DMD Fast Forward. Published on September 5, 2020 as DOI: 10.1124/dmd.120.000140
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Characterization of Seasonal Pharmacokinetic Variability in Woodchucks

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DMD Fast Forward. Published on September 5, 2020 as DOI: 10.1124/dmd.120.000140
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Running Title: Pharmacokinetics and Woodchuck Model for HBV

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No. of text pages: 17

No. of tables: 2

No. of figures: 9

No. of supplemental tables/figures: 3/1

No. of references: 41

Abstract word count: 250

Significance statement word count: 73

Introduction word count: 540

Discussion word count: 1110

ABBREVIATIONS: 3TC, lamivudine; AP, antipyrine; $AUC_{0\text{-}24h}$, area under time-concentration curve from time 0 to 24 h; AUC_{inf}, area under time-concentration curve from time 0 to infinity; BPM, beats per minute; BT, body temperature; BW, body weight; CBC, complete blood count; CHB, chronic hepatitis B; CL,

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clearance; C_{max}, maximum concentration; E_h, hepatic extraction; h, hour; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HPLC, high-performance liquid chromatography; HR, heart rate; ICG, indocyanine green; IV, intravenous; kg, kilogram; min, minute; mL, milliliter; PK, pharmacokinetics; RTG, raltegravir; SD, standard deviation; $t_{1/2}$, terminal half-life; VES, vesatolimod; V_{ss}, steady-state volume of distribution; WHV, woodchuck hepatitis virus.

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ABSTRACT

The eastern woodchuck (*Marmota monax*) is a hibernating species extensively used as an in vivo efficacy model for chronic human hepatitis B virus (HBV) infection. Under laboratory conditions, woodchucks develop a pseudo-hibernation condition and thus, the pharmacokinetics (PK) of small molecule therapeutics may be affected by the seasonal change. The seasonal PK of four probe compounds were characterized over 12 months in laboratory maintained 7 male and 9 female woodchucks. These compounds were selected to study changes in oxidative metabolism (antipyrine; AP), glucuronidation (raltegravir; RTG), renal clearance (lamivudine; 3TC) and hepatic function (indocyanine green; ICG). Seasonal changes in physiological parameters and PK were determined. Seasonal body weight increases were ≥ 30%. Seasonal changes in body temperature and heart rate were < 10%. The mean AP exposure remained unchanged from April to August 2017, followed by a significant increase (≥ 1.0-fold) from August to December and subsequent decrease to baseline at the end of study. A similar trend was observed in RTG and 3TC exposures. The ICG exposure remained unchanged. No significant sex difference in PK was observed, although female woodchucks appeared to be less susceptible to seasonal PK and body weight changes. Significant seasonal PK changes for AP, RTG and 3TC indicate decreases in oxidative metabolism, phase II glucuronidation and renal clearance during pseudo-hibernation. The lack of seasonal change in ICG exposure suggests there are no significant changes in

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hepatic function. This information can be used to optimize the scheduling of woodchuck studies to avoid seasonally-driven variation in drug PK.

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Significance Statement

Woodchuck is a hibernating species and is commonly used as a nonclinical model of hepatitis B infection. Investigation of seasonal PK changes is perhaps of greater interest to pharmaceutical industry scientists using the woodchuck model to optimize the scheduling of woodchuck studies to avoid seasonally-driven variation in drug PK and/or toxicity. This information is also valuable to drug metabolism and veterinary scientists in understanding woodchuck's seasonal metabolism and behavior under the pseudo-hibernation condition.

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Introduction

Despite the availability of an effective vaccine for the prevention of HBV infection, approximately 240 million individuals are chronically infected with HBV, and over half a million people are estimated to die each year due to liver diseases associated with chronic hepatitis B (CHB), primarily due to liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) (WHO). Approved treatment of CHB includes nucleos(t)ide analogs as well as interferon-alpha. These therapies repress viral replication and improve long-term outcome, but cure is rarely achieved. Consequently, there is an urgent need for new therapies that induce durable immune control, i.e. a functional cure, of HBV.

