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Title Page

Title: Role of the Intestinal Peptide Transporter PEPT1 in Oseltamivir Absorption: *In Vitro* and *In Vivo* Studies

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Running Title Page

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Abbreviations: ACE, angiotensin-converting enzyme inhibitors; CES1, liver carboxylesterase; CHO cells, Chinese Hamster Ovary cells; Gly-Sar, glycyl-sarcosine; OAT, organic anion transporter; PEPT1, peptide transporter 1; Trp-Gly, tryptophan-glycine; Tyr-Phe, tyrosyl-phenylalanine; Val-Ala, valine-alanine

Abstract

It was recently reported that oseltamivir (Tamiflu®) absorption was mediated by hPEPT1. Understanding the exact mechanism(s) of absorption is important in the context of drug-drug and diet-drug interactions. Hence we investigated the mechanism governing the intestinal absorption of oseltamivir and its active metabolite (oseltamivir carboxylate) in wild type (CHO-K1) and human PEPT1-transfected cells (CHO-hPEPT1), in pharmacokinetic studies in juvenile and adult rats, and in healthy volunteers. *In vitro* cell culture studies showed that the intracellular accumulation of oseltamivir and its carboxylate into CHO-hPEPT1 and CHO-K1 was always similar, under a variety of experimental conditions, demonstrating that these compounds are not substrates of hPEPT1. Furthermore, neither oseltamivir nor its active metabolite was capable of inhibiting glycyl-sarcosine (Gly-Sar) uptake in CHO-PEPT1 cells. *In vivo* pharmacokinetic studies in juvenile and adult rats showed that the disposition of oseltamivir and oseltamivir carboxylate, following oral administration of oseltamivir, was sensitive to the feed status but insensitive to the presence of milk and Gly-Sar. Moreover, oseltamivir and oseltamivir carboxylate exhibited significantly higher exposure in rats under fasted conditions than under fed conditions. In humans, oral dosing following a high fat meal resulted in a statistically significant, but moderate, lower exposure than after an overnight fasting. This change has no clinical implications. Taken together, the results do not implicate either rat or human PEPT1 in the oral absorption of oseltamivir.

Introduction:

Neuraminidase inhibitors such as oseltamivir prevent viral replication by blocking the exit of the influenza virus from the host cell and are therefore active against all strains of influenza A and B. A number of studies demonstrated the effectiveness of neuraminidase inhibitors in preventing influenza in healthy volunteers when administered in a prophylactic manner (Moscona, 2005). Oseltamivir (Tamiflu®) is an orally available ester-prodrug of its active moiety RO0640802 (oseltamivir carboxylate) (He et al., 1999b; Hoffmann et al., 2009). Following an oral dose, oseltamivir is readily absorbed and converted by the liver carboxylesterase CES1 to its active carboxylate, which is detectable within 30 min in plasma with peak levels after 3 to 5 hours (He et al., 1999b; Hill et al., 2002). The carboxylate is primarily excreted by passive glomerular filtration and active secretion into the urine possibly via the human organic anion transporters 1 and 3 (hOAT1, *SLC22A6* and hOAT3, *SLC22A8*) (He et al., 1999b; Hill et al., 2002; Ose et al., 2009).

The human peptide transporter 1 (hPEPT1, *SLC15A1*) is a high capacity, low affinity proton-coupled cotransporter expressed on the apical membranes of enterocytes located on the microvilli in the small intestine, and to a lesser degree on epithelial cells in the kidney proximal tubule (Liang et al., 1995). hPEPT1 has been implicated in the absorption of aminocephalosporins, angiotensin-converting enzyme inhibitors (ACE), β-lactam antibiotics, amino acid-conjugated antiviral drugs, L-dopa, and tri and di-peptides such as Gly-Sar (Brandsch, 2009). Several clinically relevant hPEPT1 inhibitors such as sulfonylurea antidiabetic drugs, nateglinide, glibenclamide, tolbutamide, chlorpropamide, sartans and ester prodrugs of ACE inhibitors have been identified (Faria et al., 2004; Knutter et al., 2008; Knutter et al., 2009). Di-peptides such as Gly-Sar and Val-Ala have demonstrated inhibitory potential, therefore di- and tri-peptides resulting from the digestion of milk proteins may also

inhibit hPEPT1-mediated drug absorption, causing reduced exposure of the victim drug (Fujisawa et al., 2006).

The *in vitro* transport characteristics of oseltamivir were recently examined in Caco-2 and hPEPT1 transfected HeLa cells (Ogihara et al., 2009). In these studies a significant reduction of oseltamivir uptake at 4°C and in the presence of Gly-Sar and Trp-Gly was observed. Additionally, the *in vivo* disposition of oseltamivir in rats following the oral administration of oseltamivir in the absence or presence of cow milk, casein and Gly-Sar (20 and 125 mM) was determined. Oseltamivir exposure was reduced in the presence of milk, casein and Gly-Sar compared to water. Furthermore, studies were conducted in juvenile rats (1 wk old) under fasted and breast-fed conditions and oseltamivir plasma and brain concentrations were measured at a single time point (30 min) after an oral dose. Oseltamivir plasma levels in nursed animals were significantly lower than in fasted animals (Ogihara et al., 2009). From these studies, the authors concluded that PEPT1 is involved in the absorption of oseltamivir and that this process can be reduced by milk proteins. Consequently, oseltamivir may be subject to drug and/or dietary interactions with significant implications for its disposition.

More recently, the same group reported that milk consumption affects the absorption rate of oseltamivir but not the total exposure in healthy volunteers (Morimoto et al., 2011). In previous studies, He and coworkers demonstrated that co-administration of amoxicillin, a hPEPT1 substrate (Herrera-Ruiz and Knipp, 2003), had no effect on the disposition of oseltamivir carboxylate in healthy volunteers (He et al., 1999b). Furthermore, only the pro-drug oseltamivir was studied by Ogihara and coworkers (2009) in the pharmacokinetic rat study, in which the plasma concentration-time profile of the active metabolite was not reported.

The present *in vitro* and *in vivo* studies were conducted to investigate the discrepancy with the amoxicillin drug/drug interaction clinical study and to follow oseltamivir carboxylate

exposure in rat in the presence and absence of PEPT1 inhibitors. One goal of these studies was to clarify whether oseltamivir is indeed a hPEPT1 substrate and/or inhibitor. For that reason, the intracellular accumulation of oseltamivir and its active metabolite (oseltamivir carboxylate) was tested in CHO cells (parent and hPEPT1 transfected) along with the effects of temperature (4°C), different pH values (from 4-8) and known hPEPT1 inhibitors (Gly-Sar, Trp-Gly, valacyclovir and cefadroxil). Pharmacokinetic studies in juvenile and adult rats were conducted to look into food effect (rat chow and milk) and interaction with Gly-Sar. The results of a bioequivalence study in healthy volunteers comparing fasting with a high-fat meal is discussed with regards to the results published by Morimoto and coworkers (2011). We present strong evidence that oseltamivir and its active metabolite are neither hPEPT1/rPept1 substrates nor inhibitors.

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Materials & Methods

Materials

Oseltamivir (Tamiflu®), RO0640802 (oseltamivir carboxylate) and their ^{14}C-labeled analogs were obtained from the department of Medicinal Chemistry (F.Hoffmann-La Roche, Basel, Switzerland), and Gly-Sar, Trp-Gly, Tyr-Phe, and cefadroxil were from Sigma (St. Louis, MO). [^{3}H]Gly-Sar was acquired from ARC (American Radiolabeled Chemicals, St. Louis, MO). Valacyclovir was from Kemprotec (Middlesbrough, UK) and valsartan from Apin Chemicals (Oxfordshire, UK). Pasteurized whole fat bovine milk (3.5% fat; "milk") was purchased from a local grocery store (Migros, Switzerland). 2,2-dichlorovinyl dimethyl phosphate (dichlorvos) was obtained from Riedel de Haen (Germany). Polypropylene tubes containing K_2EDTA were purchased from Milian AG (Basel, Switzerland). Acetonitrile was from Merck Chemicals (Zug, Switzerland). BSA was from Sigma-Aldrich (Buchs, Switzerland), PBS and Triton X-100 from USB (Cleveland, OH), and scintillation fluid from Perkin Elmer (Schwerzenbach, Switzerland).

Cell cultures

CHO cells (Chinese hamster ovary cells), such as CHO-K1 (control) and those transfected with human peptide transporter 1 (hPEPT1, *SLC15A1*) (CHO-PEPT1) or human peptide transporter 2 (hPEPT2, *SLC15A2*) (CHO-PEPT2) were obtained from Solvo Biotechnology (Budaörs, Hungary). The cells were maintained in HAM's F-12K medium (Gibco, Switzerland), containing 10% FCS (Sigma) and supplemented with 1:200 of penicillin-streptomycin solution (10 000 IU/µg/mL; Sigma), at 37°C in a humidified atmosphere with 5% CO_2 in tissue culture flasks (Falcon, Franklin Lakes, NJ).

Cells were plated in 24-well plates (Falcon) for *in vitro* cell-based studies. The incubation medium consisted of Henseleit-Krebs buffer (HK buffer: KCl 4.83 mM, KH_2PO_4 0.96 mM,

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$NaHCO_3$ 23.8 mM, NaCl 142 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.53 mM, MES 12.5 mM, D-Glucose 5 mM) adjusted to pH 5 or 6 after the addition of substrate and inhibitor.

***In vitro* cell-based studies**

The intracellular accumulation of the model hPEPT1 substrate [^{3}H]Gly-Sar, [^{14}C]oseltamivir and [^{14}C]RO0640802 were evaluated under various conditions such as concentration, temperature (4° and 37°C), pH, and the absence or presence of hPEPT inhibitors using the transfected cell lines CHO-PEPT1 and CHO-PEPT2, and compared to results obtained from CHO-K1 (control) cells. The experimental details have been described elsewhere (Poirier et al. 2008). Briefly, prior to initiating the experiment, cell culture medium was removed followed by a single (1 mL) wash step with HK buffer (37°C) adjusted to the corresponding pH. *In vitro* studies were initiated by replacing (aspiration) the wash buffer with 150 µL of incubation medium (temperature set according to incubation) containing the compound under investigation in the absence or presence of indicated inhibitors. After addition of the substrate ± inhibitor in the HK buffer, the pH was re-adjusted to the desired value. Plates were placed on a 37°C heating block (Eppendorf) or on ice (4°C incubation). At pre-determined time points (30 s to 15 min) plates were removed and 1 mL ice-cold PBS containing 0.2% BSA (PBS-BSA) was added to quench uptake. BSA was added to minimize non-specific binding to plastic ware. This step was followed by two wash steps (2 mL each) with PBS-BSA (37°C) followed by a third wash step (3 mL) with PBS (37°C) alone to remove BSA. Cells were solubilized with 300 µL 1% Triton X-100 for 15 min at 60°C on a shaking heating block. Two hundred microliter of the cell lysate was added to 4 mL of scintillation fluid and radioactivity was measured. Protein content was determined utilizing the BCA Protein Assay Kit with albumin as the standard (Pierce, Rockford, IL).

