermia at doses of 100 and 300 mg/kg, i.p. Clopidogrel (100, 300 mg/kg, i.p.), which is metabolized by the same carboxylesterase, tended to decrease the hypothermic effects of oseltamivir (100 mg/kg, i.p.). These results suggest that the hypothermic effects of oseltamivir are due to its hydrolytic metabolite, and that the hypothermia observed in mice has some relationship to the antipyretic effects and severe hypothermia (adverse event) observed in influenza patients after taking oseltamivir.

Key words oseltamivir; hypothermia; zanamivir; diclofenac; mouse

The anti-influenza drug oseltamivir (Ro64-0796) is hydrolyzed to the active metabolite oseltamivir calboxylate (Ro64-0802, OC) by human liver carboxylesterase (CES), and OC inhibits the influenza virus-specific neuraminidase.^{1–4)} Oral administration of the parent compound oseltamivir (OP) relieves the symptoms of influenza (cough, myalgia, nasal obstruction, sore throat, fatigue, headache, feverishness).⁵⁾ OP has strong antipyretic effects, which become apparent within 24 h after taking the drug.⁶⁾ In addition, an adverse event (unknown casual relationship)—hypothermia—after ingestion of OP has been reported. Forty-four cases of hypothermia had been reported to the MHLW (Ministry of Health, Labour and Welfare, Japan) up to March 20, 2007.⁷⁾ Data released by the Food and Drug Administration (FDA) have also described hypothermia to 34 °C in 2 cases.⁸⁾

In the present study, we investigated the effects of OP on normal core body temperature using mice, and found relatively strong hypothermic effects. Zanamivir, which is also a neuraminidase inhibitor and an anti-influenza virus drug, was compared with the effects of OP. Absorbed OP is hydrolyzed by human liver CES1 to the active metabolite OC.^{1,2)} The hydrolysis is strongly inhibited by the anti-platelet drug clopidogrel, which is metabolized by the same esterase CES1.⁹⁾ Therefore, clopidogrel was used to inhibit hydrolysis of OP in an attempt to study the involvement of OP or OC in hypothermia after OP administration.

As the antipyretic diclofenac carries a warning of possible severe hypothermia in children and the elderly with high fever in the package insert and an interview form,^{10,11)} the effects of diclofenac on body temperature and its interaction with OP were also studied.

MATERIALS AND METHODS

Animals One hundred forty-six male mice (ddY strain, SLC Shizuoka, Japan) were kept for at least 7 d under a 12/12 h light–dark cycle before experiments with full access to water and food, except those used for oral administration of OP, which were fasted for more than 15 h.

All experimental protocols used were approved by the Animal Care and Use Committee of Nagoya City University and were conducted in accordance with the guidelines of the Japanese Pharmacological Society.

Measurement of Rectal Temperature At 5 weeks of age, the mice were used to study the effects of drugs on body temperature in an experimental room for animal behavior, which was maintained at 23–25 °C. Each mouse was placed individually in a Plexiglas cage (19×12×11 cm (depth)), then removed every 10 min, held loosely in a small cloth bag, and the core body temperature was measured using a digital thermometer with a resolution of 0.1 °C (MT-132, Mother Tool, Ueda, Japan). The thermometer probe was inserted 25 mm into the rectum.¹²⁾ After each measurement, the mouse was returned to its cage. Mice whose rectal temperature before drug administration was below 37 °C were not used for experiments. Drugs were administered after the temperature became stable.