The woodchuck model of chronic hepadnavirus infection is indispensable in developing therapeutic strategies able to finally cure chronic HBV infection (Roggendorf and Tolle, 1995; Menne and Cote, 2007; Dandri and Petersen, 2017). The eastern woodchuck (*Marmota monax*), also known as the groundhog, can be naturally infected with woodchuck hepatitis virus (WHV), a hepadnavirus closely related to human HBV. The woodchuck model of CHB displays many characteristics of human disease and has provided a well-characterized mammalian model for the preclinical evaluation of most antiviral drugs now in use for treatment of chronic HBV infection (Rajagopalan et al., 1996; Genovesi et al., 1998; Mason et al., 1998; Dandri et al., 2000; Korba et al., 2000; Bryant et al., 2001; Menne et al., 2005). Most recently, this model was used to evaluate the antiviral efficacy of various novel immunomodulatory agents, including the TLR7 agonist vesatolimod (VES; GS-9620) and the TLR8 agonist selgantolimod (GS-

9688) (Menne et al., 2015; Paulsen et al., 2015; Daffis et al., 2017; Daffis et al., 2020; Mackman et al., 2020).

The eastern woodchuck is a hibernating species that undergoes large seasonal variations in metabolic state. Reduction in renal clearance and decrease in plasma atrial natriuretic factor were reported in hibernating marmots (*Marmota flaviventris*), a closely related species (Zatzman and South, 1972; Zatzman and Thornhill, 1989), and the hibernating thirteen-lined ground squirrel (*Citellus tridecemlineatus*) (Hong, 1957). In addition, seasonal changes in endocannabinoid concentrations between active and hibernating marmots were observed (Mulawa et al., 2018).

Under laboratory conditions, woodchucks develop a pseudo-hibernation condition during the winter. On the other hand, treatment duration utilizing the woodchuck model for HBV can last multiple weeks, depending on the treatment used, which makes it likely that these studies could overlap with the pseudo-hibernation period. This raises the possibility that small molecule therapeutics administered to woodchucks at different times of year will have differences in PK and metabolism, and therefore potential differences in on- and off-target effects.

There are scarce data describing the potential seasonal changes in metabolism and PK, especially if animals develop a pseudo-hibernation condition during winter. This report presents the characterization of seasonal PK changes of four probe compounds dosed intravenously once every four weeks over 12 months in male and female woodchucks. Using the exposures of the parent compounds as markers, these compounds were selected to study specific

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changes in: oxidative metabolism with AP (Balani et al., 2002), phase II glucuronidation with RTG (Kassahun et al., 2007; Liu et al., 2019), renal clearance with 3TC (Johnson et al., 1999) and hepatic function with ICG (Cooke et al., 1963; Huang and Vore, 2001; de Graaf et al., 2011; Hwang et al., 2017).

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Materials and Methods

Materials

AP, RTG, 3TC and ICG were purchased from Sigma-Aldrich (St. Louis, MO) or VWR International (West Chester, PA), and were of high-performance liquid chromatography (HPLC) or analytic grade. Structure of the probe compounds and their disposition pathways are summarized in Table 1.

Woodchucks

At the initiation of the study, a total of 16 juvenile woodchucks (Marmota monax), 7 males and 9 females, were used. All animals used in these studies were born in the laboratory facility of Northeastern Wildlife, Inc. (Harrison, ID) in the spring of 2016 and inoculated with WHV neonatally (3-5 days of age) by subcutaneous administration of 10^7 genome equivalents/mL WHV (strain WH7P2A) but had naturally cleared the infection by quantifying their serum levels of woodchuck hepatitis surface antigen throughout the infection phase. The neonatal WHV inoculation is a standard procedure in preparing WHV chronically infected woodchucks for the study of HBV cure. Woodchucks were about 1 year old at the initiation of the study. Throughout the study duration, animals were housed in floor pens containing cedar or aspen wood shavings, maintained under daily cycles of 12 hours of light and 12 hours of dark and fed with rabbit chow (Purina; 16% crude protein (≥18% for pups and pregnant animals), 2% crude fat, ~15% crude fiber, vitamin A (4500 IU), 1% calcium and 0.5% phosphate). Animals were not fasted prior to treatment.