The absence of ester hydrolysis of [^{14}C]oseltamivir to carboxylate during the incubation was confirmed by HPLC coupled with radiodetection.

In vitro studies were performed three times on different days with each data point being run in triplicate. For each experiment, Gly-Sar was incubated as a positive control.

Animals

Adult (7-8 wk old) male Sprague-Dawley rats (243–283 g each) and newborn (male and female) Sprague-Dawley pups together with their dams were obtained from Charles River Laboratories (L'Arbresle, France) and housed under a controlled environment (temperature, humidity and 12 h light/dark cycle) with access to food and water ad libitum. For the pharmacokinetic studies in adult animals, the rats were pretreated with buprenorphine (0.05 mg/kg sc) and the jugular vein was cannulated under ketamine/xylazine anesthesia (90 mg/kg ketamine and 10 mg/kg xylazine 2% diluted in 0.9% aqueous NaCl, intraperitonally). A polyethylene catheter (Portex) with a silicon tip (AMT Aromando Medizintechnik, Dusseldorf, Germany) was used for the collection of serial blood samples. Animals received meloxicam (3.0 mg/kg sc at 2, 24, and 48 h post-surgery) and were used in the pharmacokinetic studies after a recovery period of three days. Juvenile animals (7 d old, 9.7–18.9 g each) were treated orally as described below and blood was collected by cardiac puncture under deep isoflurane anesthesia. All rodent studies were conducted in strict adherence to the Swiss federal regulations on animal protection and the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), along with the explicit approval of the local veterinary authority.

Pharmacokinetic studies in adult rats

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The effect of bovine milk or Gly-Sar (125 mM) on oseltamivir and oseltamivir carboxylate maximum plasma concentration (C_{max}), time to maximum concentration (t_{max}) and area under the concentration-time profile (AUC) was evaluated in both fasted and fed adult animals. Fasting was initiated at night, 6 h prior to dosing and continued for the duration of the study (6 h). Three animals per group received a single oral dose of 30 mg/kg oseltamivir in 10 mL/kg of the three different vehicles as follows: *group 1*, compound in aqueous solution; *group 2*, compound dissolved in pasteurized whole bovine milk; *group 3*, compound in aqueous solution containing 125 mM Gly-Sar. Serial blood samples (250 µL each) were taken from the jugular vein at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, and 6 h post dosing for the fasted condition and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 24 h post dosing for the fed condition. The blood samples were collected with a syringe and added into K_2EDTA coated polypropylene tubes, containing 2.5 µl (1% v/v) of the esterase inhibitor dichlorvos 0.7% in acetonitrile, and placed on ice to prevent ester cleavage into oseltamivir carboxylate. Plasma was prepared within 30 min by centrifugation at 3000×g for 5 min at 4°C and then frozen immediately to -20°C.

Pharmacokinetic studies in juvenile rats

The influence of breast-feeding, milk and Gly-Sar on oseltamivir and oseltamivir carboxylate pharmacokinetics was evaluated in 7-day old rats. Animals (15 per group, males and females) were dosed with 30 mg/kg oseltamivir by oral gavage (10 mL/kg) under the following conditions: *group 1*, ad libitum access to mother's milk (breast-fed animals); *groups 2-4*, fasted from 8 h pre-dose and for the study duration (5 h). The compound was given as aqueous solution (*groups 1* and *2*), as solution in milk (*group 3*), or as aqueous solution containing 125 mM Gly-Sar (*group 4*). Blood samples (300 µL each) were collected at 0.25, 0.5, 1, 2, and 5 h post dosing and processed as described above for the adult rats.

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Compound analysis in rat plasma

Plasma concentrations of oseltamivir and its carboxylate were determined using a validated high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) method (Heinig and Bucheli, 2008). A column-switching system was employed, consisting of an autosampler, pumps for on-line solid-phase extraction and analytical gradient separation (Shimadzu, Kyoto, Japan) and a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA) with electrospray ionization in the positive mode. As internal standards, three-fold deuterated oseltamivir and carboxylate were used. Multiple reaction monitoring was performed at m/z 313→166 for oseltamivir, m/z 316→167 for its internal standard, m/z 285→138 for oseltamivir carboxylate and at m/z 288→139 for its internal standard. Linear regression with $1/x^2$ weighting was applied, and the range of quantitation was between 0.1 and 500 μg/L.

Rat pharmacokinetic analysis

Pharmacokinetic parameters were estimated by non-compartmental analysis with the software program ToxKin™ (Entimo AG, Berlin, Germany). The concentration at time zero following oral administration (C_0) was set equal to 0. C_{max} and t_{max} were determined graphically from the plasma concentration-time profiles. $AUC_{0\text{-}tlast}$ was calculated by linear trapezoidal rule from time zero to the last time post-dose. $AUC_{0\text{-}\infty}$ was calculated by linear trapezoidal rule and extrapolated to infinity using the apparent terminal elimination rate λz. λz was obtained by log-linear regression of the terminal phase of the plasma concentration-time curve.

Human Pharmacokinetic Study

The effect of food on the pharmacokinetics of oseltamivir and oseltamivir carboxylate was evaluated during an open-label food effect study using the market formulation of oseltamivir in healthy subjects. Details of the study have been described previously (He et al., 1999a; He et al., 1999b). Briefly, 18 healthy volunteers (9M, 9F, mean age 26 years) received an oral dose of oseltamivir 150 mg fasting (from midnight) and after a high-fat, high-calorie breakfast (FDA guideline). This meal included eggs, bacon, toast with butter, hash brown potatoes and whole milk. Treatments were separated by a 7–10 days washout period. Plasma and urine samples (0–72 h) were analyzed for oseltamivir and oseltamivir carboxylate by HPLC/MS/MS. The primary statistical objective was to compare the plasma AUC and C_{max} (log transformed) of oseltamivir and oseltamivir carboxylate under fasted *vs* fed status. Sample size was determined *a priori* to ensure a balanced overall crossover study design and 80% power for having the confidence interval on the metabolite AUC and C_{max} fall completely between 80% and 125% if any of the treatments truly differed by 10% or less. Analyses of variance appropriate for this design was performed including terms for sequence, subjects within sequence, period and regimen. Analysis was done on both the raw and natural logarithm transformed scale for AUC parameters and C_{max}. Confidence intervals (90%) for the difference in computed parameter least square means were calculated and expressed as a percentage of the reference. The analysis of variance was performed using SAS software (version 6.12). Microsoft Excel (version 4 or equivalent) was used to calculate the pharmacokinetic parameters.

This study was conducted in full compliance with the principles of the 'Declaration of Helsinki' or with the laws and regulations of the country in which the research was conducted, whichever afforded the greater protection to the individual. All subjects gave written informed consent prior to enrolment in the study after adequate explanation of the aims, methods, anticipated benefits and potential hazards of the study.

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Statistical Analysis

The *in vitro* experiments were analyzed with hierarchical models, where the technical replicates were nested within days. The sample size (n) was the number of independent replications of the experiment (between 3 and 6), which were conducted on different days, with typically three technical replicates per experimental condition per day (Cumming et al., 2007). No corrections for multiple testing were performed.

A power analysis was performed to estimate the number of rats needed to obtain significant results (using α=0.05) for various effect sizes. Based on the $AUC_{0\text{-}6h}$ values for the distilled water and milk groups from Table 1 of Ogihara et al (2009), n=3 rats per group gave sufficient power (greater than 99%) to detect a difference between the means of the two groups as large as observed in the Ogihara study. If the difference between the means would be 50% smaller than the Ogihara study (but assuming that the variance in the data is the same), power would still be 98% with n=3 rats per group.

PK parameters from the studies in adult rats were analyzed with a one-way ANOVA (water *vs* milk *vs* Gly-Sar). In case of a significant overall effect, Tukey HSD post-hoc tests were used for pair-wise comparisons, with the main comparisons of interest being milk *vs* water and Gly-Sar *vs* water. The same parameters were also analyzed with a two-way ANOVA, with status (fed *vs* fasted) and group (milk *vs* Gly-Sar *vs* no milk/no Gly-Sar) as the two variables. The juvenile rats only provided a sample at one time point, and therefore PK parameters could not be estimated for each animal. Nevertheless, differences in $AUC_{0\text{-}5h}$ between groups could be estimated. It was performed with the PK package (http://cran.r-project.org/web/packages/PK) for R (www.r-project.org). The main comparisons of interest were fasted milk (group 3) and fasted Gly-Sar (group 4) *vs* fasted water (group 2), and fasted water *vs* fed water (group 1). Corrections for multiple testing were done with Holm's method.

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For all analyses, oseltamivir and oseltamivir carboxylate data were analyzed separately.

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Results

Effect of hPEPT1 expression in CHO cells on Gly-Sar, oseltamivir and oseltamivir carboxylate intracellular accumulation

To evaluate the functionality of the cell system, active uptake of [^{3}H]Gly-Sar was measured. In the control cells (CHO-K1), after a 10 min incubation at pH 6, [^{3}H]Gly-Sar was barely detectable (1.1 ± 0.1 pmol/mg protein) in the cell lysates. In contrast, in CHO-PEPT1 cells, intracellular [^{3}H]Gly-Sar level reached 182 ± 31 pmol/mg protein ($p < 0.0001$, Figure 1). Tested from 30 s to 15 min, [^{3}H]Gly-Sar intracellular concentration continuously increased over time in CHO-PEPT1 cells but not in CHO-K1 cells (data not shown). These results are consistent with a hPEPT1 mediated uptake of Gly-Sar in CHO-PEPT1 cells (Fujisawa et al., 2006). Using the same conditions, no difference in intracellular accumulation was observed between parental and transfected cells for oseltamivir or its carboxylate at 5 or 100 µM (Figure 1). Different experimental conditions such as pH (from 5 to 6), incubation time (from 30 s to 15 min) and buffers (HK and HBSS) were tested *in vitro* (data not shown); under all conditions, no statistically significant difference was observed between CHO-PEPT1 and CHO-K1 cells for oseltamivir or oseltamivir carboxylate. These observations suggest that oseltamivir and oseltamivir carboxylate permeation into CHO cells is independent of hPEPT1. hPEPT2 substrate properties were also tested in parallel using the same conditions (data not shown) and similarly no active transport of oseltamivir or its carboxylate could be observed.