Drugs The drugs used were oseltamivir phosphate (Tamiflu capsule, Chugai Pharmaceutical Co., Tokyo, Japan), zanamivir hydrate (Relenza, dry powder inhaler, Glaxo-SmithKline Co., Tokyo, Japan), clopidogrel sulphate (Plavix tablet, Sanofi-Aventis Co., Tokyo, Japan) and diclofenac sodium (Sigma, St. Louis, MO, U.S.A.). A Tamiflu capsule (75 mg) contains 98.5 mg of oseltamivir phosphate and 64.5 mg of additives. The soluble additive is povidone, and the insoluble additives are pregelatinized starch, croscarmellose sodium, talc and stearyl fumarate sodium. The content of the

* To whom correspondence should be addressed. e-mail: hiono@phar.nagoya-cu.ac.jp

capsule was suspended in saline. Five hundreds milligrams of oseltamivir phosphate are dissolved in 1 ml of water,³⁾ and the maximal concentration of OP used for oral administration (1000 mg/kg) was 100 mg/ml. Thus, insoluble substances were considered to be additives, and not OP, and the suspension was injected after shaking. A Relenza blister (5 mg) contains zanamivir hydrate and lactose, and these were completely dissolved in saline. A Plavix tablet, which contains 75 mg of clopidogrel and additives, was made into an emulsion by grinding in a mortar and pestle containing saline. Diclofenac sodium was dissolved in saline. Doses of drugs were expressed as a free base and administered intraperitoneally (i.p.), subcutaneously (s.c.) or orally (p.o.) at 0.1 ml volume/10 g body weight.

Statistical Analysis Mean core body temperature before drug administration was $38.2 \pm 0.03^\circ\text{C}$ (S.E.M., $n=146$), the range was $37\text{--}39.3^\circ\text{C}$, and drug effects were expressed as the decrease in body temperature ($\Delta^\circ\text{C}$). All data were expressed as mean $\pm$ S.E.M. ($n=6$ or 8). Multiple t -test with Bonferroni correction following ANOVA was used for multiple comparison between control and treated groups.¹³⁾ Student's t -test or Welch's procedure was also applied to the same group because multiple comparison can overslip side effects (adverse reaction) (known casual relationship) of drugs.¹⁴⁾ Differences at $p<0.05$ (two-tailed) were considered to be significant.

RESULTS

Effects of OP on Core Body Temperature OP (30, 100, 300 mg/kg, i.p.) dose-dependently lowered the body temperature (Fig. 1). The peak effects were observed 10, 20 and 30–40 min after administration of 30, 100 and 300 mg/kg of OP, respectively. Variations in the effects of intraperitoneal OP were smaller than those of oral OP (Fig. 2), and there were many significant time points between the saline and OP groups (multiple t -test with Bonferroni correction) (Fig. 1). $AUC_{0-60\text{ min}}$ of hypothermia ($\Delta^\circ\text{C} \times \text{min}$) values were: -3.2 ± 3.6 ($n=6$) (saline), -20.7 ± 6.4 (30 mg/kg), -79.3 ± 5.7 (100 mg/kg) and -164.1 ± 15.9 (300 mg/kg).

Fig. 1. Oseltamivir (30–300 mg/kg, i.p.) Decreases Core Body Temperature in a Dose-Dependent Manner in Mice

Each point represents the mean $\pm$ S.E.M. of 6 mice. Ordinate: decrease in body temperature from the baseline (mean of $-30\text{--}0$ min). Abscissa: time in minutes after administration of the drug. Significance of differences between control and test values was determined by the two-tailed multiple t -test with Bonferroni correction following one-way analysis of variance (3 comparisons in 4 groups). $*p<0.05$ and $**p<0.01$. OP, oseltamivir.

Significant differences in effects were observed between saline and the 100 mg ($p<0.05$) and 300 mg/kg ($p<0.01$) groups (multiple t -test with Bonferroni correction).

Oral administration of OP (100, 300, 1000 mg/kg) also lowered the core body temperature in a dose-dependent manner (Fig. 2). Saline lowered the body temperature, but significant hypothermia was observed at doses of 300 and 1000 mg/kg, p.o. (Fig. 2, multiple t -test with Bonferroni correction). When non-corrected Student's t -test was employed, the effect of 100 mg/kg OP was statistically significant at some time points (Fig. 2). The peak effects were observed at 30–60 min after administration, and recovery was not evident at 2 h after administration. $AUC_{0-120\text{ min}}$ of hypothermia ($\Delta^\circ\text{C} \times \text{min}$) values were: -93.9 ± 10.9 ($n=8$) (saline), -149.5 ± 27.9 (100 mg/kg), -219.2 ± 51.3 (300 mg/kg) and -300.6 ± 39.0 (1000 mg/kg). Significant effects were observed between saline and the 300 ($p<0.05$) and 1000 mg/kg ($p<0.01$) groups (multiple t -test with Bonferroni correction). Non-corrected Student's t -test showed the same degrees of significance as those from the multiple t -test. When compared by peak effects, approximately triple doses of oral oseltamivir were needed to produce the same peak effects as intraperitoneal oseltamivir.