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Seasonal single dose PK studies in woodchucks

The animal protocol and all procedures involving woodchucks were reviewed and approved by the Northeastern Wildlife IACUC and adhered to the national guidelines of the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the American Veterinary Medical Association. A pre-study was conducted on March 16, 2017 to ensure animal safety and to safeguard any potential adverse event due to a four-drug cocktail co-administration. In that study, five separate vials of formulated solution were provided including four vials containing the individual compounds formulated separately and the fifth vial with all four compounds co-formulated into a single formulation. The four compounds, AP, RTG, 3TC and ICG, were either individually formulated or co-formulated as a solution, each at a dose concentration of 0.5 mg/mL and a dose volume of 1 mL/kg in 10% ethanol, 10% polyethylene glycol 300 and 80% water. The formulated solution was administered by a 2-minute intravenous (IV) slow bolus injection via an indwelling catheter in a cephalic vein at a dose of 0.5 mg/kg for each compound. The formulations were provided in 25-mL single-use sterile vials, refrigerated at 2-8ºC and protected from light. Animals were divided into 5 groups of n = 3 for each. ICG, RTG, 3TC, AP and the four-compound co-formulation were administered to woodchucks in Groups 1 to 5, respectively.

The main study involving a monthly administration of the four-drug cocktail occurred from April 12, 2017 to April 18, 2018. A snapshot of the PK study is summarized in Table 2. A co-formulated cocktail of all four compounds consisted

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of each compound formulated at the same dose, volume, dosing concentration and vehicle as described above. The cocktail was provided in 75-mL single-use sterile vials, refrigerated at 2-8ºC and protected from light. One vial was shipped to Northeastern Wildlife, Inc. (Harrison, ID) for each monthly dosing.

The monthly dosing was scheduled to occur once every four weeks. However, each dosing day could be potentially moved by up to 4 days before or after the date to accommodate scheduling conflicts. Body weight, body temperature, and heart rate were measured in each animal prior to dosing each month. Heart rate was determined prior to the induction of anesthesia for blood collections using a ketamine/xylazine combination approach. K_2EDTA was used for PK and hematology sample collection. No anticoagulant was used for samples collected for clinical chemistry. Roughly 1.0 mL blood was collected for complete blood count (CBC) and hematology. PK samples were collected at 5 min, 10 min, 1 h, 2 h, 4 h, 8 h, 24 h postdose. Blood for PK (0.5 mL) and hematology (1.0 mL) was protected from light and maintained on wet ice, in chilled cryoracks or at approximately 5°C prior to centrifugation to obtain plasma. Centrifugation began within 1 hour of collection. Individual plasma samples were harvested, placed into 96-well tubes and maintained on dry ice prior to storage at approximately -70°C. Samples for clinical chemistry, CBC and hematology were handled according to the contract research organization standard operating procedures. The clinical pathology data were included in the final study report as an appendix for informational purposes. Urine sample was not collected to minimize disruption of the animals.

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Tolerability was assessed on-site via in-life parameters including daily visual observation of the woodchucks, as well as body weight and body temperature. Clinical pathology was assessed by analyzing serum chemistry, hematology and coagulation samples at Cornell University, Animal Health Diagnostic Center (Ithaca, NY).

Bioanalytical and PK analysis

Plasma samples were protected from light and stored at -20°C until shipped to Charles River Laboratories (1 Innovation Drive, Worcester, MA) for bioanalysis. Plasma concentrations of all four administered compounds were quantified by liquid chromatography–tandem mass spectrometry methods. The representative bioanalytical methods and assay performance are detailed in supplemental materials (Table S1-S2). Analyst software (AB Sciex, Framingham, MA) was used for data acquisition and chromatographic integration. Analyst data were exported to Watson® LIMS software version 7.4.1 (Thermo Fisher Scientific, Waltham, MA) for regression analysis, concentration calculations and descriptive statistics.

Pharmacokinetic parameters, including area under the plasma concentration–time curve from time 0 to 24 hour ($AUC_{0\text{-}24h}$), area under the plasma concentration–time curve from time 0 to infinity (AUC_{inf}), maximal concentration (C_{max}), terminal half-life ($t_{1/2}$), steady-state volume of distribution (V_{ss}) and clearance (CL), were determined by non-compartmental analysis using Phoenix WinNonlin 6.4 (Pharsight Corporation, Princeton, NJ).