Effect of extracellular pH on Gly-Sar and oseltamivir intracellular accumulation into CHO-K1 and CHO-PEPT1 cells

To investigate the contribution of a proton gradient on intracellular accumulation, the pH was varied between 4 and 8. Gly-Sar permeation into control cells was independent of the pH in

the incubation medium (Figure 2a). In contrast, in hPEPT1 expressing cells, Gly-Sar active uptake was strongly pH dependent and exhibited a bell shaped curve with its highest uptake at pH 6, which was approximately 200-fold higher than that in control cells ($p < 0.001$, Figure 2a). Our results are in excellent agreement with earlier reports (Fujisawa et al., 2006). The permeation of oseltamivir displayed a sigmoidal shaped curve in both control and hPEPT1 expressing cells, and appeared to reach a plateau value at higher pH as expected from passive permeation of a basic compound (Neuhoff et al., 2003). This curve was distinctively different from the bell shaped curve for Gly-Sar (Figure 2b) and further supports the contention that oseltamivir is not a substrate of hPEPT1.

Effect of hPEPT1 model inhibitors on Gly-Sar and oseltamivir intracellular accumulation into CHO-PEPT1 cells

To further establish the involvement of hPEPT1 in Gly-Sar or oseltamivir uptake, we examined the effect of known hPEPT1 inhibitors (Trp-Gly, valacyclovir and cefadroxil (Faria et al., 2004)) at concentrations of 20 mM on Gly-Sar and oseltamivir accumulation in CHO-K1 and CHO-PEPT1 cells. As illustrated in Figure 3a, the addition of hPEPT1 inhibitors decreased the intracellular accumulation of Gly-Sar in CHO-PEPT1 cells by 85-96% down to the background level in CHO-K1 cells ($p < 0.0001$), thereby demonstrating hPEPT1 mediated transport. Even though the addition of Trp-Gly, valacyclovir and cefadroxil significantly decreased the oseltamivir levels in CHO-PEPT1 cells ($p < 0.05$) this change was similar in control and hPEPT1 transfected cells (Figure 3b). Therefore, the effect of Trp-Gly, valacyclovir and cefadroxil was non-specific, providing additional evidence that hPEPT1 does not play a role in oseltamivir uptake.

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Effect of temperature (4°C) on Gly-Sar and oseltamivir intracellular accumulation into CHO-PEPT1 cells

The inhibitory effect of low temperature (4°C) on Gly-Sar and oseltamivir accumulation was compared to a 37°C incubation in both CHO-K1 and CHO-PEPT1 cells. As illustrated in Figure 3a, lowering the temperature to 4°C decreased the intracellular accumulation of Gly-Sar in CHO-PEPT1 cells by 96% down to the background level in CHO-K1 cells ($p < 0.0001$). As shown in Figure 3b, the intracellular accumulation of oseltamivir at 4°C was also significantly decreased ($p < 0.0001$), as compared to 37°C. However, the extent of change was similar in control and hPEPT1 expressing cells at both temperatures. Therefore, for oseltamivir this reduction was not due to a reduced activity of hPEPT1 expressed in CHO-PEPT1 cells.

Effect of oseltamivir and oseltamivir carboxylate on the active uptake of Gly-Sar in CHO-PEPT1 cells

It is known that different substrates for the same transporter will compete for the available binding site(s). Therefore, to elucidate whether oseltamivir and oseltamivir carboxylate are capable of competing with Gly-Sar for uptake by hPEPT1, we performed competition experiments across a 30-fold concentration range (0.3 to 10 mM). No statistically significant reduction on Gly-Sar uptake was noticeable in the presence of oseltamivir or oseltamivir carboxylate. Only a slightly significant ($p = 0.045$) increase of Gly-Sar uptake was observed in the presence oseltamivir carboxylate at 10 mM. Incubation with Tyr-Phe (0.5 mM), a substrate and inhibitor of hPEPT1, resulted in approximately a 4-fold reduction of Gly-Sar uptake ($p < 0.0001$, Figure 4).

Pharmacokinetics of oseltamivir and oseltamivir carboxylate in adult rats

Oseltamivir was dosed orally at 30 mg/kg (calculated as a free base) to rats in various vehicles (water, bovine milk and aqueous 125 mM Gly-Sar) under both fasted and fed conditions. The plasma concentration-time profiles and the derived pharmacokinetic parameters, including statistics, for the fasted condition are depicted in Figure 5 and Table 1. Over 90% of the overall oseltamivir and oseltamivir carboxylate exposure ($AUC_{0-\infty}$) were covered by the six hours observation interval. Comparing the different conditions, there was no statistically significant influence of milk or Gly-Sar on the C_{max}, t_{max}, and plasma exposure (AUC) of both oseltamivir and oseltamivir carboxylate.

Plasma concentration-time profiles and pharmacokinetic parameters, including statistics, for the fed condition are depicted in Figure 5 (truncated to 6 h for better comparison to the fasted condition) and Table 2. The 24 h observation period (AUC_{0-24h}) covered more than 99% of the overall oseltamivir and oseltamivir carboxylate exposure ($AUC_{0-\infty}$). The statistical analysis of exposures following single oral dose of 30 mg/kg oseltamivir to adult fed rats in the presence and absence of milk or Gly-Sar show no significant differences in oseltamivir or carboxylate C_{max}, t_{max}, and plasma exposure (AUC) between the various co-administrations.

Comparison of drug plasma exposure in fasted *vs* fed adult rats

The effect of food intake on oseltamivir and oseltamivir carboxylate plasma exposure in rats was evaluated by a two-way ANOVA statistical analysis and is reported in Table 3. For oseltamivir, the exposure (AUC_{0-6h}) was significantly lower in fed *vs.* fasted rats (p=0.0051). This negative food effect was similar across groups (interaction effect: p=0.199). However no statistically significant food effect was seen for $AUC_{0-\infty,}$ likely due to the large percentage of extrapolated AUC from the 6 h time point to infinity in the fed state. Indeed a comparably larger fraction of exposure was observed after the 6 h time point (t_{last} in fasted state) in fed

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animals than in fasted animals. Oseltamivir C_{max} concentration was significantly lower in fed *vs.* fasted rats (p=0.0007), with an even larger negative food effect in the presence of Gly-Sar (interaction effect: p=0.0287). The exposure of carboxylate (AUC_{0-6h}) was lower in fed *vs.* fasted rats (p<0.0002) and this negative food effect was larger in the presence of Gly-Sar (interaction effect: p=0.0243). However, for the exposure of oseltamivir carboxylate ($AUC_{0-\infty}$) a significant negative food effect (p=0.049) was seen only in the presence of Gly-Sar. Also the carboxylate C_{max} concentration was significantly lower in fed *vs.* fasted rats (p<0.0001) and this negative food effect was larger in the presence of Gly-Sar (interaction effect: p=0.0038). No statistically significant discrepancy in t_{max} value for both oseltamivir and oseltamivir carboxylate was observed between fasted and fed conditions (*group 1* "fasted" *vs. group 1* "fed").

Plasma exposure of oseltamivir and oseltamivir carboxylate in juvenile rats

Oseltamivir was dosed at 30 mg/kg (calculated as a free base) by oral gavage to juvenile male and female rats in various formulations (water, bovine milk and 125 mM Gly-Sar in water) under breast-fed (residing with the dams, oseltamivir administered in water only) or fasted conditions (pups removed from the dams, all three treatments). Plasma concentration-time profiles and pharmacokinetic parameters are depicted in Figure 6 and Table 4. The $AUC_{0-\infty}$ was not calculated in this study due to the limited coverage or complete absence of a terminal phase for oseltamivir and oseltamivir carboxylate, respectively. Within the five hour observation period, breast-fed pups showed significantly lower exposures than fasted pups for oseltamivir (-30%, p=0.002) and oseltamivir carboxylate (-60%, p < 0.001) after administration as aqueous solution. In fasted pups, no impact of Gly-Sar co-administration was observed and co-administration of milk reduced the exposure of oseltamivir carboxylate (-24%, p=0.028) but not oseltamivir. The presence of maternal or bovine milk in the

gastrointestinal tract of juvenile rats tended to delay the t_{max} of oseltamivir and consequently of the active metabolite when compared to the t_{max} values obtained under fasted conditions (*group 1* and *3 vs. group 2* and *4*).

Pharmacokinetics of oseltamivir and oseltamivir carboxylate in healthy volunteers following a single oral dose under fed or fasted condition

Mean plasma concentration-time profiles in healthy volunteers for oseltamivir and oseltamivir carboxylate, after administration of oseltamivir following overnight fasting and with food, are shown in Figure 7. Mean (SD) pharmacokinetic parameters are summarized in Table 5. Food had a substantial effect on oseltamivir plasma concentrations, resulting in an 18% decrease in C_{max}, an 18% increase in $AUC_{0-\infty}$, a 2.6-fold increase in t_{max} and a 33% increase in half-life. Oseltamivir carboxylate plasma concentrations increased rapidly in all subjects, reaching maximal levels at about 4.5 hours for the fasted group and 5.4 hours with food. The presence of food resulted in a small reduction in oseltamivir carboxylate C_{max} (about 20%) and AUC_{0-24h} (about 9%), a delay of approximately 1 hour in achieving maximal plasma concentrations and an increase of 1 hour in half-life. However, for the primary pharmacokinetic parameters of the active metabolite, the 90% confidence intervals for the ratios of means for the two treatments were within the 80-125% ranges for log-transformed $AUC_{0-\infty}$ (93.3-100.7%), and within the 70-143% ranges for log-transformed C_{max} (75.6-86.2%), indicating equivalence of the two treatments.