Fig. 2. Oseltamivir (100–1000 mg/kg, p.o.) Decreases Core Body Temperature in a Dose-Dependent Manner in Mice

Each point represents the mean $\pm$ S.E.M. of 8 mice. Ordinate: decrease in body temperature from the baseline (mean of $-30\text{--}0$ min). Abscissa: time in minutes after administration of the drug. $*p<0.05$ and $**p<0.01$ (multiple t -test between control and test values). $\#p<0.05$ (non-corrected Student's t -test was applied to those groups (see Materials and Methods)). OP, oseltamivir.

Fig. 3. Zanamivir (100, 300 mg/kg, i.p.) Does Not Alter Core Body Temperature in Mice

Each point represents the mean $\pm$ S.E.M. of 6 mice. Ordinate: decrease in body temperature from the baseline (mean of $-30\text{--}0$ min). Abscissa: time in minutes after administration of the drug. No significant differences were seen by multiple t -test or Student's t -test. Zana, zanamivir.

Effects of Zanamivir on Core Body Temperature
Zanamivir (100, 300 mg/kg, i.p.) slightly lowered the core body temperature (Fig. 3). No statistical significance was observed (multiple *t*-test with Bonferroni correction) and Student's *t*-test).

Effects of Clopidogrel on Hypothermic Effects of OP

Fig. 4. Effects of Clopidogrel (100, 300 mg/kg, s.c.) on Hypothermia Induced by Oseltamivir (100 mg/kg, i.p.)

Each point represents the mean $\pm$ S.E.M. of 6 mice. Ordinate: decrease in body temperature from the baseline (mean of -40 – 0 min). Abscissa: time in minutes after administration of the oseltamivir. Clopidogrel was administered at the point shown by the upward arrow (-15 min). No significant differences were seen by multiple *t*-test or Student's *t*-test. OP, oseltamivir, Clo, clopidogrel.

Fig. 5. Effects of Diclofenac on Core Body Temperature and the Interaction with Oseltamivir

Diclofenac (1 – 30 mg/kg, i.p.) does not alter core body temperature in mice (A). Diclofenac (30 , 60 mg/kg, s.c.) does not affect the hypothermic effects of oseltamivir (100 mg/kg, i.p.) (B). Each point represents the mean $\pm$ S.E.M. of 6 mice. Ordinates: decrease in body temperature from the baseline (mean of -30 – 0 min (A) and -40 – 0 min (B)). Abscissae: time in minutes after the administration of oseltamivir. Diclofenac was administered at the point shown by the upward arrow (-15 min). #*p* < 0.05 (non-corrected Student's *t*-test). Dic, diclofenac.

Since the hydrolysis of OP is strongly inhibited by clopidogrel,⁹⁾ clopidogrel was used to inhibit the hydrolysis of OP. Clopidogrel (300 mg/kg, s.c.), which alone slightly lowered body temperature, only tended to reduce the hypothermic effects of OP (Fig. 4).

Effects of Diclofenac on Core Body Temperature and Its Interaction with OP Diclofenac failed to produce hypothermia at doses of 1 – 30 mg/kg, i.p. (Fig. 5A). Since non-steroidal anti-inflammatory drugs (NSAIDs) are sometimes prescribed with OP for influenza infection in Japan, the drug interaction between OP and the strong NSAID diclofenac was studied. Diclofenac (30 , 60 mg/kg, s.c.) administered 15 min before OP did not affect the latter's hypothermic effects (100 mg/kg, i.p.) (Fig. 5B).