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Three woodchucks died during the study for reasons that were judged by the veterinarian to be unrelated to the study drugs. Details of the clinical observations were summarized in supplemental materials.

Statistical analysis

Student's t-test was performed with GraphPad Prism version 7.03 (GraphPad Software, San Diego, CA) using an unpaired, two-tailed, parametric method with 95% confidence level assuming same standard deviation for both populations. The p values less than 0.05 were considered as statistically significant. At least two values were required to calculate mean. At least three values were required to report standard deviation (SD).

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Results

Woodchuck baseline characteristics

The mean body weight was 3.3 (SD 0.4) kg (range 2.6-3.9 kg). The mean (SD) body temperature was 37.7 (0.9) °C (range 37.0-38.7 °C). The mean heart rate under restrained condition was 244 (50) beats per minute (BPM) (range 126-312 BPM). The mean heart rate under anesthesia condition was 130 (21) BPM (range 105-156 BPM). All animals had Gamma-glutamyl transferase < 10 international units per liter at the time of study initiation and were in good overall health.

Seasonal changes of physiological parameters

Seasonal changes in body weight, body temperature and heart rate for both male and female woodchucks from March 16, 2017 to April 18, 2018 are plotted in Fig. 1. A trend of weight gain from April to June, reaching a plateau from June to October and back to baseline on January was observed in male woodchucks. The body-weight trend in female woodchuck was slightly different: a slow increase was observed from April to August, reaching a peak in August followed by a decline to baseline in November (Fig. 1A). The body weight increases from April 12, 2017 to August 14, 2017 were statistically significant for both male ($p < 0.0001$) and female ($p < 0.0001$) woodchucks even though the magnitude of change in males (42% ± 12%) was greater than that in females (30% ± 18%). No significant changes (< 10%) in body temperature and heart rate were observed

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throughout the study duration for either male or female woodchucks maintained under lab conditions (Fig. 1B and 1C).

Seasonal changes of PK parameters

Representative plasma concentration-time profiles following an IV co-injection of AP, RTG, 3TC and ICG in woodchucks on November 23, 2017 are shown in Fig. 2. Seasonal $AUC_{0\text{-}24h}$ changes from April 13, 2017 to March 18, 2018 are plotted in Fig. 3-6. Tabulated summary of the $AUC_{0\text{-}24h}$ values of AP, RTG, 3TC, ICG and plots of statistical analysis from the pre-study and the seasonal PK study are available in supplemental materials (Table S3 and Fig. S1). Additional PK parameters such as AUC_{inf}, C_{max}, $t_{1/2}$, V_{ss} and CL from pre-study and representative PK data from May 11 and November 23, 2017 are summarized in Table S3 and plotted in Fig. 9, respectively.

The pre-study results (Table S3) did not show any significant drug-drug interactions when comparing the corresponding $AUC_{0\text{-}24h}$ values of AP, RTG, 3TC and ICG following the IV administration of individual formulation vs. four-drug co-formulation.

The $AUC_{0\text{-}24h}$ of AP remained relatively unchanged from April to August, then gradually increased from August to the end of November (3.1 ± 2.4 -fold increase in males and 1.0 ± 1.8 -fold increase in females), reaching the maximum by the end of November and subsequently returned to baseline in March of the following year (Fig. 3A). A similar trend was observed in female woodchucks, but the peak exposure was reached at the end of October (Fig. 3B). The exposure increases from May 11 to November 23 in both male (Fig. 7A) and female (Fig. 8A)

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woodchucks were statistically significant. The magnitude of exposure change was less pronounced in females (2.1 ± 2.6 -fold) than in males (9.0 ± 4.3 -fold). Although, in general, no significant sex difference was observed throughout the study duration, a statistically significant seasonal sex difference ($p = 0.0011$) was observed at the end of November (Fig. 2A and S1A).