Food caused an increase in the amount of renally secreted oseltamivir compared to fasted as evidenced by an increase in excreted drug from 3.09 ± 1.22 to 4.32 ± 1.53%. The high fat meal had no effect on apparent clearance and/or renal clearance for oseltamivir or its carboxylate (Table 5).

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Discussion

Oseltamivir is an orally available ester-prodrug of RO0640802 (carboxylate), clinically used as a neuraminidase inhibitor to treat influenza. Upon absorption, oseltamivir is converted to its pharmacologically active carboxylate in the liver by carboxylesterase CES1 (Shi et al., 2011). Ogihara and co-workers reported that oseltamivir transport *in vitro* and *in vivo*, in rats and healthy volunteers, is mediated by PEPT1 (Ogihara et al., 2009; Morimoto et al., 2011). In the present report, we provide an in-depth systematic examination of the pharmacokinetic parameters of oseltamivir and its carboxylate in adult and juvenile rats, as well as mechanistic studies in hPEPT1 expressing CHO cells. Furthermore, we compared food effect data obtained from a human bioequivalence study to the observation by Morimoto and co-workers and conclude the intestinal absorption of oseltamivir is not limited by rPept1 or hPEPT1.

Oseltamivir and oseltamivir carboxylate are not hPEPT1 substrates *in vitro*

Recently, we emphasized the importance of controlling the experimental conditions to quantify active transport *in vitro* (Poirier et al., 2008). In the present report we implemented thorough procedures for the evaluation of oseltamivir and oseltamivir carboxylate as hPEPT1 substrates. Instructions detailed by Brandsch (2009) to avoid false negatives and positives in PEPT *in vitro* experiments were strictly followed, especially with regards to pH monitoring, drug stability, comparison to control cells and inhibitor effects.

First, we established the functionality of CHO-PEPT1 cells by demonstrating a 200-fold higher uptake of Gly-Sar in CHO-PEPT1 compared to CHO-K1 cells, an optimal pH, and a strong inhibition of Gly-Sar accumulation in the presence of typical inhibitors (Figures 1 to 3). These results implicate hPEPT1 mediated uptake for Gly-Sar and are in accordance with studies by Fujisawa and collaborators (2006). Oseltamivir is an ethyl ester, which potentially

can be hydrolyzed in the incubation medium during the experiment. We monitored for and confirmed ester stability during the incubation period.

hPEPT1 favors a pH gradient for optimal function. The efficiency of hPEPT1 mediated transport depends on an acidic extracellular pH environment and the optimum pH seems to be substrate specific (Steel et al., 1997). Therefore, we performed a pH-dependent experiment which clearly showed that: *i.* as opposed to Gly-Sar, oseltamivir lacks an optimum pH for accumulation in CHO-PEPT1 cells and *ii.* as opposed to Gly-Sar, oseltamivir accumulation in CHO-K1 closely follows that in CHO-PEPT1 cells (Figure 2). Since no difference between hPEPT1 and control cells was seen, our data do not support hPEPT1 mediated uptake of oseltamivir.

As observed by Ogihara and co-workers, when incubated at 4°C oseltamivir intracellular accumulation decreased drastically in CHO-PEPT1 cells (Figure 3). However similar observation was noted in CHO-K1 cells, demonstrating a non-specific effect as previously described (Poirier et al., 2008). Decrease of permeation at 4°C is not a proof of active uptake.

To further corroborate the lack of interaction of oseltamivir and its carboxylate with hPEPT1, a series of inhibition experiments was conducted. As suggested by Brandsch (2009), the inclusion of CHO-K1 cells is essential for a correct results interpretation. Using typical hPEPT1 inhibitors, we noticed an identical degree of interference with accumulation in CHO-PEPT1 and CHO-K1 cells for oseltamivir (Figure 3b). Care was taken in re-adjusting the pH after dissolving substrate and inhibitors.

An additional piece of evidence for lack of interaction with hPEPT1 comes from results demonstrating that Gly-Sar uptake in CHO-PEPT1 cells was not inhibited by up to 10 mM oseltamivir or oseltamivir carboxylate (Figure 4).

In summary, the evidence from our *in vitro* studies supports the validity of the CHO-PEPT1 cell line. Moreover, oseltamivir and oseltamivir carboxylate are neither substrates nor inhibitors of hPEPT1 (or hPEPT2, data not shown).

These *in vitro* findings are supported by the independent work of Hu and co-workers (2012) who demonstrated, using transfected yeast, that oseltamivir was not a substrate for human, mouse and rat PEPT1.

Pept1 inhibitors do not significantly impact oseltamivir and oseltamivir carboxylate rat pharmacokinetics

The present rat studies were designed and powered to confirm data from a previous report on effects of milk and PEPT1 inhibitors on oseltamivir pharmacokinetics (Ogihara et al., 2009). The pharmacokinetics ($AUC_{0\text{-}6h}$ and C_{max}) in fasted rats treated with aqueous oseltamivir matched very well those reported previously (Ogihara et al., 2009). However, we found no reduction of oseltamivir exposure ($AUC_{0\text{-}\infty}$) in fasted rats (Table 1; Fig 5 open circles) when administered in milk or in Gly-Sar.

The same vehicles were also investigated in fed rats (rat chow ad libitum; Table 2; Fig 5 closed circles). This allowed a comparison of the effects of putative (bovine milk) or known (Gly-Sar) PEPT1 inhibitors in fasted and fed rats. As in the fasted rats, the different vehicles did not affect oseltamivir exposure in fed rats.

In this study, the active drug (oseltamivir carboxylate) was monitored in parallel to the prodrug in all plasma samples to evaluate the effect of the co-administered vehicles on the pharmacologically active oseltamivir carboxylate. All blood samples were treated with dichlorvos immediately after sampling to prevent *ex vivo* conversion of oseltamivir to oseltamivir carboxylate (Chang et al., 2009). Consistent behavior of prodrug and carboxylate

across treatments indicates adequate stabilization of blood samples and corroborates the findings with the prodrug.

Administering oseltamivir in water, milk or aqueous Gly-Sar had no effect on oseltamivir carboxylate exposure in fasted or fed rats (Table 1 and 2; Fig 5 open and closed circles, resp.). Overall, we confirmed previously reported exposures of oseltamivir when administered as aqueous solution to fasted rats but could not observe an effect of co-administration of milk or aqueous Gly-Sar on oseltamivir or oseltamivir carboxylate exposure in male Wistar rats.

Application of oseltamivir in the three vehicles to fasted and fed rats also allows for a direct comparison of the exposures under the two feeding conditions (Table 3 and Figure 5 open *vs* closed circles). In contrast to the lack of impact of the PEPT1 inhibitor co-administration, fed rats showed moderately but consistently lower exposure towards oseltamivir carboxylate compared to fasted rats while the reduction of exposure towards oseltamivir was not statistically significant. The reduced exposure may be due to slower absorption of oseltamivir together with a slower conversion to oseltamivir carboxylate in fed rats favoring competitive elimination (other than conversion to the active carboxylate) in the fed state of rodents. A slight trend towards a larger fraction unchanged excreted in the urine is observed in fed *vs* fasted humans (Table 5) and this effect may be more pronounced in the preclinical species.

The pharmacokinetics of oseltamivir and its carboxylate were further examined in juvenile rats either breast-fed or fasted in the presence and absence of bovine milk or Gly-Sar (Table 4, Figure 6). Similar as in the adult animals, Gly-Sar had no statistically significant effect on oseltamivir and active metabolite plasma exposure in juvenile rats. Also the presence of bovine milk in the formulation had no significant effect on the exposure of oseltamivir, but reduced the exposure of the active metabolite. Breast feeding resulted in a reduction of oseltamivir and oseltamivir carboxylate exposure ($AUC_{0\text{-}5h}$). Similar results were observed by

Ogihara and co-workers determining oseltamivir exposure at a single time point (30 min after dosing) (Ogihara et al., 2009). Apparently, breast feeding in juvenile rats had a very similar effect on the absorption and disposition of oseltamivir and oseltamivir carboxylate as food in adult rats. It is noteworthy that oseltamivir and oseltamivir carboxylate exposure in adult rats were lower than in juvenile rats irrespective of feed status. A very similar age-dependency for exposure of oseltamivir and oseltamivir carboxylate was recently reported for marmosets (Parrott et al., 2011).

The rodent *in vivo* studies show that co-administration of the PEPT1 inhibitor Gly-Sar or bovine milk has no relevant effect on oseltamivir and oseltamivir carboxylate exposure. A moderate food effect of rat chow in adult rats and breast feeding in juvenile animals is, however, observed. Together, the *in vivo* data in rodents support the *in vitro* studies with hPEPT1 that oseltamivir is not a substrate for peptide transporter 1.

Food effect study in human healthy volunteers

The relative bioavailability of oseltamivir was examined and compared in healthy volunteers under fasted and fed (standard high-fat high-calorie breakfast including milk) conditions (Table 5, Figure 7). Food had a substantial effect on oseltamivir prodrug plasma concentrations, however, these changes are not clinically relevant. In contrast, food had a relatively small effect on the active metabolite. The active metabolite $AUC_{0-\infty}$ ratio after oral administration of oseltamivir with food, relative to fasting, was 98%. The $AUC_{0-\infty}$ values demonstrated that the fasting and fed treatment regimens were equivalent, with a 90% CI of 93% to 101% on the log-transformed scale. Consequently, from a clinical perspective, oseltamivir may be given in the fed or fasted state without impact on its clinical efficacy. Whereas overall exposure ($AUC_{0-\infty}$) of oseltamivir carboxylate was not affected by high fat food, the AUC_{0-24h}, C_{max}, t_{max} and half-life were modestly, but significantly altered,

suggesting a mechanistic interaction with carboxylesterases responsible for pro-drug conversion.