DISCUSSION

Tamiflu interview form and New Drug Application (NDA) data summary describe that OP at 7.6 , 76.1 and 761 mg/kg, *p.o.* does not affect body temperature in adult rats,^{3,15)} whereas at 533 mg/kg, *p.o.* it lowered the body temperature of rats aged 7 or 14 d.¹⁶⁾ A recent study by Izumi *et al.*¹⁷⁾ has provided supplementary data indicating that OP at 50 mg/kg, i.p. significantly augmented the hypothermic effects of ethanol in 30 -d-old rats. Here, we have demonstrated that OP alone generates potent hypothermic effects in mice, consistent with our preliminary study obtained using adult rats (data not shown).

Brain/plasma C_{max} ratios for OP and OC in mice administered OP at 10 mg/kg, *p.o.* were 0.42 and 0.22 , respectively.¹⁸⁾ Thus, it is considered that both OP and OC penetrated the blood–brain barrier in the present study using a high dose of OP. Body temperature is usually regulated by opposing controls of heat production and heat loss. The preoptic anterior hypothalamus (POAH) is a thermoregulation center.¹⁹⁾ The organum vasculosum laminae terminalis (OVLT), part of the circumventricular organs (CVO), is located near the POAH and is the target site of endogenous pyrogens.²⁰⁾ In addition, the CVO lacks the blood–brain barrier.^{21,22)} These circumstances suggest that OP or OC can affect body temperature regardless of whether or not the target site of either drug is located within the blood–brain barrier.

OP (30 – 300 mg/kg, i.p. and 100 – 1000 mg/kg, *p.o.*) dose-dependently lowered the normal core body temperature in mice (Figs. 1, 2). The Tamiflu NDA data summary describes very rapid hydrolysis of OP in mice,²³⁾ and the present study indicated that the hypothermic effects of OP were relatively sustained (Figs. 1, 2). Therefore, it is suggested that the active compound that lowered body temperature was metabolized OC, and not the parent compound OP. However, a further study using OC powder will be required before a final conclusion can be reached. Tamiflu capsule contains insoluble additives. In our preliminary study, a filtrate of Tamiflu suspension lowered the body temperature (data not shown). As the soluble additive povidone is a very high-polymer compound (molecular weight several tens of thousands) and its molar dose included in a Tamiflu capsule is very low, it seems unlikely that additives other than OP or OC lowered the body temperature. Oral saline (10 ml/kg) administration unexpectedly produced hypothermia (Fig. 2). In the preliminary study ($n=6$ or 7), oral water administration to fasted

mice and oral saline administration to nonfasted mice lowered the body temperature by about 1 °C. In addition, insertion alone of an oral probe to the stomach *via* esophagus produced mild hypothermia (0.5 °C). Although pyrogenic messages *via* peripheral (largely vagal) afferent nerves activated by the cytokines induces hyperthermic response by direct afferent transmission to the POAH,²⁴ evidences of reflex hypothermia by mechanical vagal afferent stimuli could not be found in literatures. Nevertheless, these findings suggest that mechanical messages from esophagus and stomach *via* vagal afferents reflexly lower body temperature.

Zanamivir as well as OC inhibit the influenza virus-specific neuraminidase and also the release of virus from host cells.²⁵ As the hydrolyzed compound zanamivir cannot be absorbed by the gastrointestinal tract, a fine powder of zanamivir is inhaled (10 mg, twice a day) when used for treatment of humans. After inhalation of zanamivir powder, distribution of the drug is restricted to an upper respiratory tract, a target site of influenza virus. In order to elevate its plasma concentration, the same doses of zanamivir (100, 300 mg/kg) as OP were intraperitoneally administered. Zanamivir (100, 300 mg/kg, i.p.) did not produce hypothermia (Fig. 3). From these results, it is considered that hypothermia after OP administration is not due to neuraminidase inhibition at a thermoregulation center or a peripheral organ that is involved in thermoregulation.