Most recently, Morimoto and collaborators (2011) reported an initial reduction of absorption of oseltamivir in the presence of milk in six healthy volunteers as evidenced by the delayed onset of plasma concentration of oseltamivir. Striking are the similarities between the plasma concentration profiles following dosing in milk or high-fat meal (including milk) in Morimoto's and the present study, respectively. The authors attributed the early reduction in absorption to the inhibition of hPEPT1 mediated uptake of oseltamivir. However, *i.* we observed in the present report that neither oseltamivir nor its carboxylate are substrates of hPEPT1 *in vitro*; *ii.* recently a monkey PBPK model using exclusively passive permeability to predict absorption without any active component could describe oseltamivir and oseltamivir carboxylate pharmacokinetic profiles (Parrott et al., 2011); *iii.* He and coworkers demonstrated that co-administration of amoxicillin, a known hPEPT1 substrate (Herrera-Ruiz and Knipp, 2003), had no effect on the disposition of the oseltamivir carboxylate (He et al., 1999b). Taken together, the results point away from a hPEPT1-mediated absorption of oseltamivir. Instead, the results point toward a significant but moderate food effect on oseltamivir that is mediated by milk and/or food not affecting the overall exposure ($AUC_{0-\infty}$) of the active drug, oseltamivir carboxylate.

In concluding, we present evidence from *in vitro* studies, using a multitude of conditions, and *in vivo* pharmacokinetic (animal and human) experiments that oseltamivir and its carboxylate are neither substrates nor inhibitors of rat or human PEPT1.

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Authorship Contributions:

Participated in research design: Poirier, Belli, Funk, Otteneder, Prinssen, Lazic, Rayner, Hoffmann, Singer, Smith, Schuler

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Contributed new reagents or analytical tools: Heinig

Performed data analysis: Poirier, Belli, Funk, Lazic, Rayner, Schuler

Wrote or contributed to the writing of the manuscript: Poirier, Belli, Funk, Lazic, Rayner, Smith, Schuler

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Footnotes

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Figure legends

Figure 1: Gly-Sar, oseltamivir and oseltamivir carboxylate intracellular accumulation in CHO-K1 cells (white bars) and CHO-PEPT1 cells (black bars) after a 10 min incubation at 37°C, pH6. Values are mean ± SD of three independent experiments (except Gly-Sar n=6). Statistical analysis was performed on intracellular accumulation; data for CHO-K1 cells were compared to those for CHO-PEPT1 cells: **** $p < 0.0001$

Figure 2: Effect of pH on (a) Gly-Sar (2 µM) and (b) oseltamivir (100 µM) intracellular accumulation in CHO-K1 cells (open squares) and CHO-PEPT1 cells (closed squares) after a 10 min incubation at 37°C. Values are mean ± SD of three independent experiments. Statistical analysis was performed on intracellular accumulation; data for CHO-K1 cells were compared to those for CHO-PEPT1 cells: *** $p < 0.0001$

Figure 3: Effect of Trp-Gly, Gly-Sar, valacyclovir, cefadroxil (all 20 mM) and 4°C temperature on (a) Gly-Sar (2 µM) and (b) oseltamivir (100 µM) intracellular accumulation in CHO-K1 cells (white bars) and CHO-PEPT1 cells (black bars) after a 10 min incubation at 37°C, pH6. Values are mean ± SD of three independent experiments. Statistical analysis was performed on intracellular accumulation in CHO-PEPT1 where data in control condition (37°C) were compared to those in presence of inhibitors or at 4°C: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Statistical analysis was also performed on intracellular accumulation where data for CHO-K1 cells were compared to those for CHO-PEPT1 cells: no statistically significant difference was observed for oseltamivir.

Figure 4: Effect of oseltamivir, oseltamivir carboxylate and Tyr-Phe (as a positive control) on Gly-Sar (2 µM) intracellular accumulation in CHO-PEPT1 cells after a 10 min incubation at 37°C, pH6. Values are mean ± SD of three independent experiments. Statistical analysis was performed on intracellular accumulation in CHO-PEPT1; data in presence of oseltamivir, oseltamivir carboxylate and Tyr-Phe were compared to those in the control condition: * $p < 0.05$, [a] $p=0.045$, **** $p < 0.0001$.

Figure 5: Mean plasma concentration-time profiles for (a, c, e) oseltamivir and (b, d, f) oseltamivir carboxylate following a single oral dose of oseltamivir (30 mg/kg) to adult rats. Oseltamivir was either dosed (a, b) as a water solution, (c, d) as a solution in bovine milk or (e, f) as a water solution containing 125 mM of Gly-Sar. The rats were either fed (closed circles), or fasted (open circles). Values are mean ± SD (n=3 per group).

Figure 6: Mean plasma concentration-time profiles for (a) oseltamivir and (b) oseltamivir carboxylate following a single oral dose of oseltamivir (30 mg/kg) to juvenile rats. Oseltamivir was either dosed as a water solution (circles), as a solution in bovine milk (open triangles) or as a water solution containing 125 mM of Gly-Sar (open squares). The juvenile rats were either breast-fed (closed circles), or fasted (open symbols). Values are mean ± SD (n=3 per time points).

Figure 7: Mean plasma concentration-time profiles of (a) oseltamivir and (b) oseltamivir carboxylate following a single oral dose of oseltamivir (150 mg) to healthy volunteers before (open circles) and after (closed circles) a

standardized high-fat and high-calorie breakfast. Values are mean ± SD (n=18). Asterisks indicate a significant difference between treatments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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Tables

Table 1: Summary of the mean (SD) pharmacokinetic parameters of oseltamivir and oseltamivir carboxylate following a single oral dose of oseltamivir (30 mg/kg) to adult fasted rats (n=3 per group). Oseltamivir was either dosed as a water solution, as a solution in milk or as 125 mM aqueous Gly-Sar. Statistical analysis was performed on C_{max}, t_{max}, AUC_{0-6h} and $AUC_{0-\infty}$ values; bovine milk (group 2) and Gly-Sar (group 3) treatments were compared to water (group 1). No statistically significant difference was observed.

PK parameter	**Oseltamivir**			**Oseltamivir carboxylate**		
	Water group 1	Bovine milk group 2	Gly-Sar group 3	Water group 1	Bovine milk group 2	Gly-Sar group 3
C_{max} (µg/L)	1690 (452)	1630 (200)	2070 (101)	2290 (283)	1630 (236)	2560 (533)
t_{max} (h)	0.83 (0.57)	0.5 (0)	0.25 (0)	1.08 (0.38)	1.92 (1.13)	1.17 (0.72)
AUC_{0-6h} (µg/L·h)	2680 (262)	3010 (533)	3780 (821)	5910 (429)	5810 (1280)	8350 (1850)
$AUC_{0-\infty}$ (µg/L·h)	2710 (271)	3060 (510)	3810 (795)	6140 (502)	6300 (1310)	8860 (1420)

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Table 2: Summary of the mean (SD) pharmacokinetic parameters of oseltamivir and oseltamivir carboxylate following a single oral dose of oseltamivir (30 mg/kg) to adult fed rats (n=3 per group). Oseltamivir was either dosed as a water solution, as a solution in milk or as 125 mM aqueous Gly-Sar. Statistical analysis was performed on C_{max}, t_{max}, $AUC_{0\text{-}6h}$ and $AUC_{0\text{-}\infty}$ values, bovine milk (group 2) and Gly-Sar (group 3) treatments were compared to water (group 1). No statistically significant difference was observed.

PK parameter	**Oseltamivir**			**Oseltamivir carboxylate**		
	Water group 1	Bovine milk group 2	Gly-Sar group 3	Water group 1	Bovine milk group 2	Gly-Sar group 3
C_{max} (µg/L)	1270 (325)	1430 (250)	926 (180)	1110 (136)	1310 (374)	693 (104)
t_{max} (h)	0.41 (0.14)	0.41 (0.14)	0.66 (0.14)	0.75 (0)	0.75 (0)	1.42 (0.63)
$AUC_{0\text{-}6h}$ (µg/L·h)	2200 (185)	2510 (606)	2280 (383)	3540 (562)	4510 (1440)	2960 (469)
$AUC_{0\text{-}24h}$ (µg/L·h)	2510 (172)	2800 (597)	2830 (507)	5280 (861)	6330 (1680)	4860 (998)
$AUC_{0\text{-}\infty}$ (µg/L·h)	2510 (175)	2810 (589)	2840 (506)	5290 (862)	6340 (1670)	4870 (999)

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Table 3: Two-way ANOVA statistical analysis of PK parameters with status (fed *vs* fasted) and group (milk *vs* Gly-Sar *vs* no milk no Gly-Sar) as categorical variables.

p values	C_{max}	t_{max}	$AUC_{0\text{-}6h}$	$AUC_{0\text{-}\infty}$
Oseltamivir				
status (fed *vs* fasted)	**0.000669**	0.822	**0.00514**	0.0745
group (milk *vs* Gly-Sar *vs* water)	0.954	0.455	0.177	0.0959
status:group interaction	**0.0287**	**0.0467**	0.199	0.380
Oseltamivir carboxylate				
status (fed *vs* fasted)	**0.00000624**	0.182	**0.000118**	**0.0718**
group (milk *vs* Gly-Sar *vs* water)	0.453	0.467	0.402	0.418
status:group interaction	**0.00381**	0.184	**0.0243**	**0.0489**

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Table 4: Summary of the mean pharmacokinetic parameters of oseltamivir and oseltamivir carboxylate following a single oral dose of oseltamivir (30 mg/kg) to juvenile rats (composite design, n=3 per time point). Oseltamivir was either dissolved in water, bovine milk or aqueous Gly-Sar (125 mM). The juvenile rats were either breast-fed or fasted. Statistical analysis was performed on $AUC_{0\text{-}5h}$ values; data for fasted juvenile rats receiving bovine milk (group 3) or Gly-Sar (group 4) were compared to those of fasted animals receiving the drug in water solution (group 2). In addition, group 2 was compared to group 1 (breast-fed juvenile rats).

Drug:	Oseltamivir				Oseltamivir carboxylate			
Condition:	breast-fed	fasted			breast-fed	fasted		
Treatment:	water	water	bovine milk	Gly-Sar	water	water	bovine milk	Gly-Sar
Group:	1	2	3	4	1	2	3	4
PK parameters:								
C_{max} (µg/L)	6140	10500	7990	10400	1500	3100	2460	2710
t_{max} (h)	1.0	0.50	1.0	0.50	5.0 [#]	2.0	5.0 [#]	2.0
$AUC_{0\text{-}5h}$ (µg/L·h)	20300	29500 [a]	26900	27100	5070	12100 [a]	9260 [b]	10900

[a] $p < 0.002$ *vs* group 1; [b] $p = 0.028$ *vs* group 2; [#] 5 h time point was last sampling time point, actual t_{max} may be later.

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Table 5: Summary of the mean (SD) pharmacokinetic parameters of oseltamivir and oseltamivir carboxylate following a single oral dose of oseltamivir 150 mg to healthy volunteers (n=18) under fasting conditions and with food. Statistical analysis was performed on PK parameters; data for fasted volunteers were compared to those receiving food.

PK parameter	oseltamivir			oseltamivir carboxylate		
	fasted	fed	ratio (%) fed:fasted	fasted	fed	ratio (%) fed:fasted
C_{max} (µg/L)	124 (63.2)	101 (32.2)	81.4	551 (204)	441 (142) ***	80.0
t_{max} (h)	0.806 (0.572)	2.06 (0.937) ***	256	4.37 (1.21)	5.42 (0.772) **	124
$t_{½\beta}$ (h)	1.61 (0.529)	2.15 (1.17)	134	6.87 (1.39)	8.20 (1.63) ***	120
CL/F (L/min)	13.0 (11.1)	9.54 (3.96)	73.4	0.371 (0.0427)	0.385 (0.0665)	103.9
$AUC_{0\text{-}24h}$(µg/L*h)	238 (82.0)	280 (71.4) ***	118	5543 (688)	5064 (865) **	91.3
$AUC_{0\text{-}\infty}$ (µg/L*h)	244 (81.9)	287 (71.6) ***	118	6218 (756)	6069 (1029)	97.6
f_e (%)	3.09 (1.22)	4.32 (1.53) ***	140	58.5 (10.9)	55.5 (9.59)	94.9
CL_R (L/h)	20.4 (7.20)	23.1 (5.95)	113	13.4 (2.76)	13.1 (2.65)	97.7

** $p < 0.01$, *** $p < 0.001$

Figure 1

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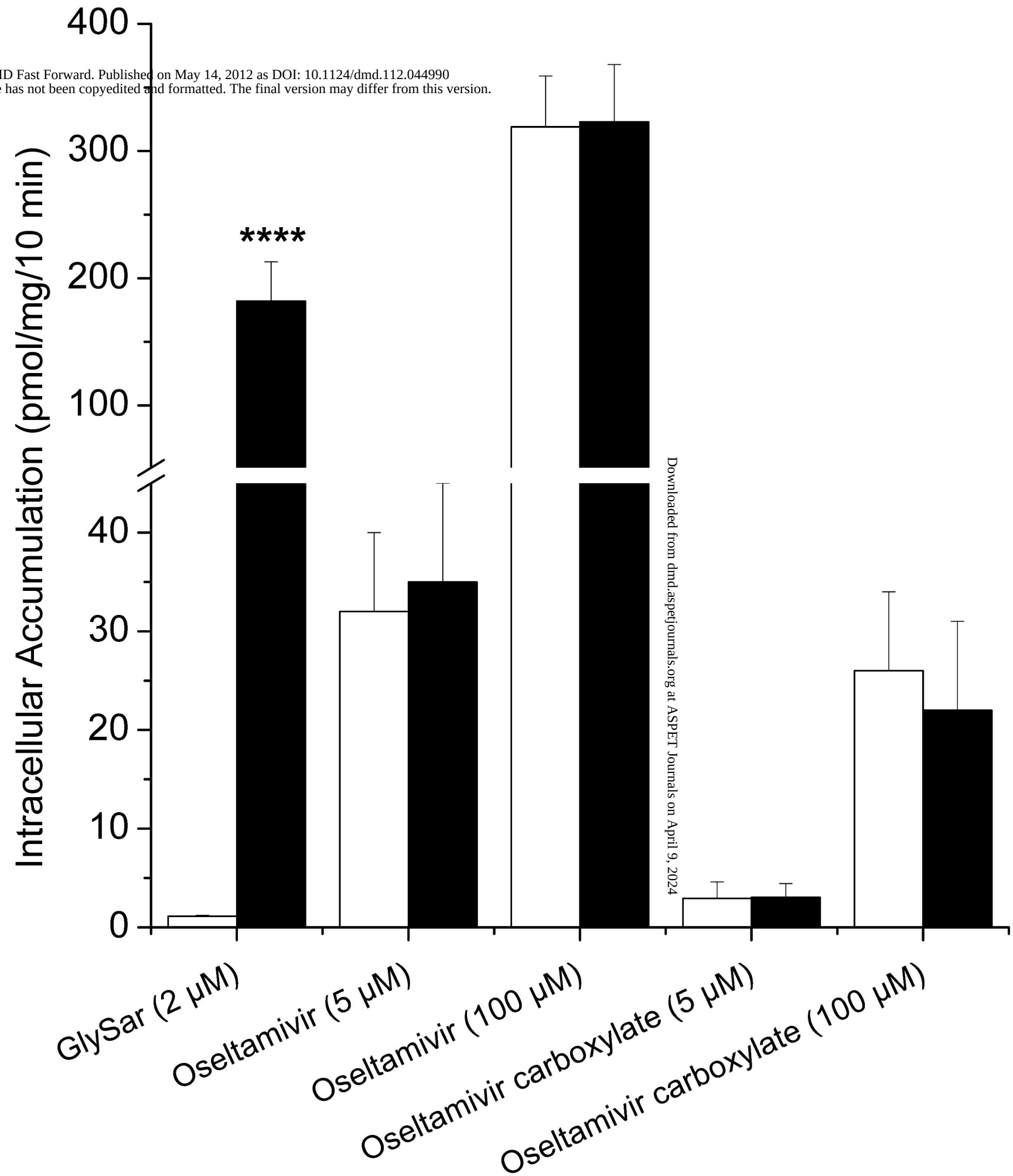


Figure 2

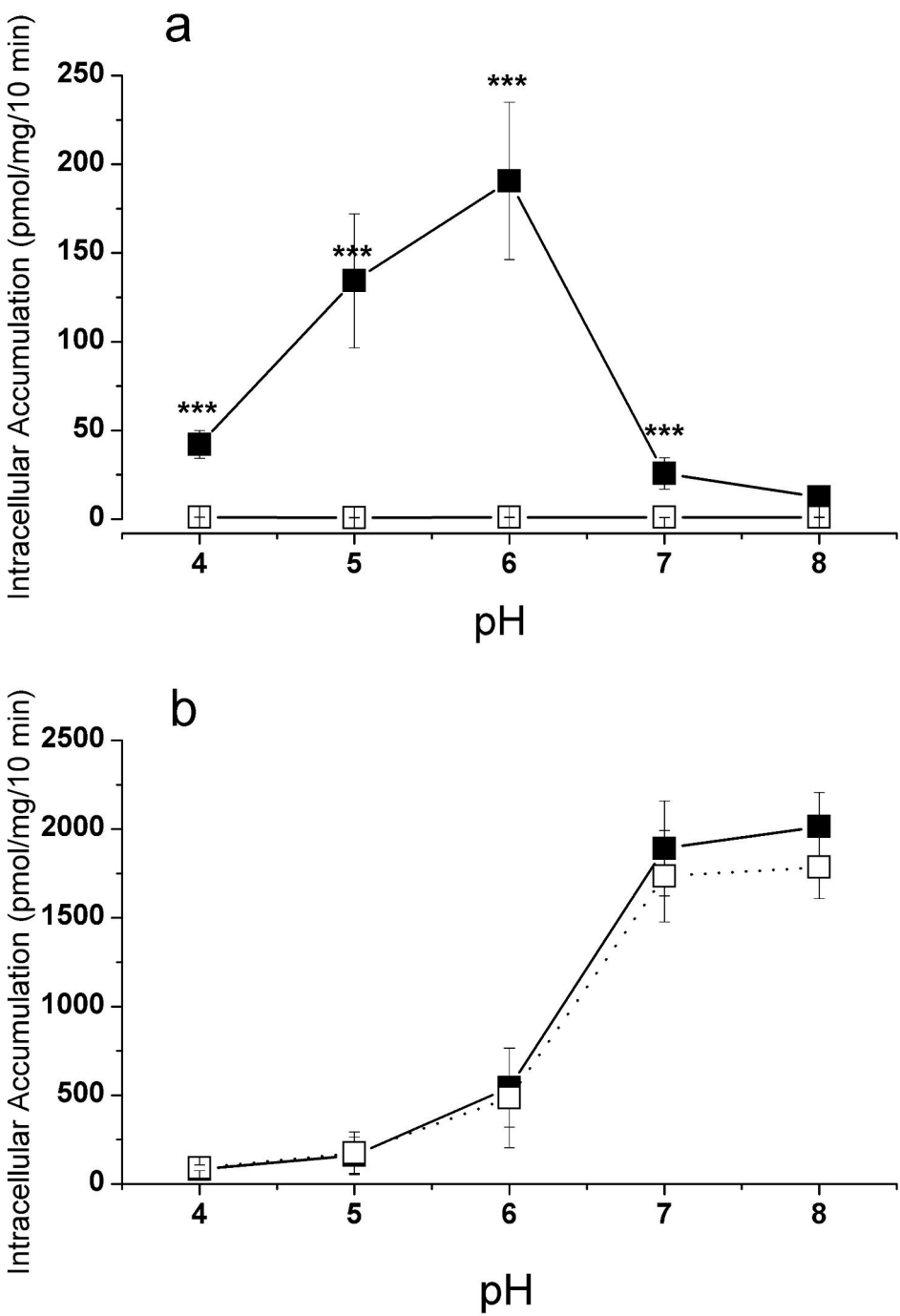

a
Intracellular Accumulation (pmol/mg/10 min)
250
200
150
100
50
0
***
***
***
***
4
5
6
7
8
pH
b
Intracellular Accumulation (pmol/mg/10 min)
2500
2000
1500
1000
500
0
4
5
6
7
8
pH