CESs are classified into 5 subfamilies (CES1—CES5), and CES1 is subclassified into CES1A—CES1H.²⁶ The CESs that hydrolyze OP are suggested to be the CES1A and CES1B isozymes in human liver and mouse plasma, respectively.^{4,26,27} Clopidogrel, a substrate of CES1, inhibits the hydrolysis of OP to OC *in vitro*: hydrolysis of OP (50 μ M) is inhibited by 5 and 50 μ M clopidogrel to 50% and 10%, respectively, in human CES1-expressing cells.⁹ *In vivo*, a large proportion of clopidogrel is rapidly metabolized by CES to the non-active metabolite SR26334 in humans,^{28,29} and $T_{\max}$ of the hydrolyzed metabolite SR26334 after oral administration of clopidogrel (75 mg) is 1.9 h in humans.^{29,30} $T_{\max}$ of OP administered orally at a dose of 75 mg is 1 h, and $T_{\max}$ of its metabolite OC is 4 h,^{3,31} suggesting that clopidogrel competitively inhibits the hydrolysis of OP to OC in humans. Clopidogrel 300 mg/kg, s.c., which alone slightly lowered body temperature and did not affect behavior of mice, tended to inhibit the hypothermic effects of OP, although not to a significant degree (Fig. 4). Conversion of OP to OC is very rapid in mouse plasma: the concentration of OC after oral OP administration in mice attains a near $C_{\max}$ value within 15 min after administration²³ and high amounts of CES are present in mouse plasma.²⁷ These findings support the negative non-significant interaction of clopidogrel with OP in the present *in vivo* mouse study. The tendency for clopidogrel to exert inhibitory effects on OP-induced hypothermia (Fig. 4) and the prolonged effect of OP on temperature (Figs. 1, 2) suggest that hypothermia is induced by OC, but hypothermic effects induced by OP also cannot be ruled out. In fact, it has been demonstrated that both OP and OC facilitate neuronal firing in hippocampal slices, OC being 30 times more potent than OP in this respect.¹⁷

Diclofenac, a strong NSAID, is used for treating high fever. Although diclofenac does not lower the normal body temperature in animals, it effectively reduces fever due to py-

rogens; the ED₅₀ is 0.13 mg/kg, *p.o.* in rats.³² Voltaren package insert and interview form give a warning of severe hypothermia if used in children and the elderly with high fever.^{10,11} In this study, very high doses of diclofenac (1—30 mg/kg, i.p.) did not decrease the body temperature (Fig. 5A) and at 30 and 60 mg/kg, s.c. it did not interact with the hypothermic effects of OP (100 mg/kg, i.p.) (Fig. 5B). Thus, no drug interaction between diclofenac and OP was evident, at least in terms of normal body temperature. However, as diclofenac can be used in patients with high fever, further studies using pyrexia mice are needed to investigate drug interaction between diclofenac and OP.

In the present study, intraperitoneal and oral administration of OP induced dose-dependent hypothermic effects in normal mice. However, since recent clinical studies have shown that the antipyretic effect of OP on type A influenza is stronger than that on type B influenza,^{33,34} the antipyretic effect of OP is considered to be due to not only direct pharmacological effects on thermoregulation, but also anti-influenza virus activity.

Severe hypothermia as an adverse event has been reported to the MHLW from manufacturer of OP and also from medical institutions.⁷ The proportion of hypothermia cases in Japanese patients below 10 years old relative to all reported cases is 40.1% (18/44 cases), and this ratio is higher than those for other adverse reactions (*i.e.* 16.7% for anaphylaxis (6/36 cases)), based on initial data made available to the public by the MHLW.⁷ Since the body weight of children below 10 years old is low, it is considered that more severe hypothermia may occur in comparison with that in the elderly when heat production decreases or heat loss increases after OP ingestion. Thus, it is possible that the hypothermic effects observed in mice are related to the severe hypothermia in humans after OP ingestion. Further studies are needed to elucidate the mechanisms of hypothermia in mice and their relationship to the adverse events reported in humans.

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