Figure 3

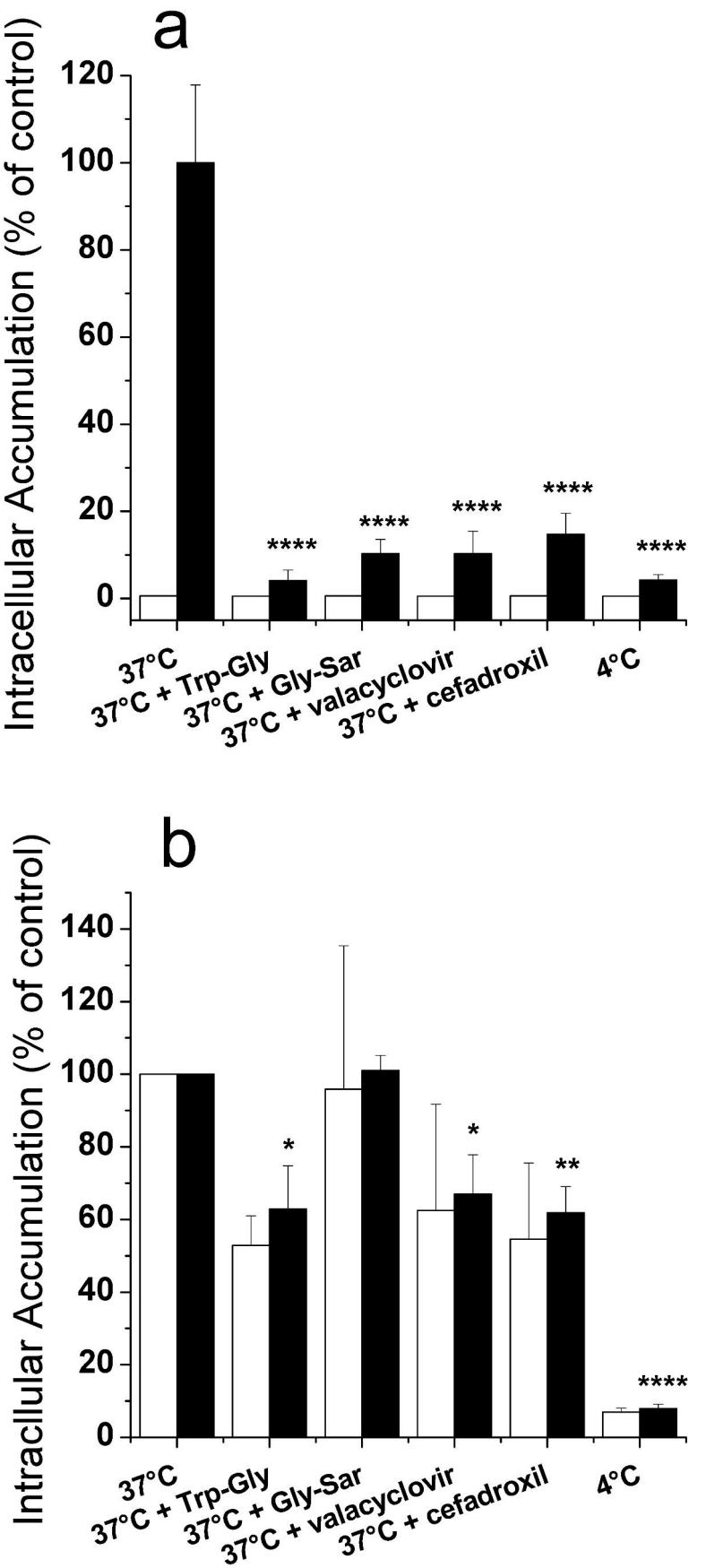

a
Intracellular Accumulation (% of control)
120
100
80
60
40
20
0
****
****
****
****
****
37°C
37°C + Trp-Gly
37°C + Gly-Sar
37°C + valacyclovir
37°C + cefadroxil
4°C
b
Intracllular Accumulation (% of control)
140
120
100
80
60
40
20
0
*
*
**
****
37°C
37°C + Trp-Gly
37°C + Gly-Sar
37°C + valacyclovir
37°C + cefadroxil
4°C

Figure 4

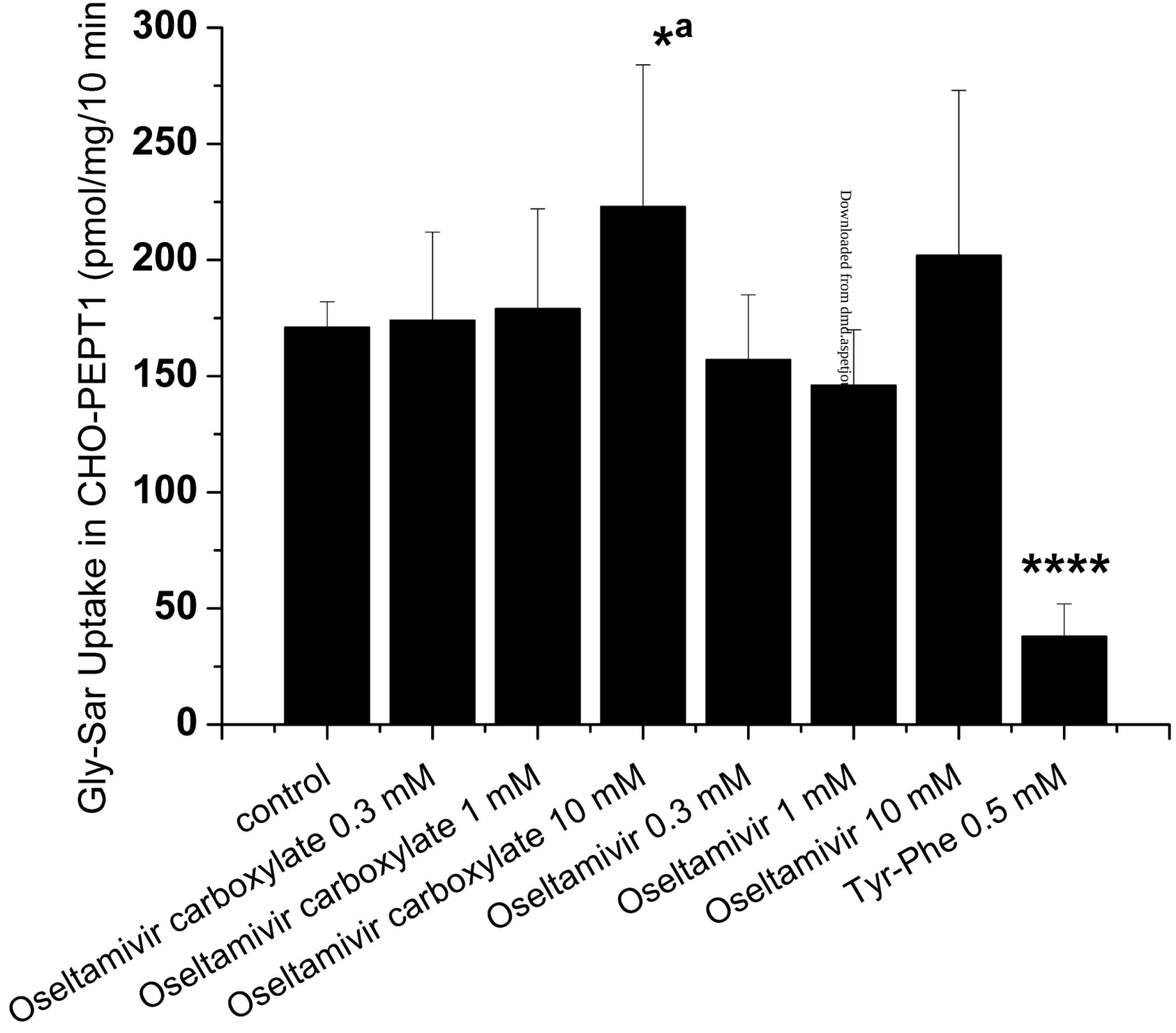


Figure 5

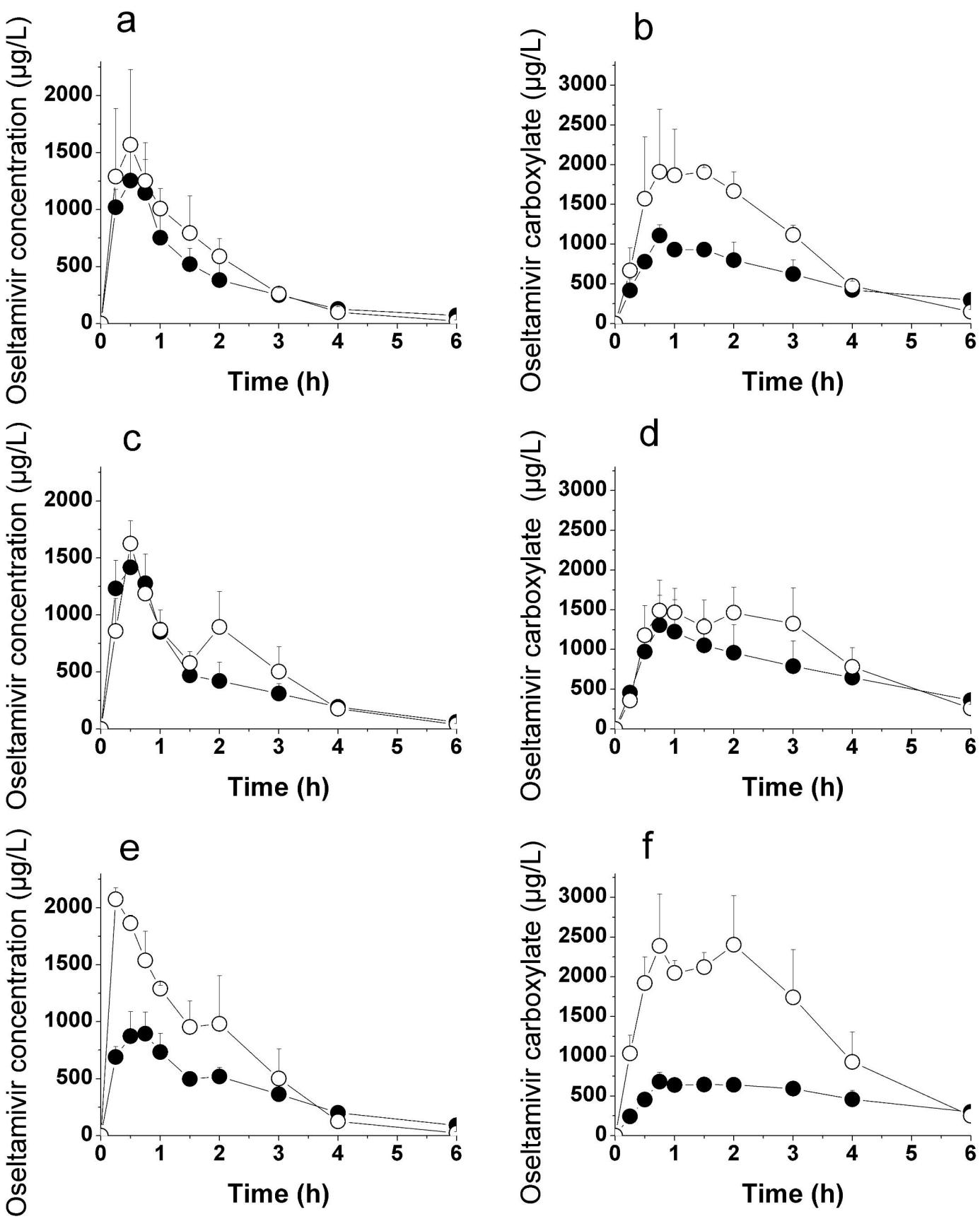
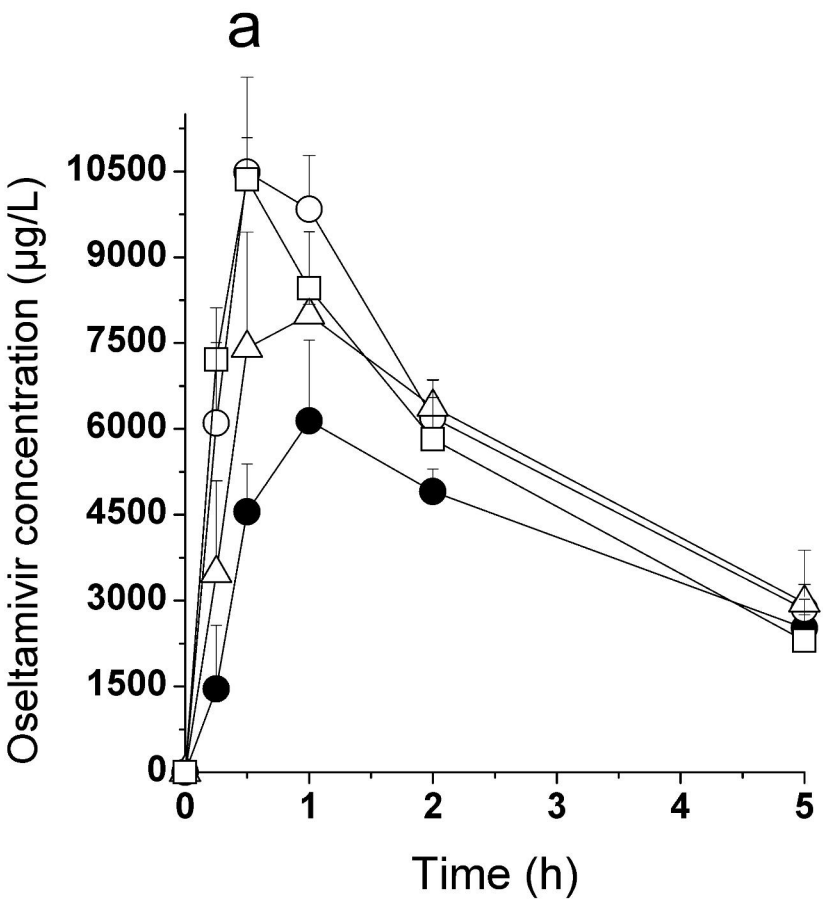

a
Oseltamivir concentration (µg/L)
2000
1500
1000
500
0
0 1 2 3 4 5 6
Time (h)
b
Oseltamivir carboxylate (µg/L)
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6
Time (h)
c
Oseltamivir concentration (µg/L)
2000
1500
1000
500
0
0 1 2 3 4 5 6
Time (h)
d
Oseltamivir carboxylate (µg/L)
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6
Time (h)
e
Oseltamivir concentration (µg/L)
2000
1500
1000
500
0
0 1 2 3 4 5 6
Time (h)
f
Oseltamivir carboxylate (µg/L)
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6
Time (h)

Figure 6


a
Oseltamivir concentration (µg/L)
10500
9000
7500
6000
4500
3000
1500
0
0
1
2
3
4
5
Time (h)

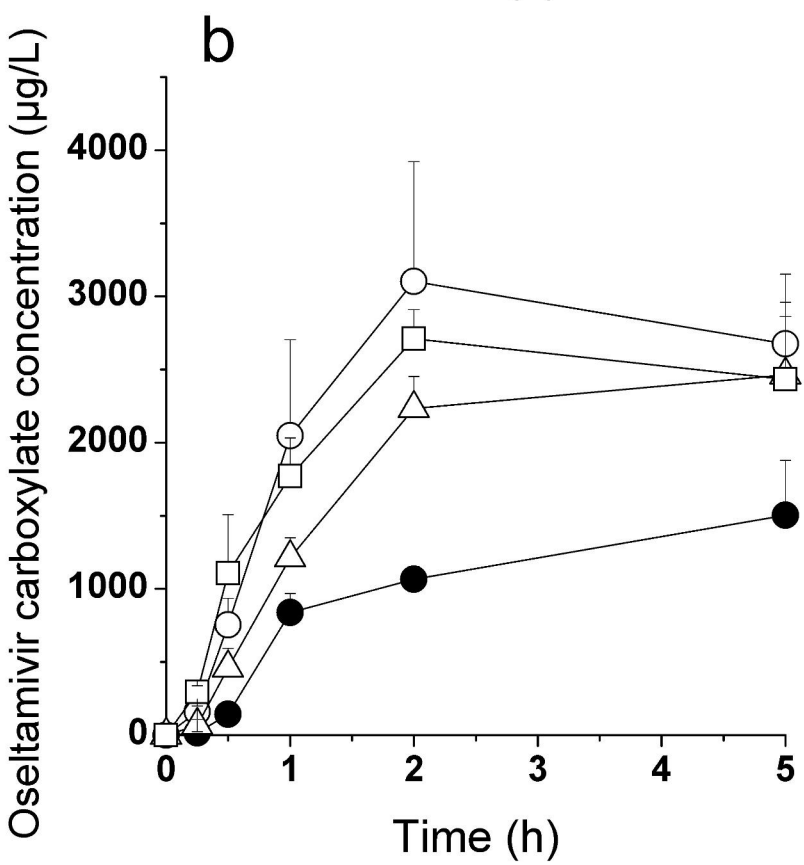
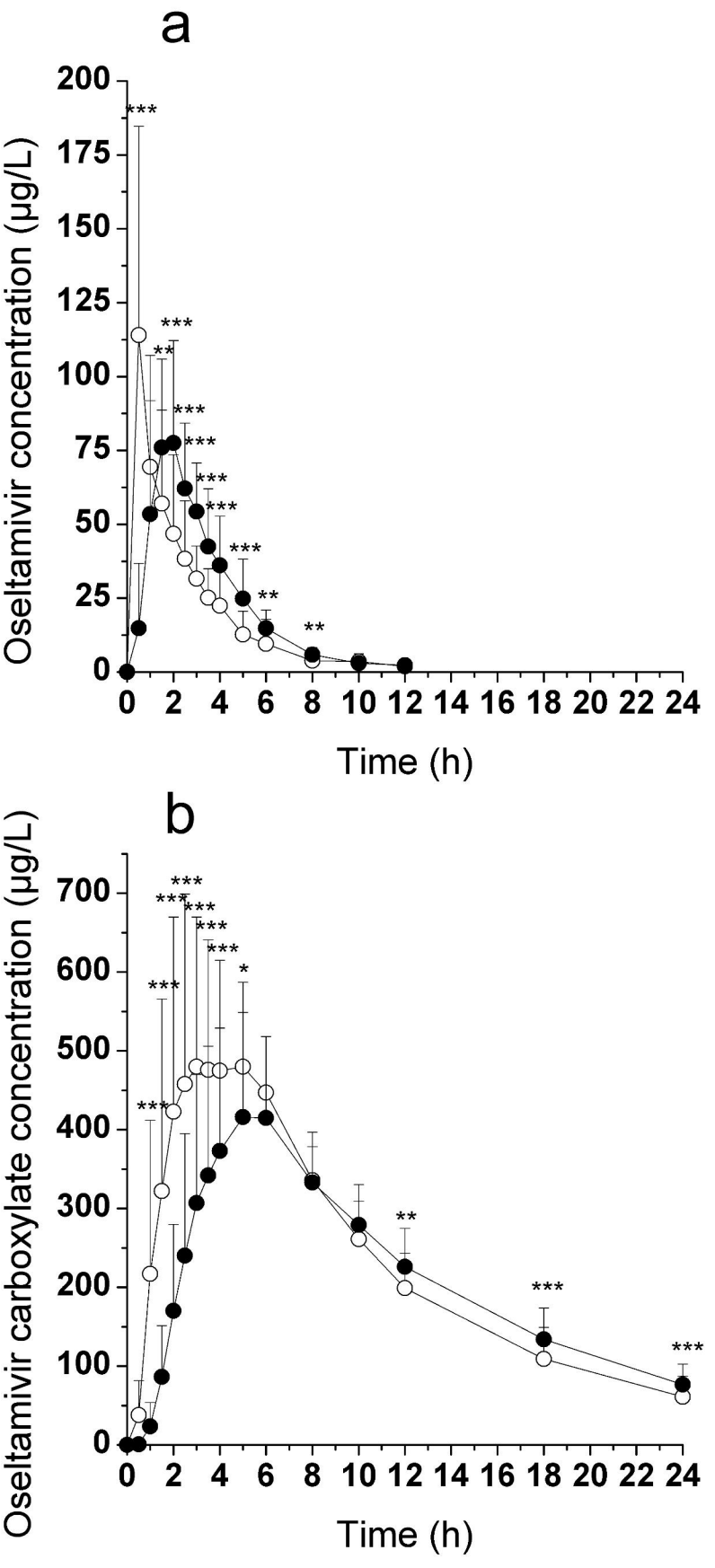

b
Oseltamivir carboxylate concentration (µg/L)
4000
3000
2000
1000
0
0
1
2
3
4
5
Time (h)

Figure 7


a
Oseltamivir concentration (μg/L)
200
175
150
125
100
75
50
25
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)
b
Oseltamivir carboxylate concentration (μg/L)
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h)