The trends of seasonal exposure changes for both RTG and 3TC in male (Fig. 4A and 5A) and female (Fig. 4B and 5B) woodchucks were very similar to those corresponding changes for AP (Fig. 3A and 3B). Statistically significant increases of RTG and 3TC exposures from May 11 to November 23 were observed in both male (Fig. 7B and 7C) and female (Fig. 8B and 8C) woodchucks. No significant sex differences in $AUC_{0\text{-}24h}$ values were observed in the year-round study with the exception that statistically significant seasonal sex differences ($p = 0.011$ for RTG and $p = 0.012$ for 3TC) were observed at the end of November (Fig. 2B, 2C, S1B and S1C). No significant ICG $AUC_{0\text{-}24h}$ changes were observed in either male (Fig. 6A and 7D) or female (Fig. 6B and 8D) woodchucks and no sex difference was observed (Fig. 2D and S1D) throughout the study duration.

Representative AUC_{inf}, C_{max}, $t_{1/2}$, V_{ss} and CL values between May 11 and November 23, 2017 were compared in Fig. 9. In both male and female woodchucks, AUC_{inf} increases were statistically significant for AP ($p < 0.0001$ for male and $p = 0.026$ for female), RTG ($P < 0.0001$ for both male and female) and 3TC ($p < 0.0001$ for male and $p = 0.041$ for female) but not for ICG ($p = 0.69$ for male and $p = 0.77$ for female). No statistically significant V_{ss} changes ($p \geq 0.22$)

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were observed for any of the four compounds tested in either male or female woodchucks. In both male and female woodchucks, CL decreases were statistically significant for AP ($p < 0.0001$ for male and $p = 0.0047$ for female), RTG ($p < 0.0001$ for both male and female) and 3TC ($p < 0.0001$ for male and $p = 0.016$ for female) but not for ICG ($p = 0.29$ for male and $p = 0.48$ for female). In male, increases of C_{max} were statistically significant for AP ($p = 0.0017$), RTG ($p < 0.0001$) and 3TC ($p < 0.0001$) but not for ICG ($p = 0.68$). In female, C_{max} increases were statistically significant for ICG ($p = 0.037$) but not for AP ($p = 0.14$), RTG ($p = 0.28$) or 3TC ($p = 0.36$). In male, increases of $t_{1/2}$ were statistically significant for AP ($p < 0.0001$) and 3TC ($p = 0.0003$) but not for RTG ($P = 0.061$) or ICG ($p = 0.48$). In female, $t_{1/2}$ increases were statistically significant for AP ($p = 0.039$) but not for RTG ($p = 0.30$), 3TC ($p = 0.14$) or ICG ($p = 0.70$). Increased $t_{1/2}$ led to higher percentage of extrapolated AUC_{inf} values which were used to calculate CL values. As such, seasonal $AUC_{0\text{-}24h}$ values were selected as the most representative PK parameters for discussion.

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Discussion

Under wild conditions, it is well known that hibernating animals need to store energy by gaining weight before the winter and slow their metabolism, lower body temperature, slow breathing and heart rates during the winter to last through the duration of their dormant period (Geiser, 2004). Reduced cardiac output was reported in thirteen-lined ground squirrel during hibernation (Bullard and Funkhouser, 1962). Elevated plasma concentrations of persistent organic pollutant and prolonged accumulation of highly reactive metabolites were observed in grizzly bears during hibernation (Christensen et al., 2007). Sex differences in PK were generally attributed to differences in expression of hepatic enzymes (Czerniak, 2001) and hibernating animals such as woodchuck were not typically used as animal models to explore sex differences in toxicology studies (Curry 3rd, 2001).

The eastern woodchuck is a hibernating species that is being extensively used in laboratory to study the antiviral efficacy for treatment of chronic HBV infection (Rajagopalan et al., 1996; Genovesi et al., 1998; Mason et al., 1998; Dandri et al., 2000; Korba et al., 2000; Bryant et al., 2001; Menne et al., 2005), functional cure of HBV (Menne et al., 2015; Paulsen et al., 2015; Daffis et al., 2017; Daffis et al., 2020) and treatment of WHV associated HCC (Tennant et al., 2004; Iyer et al., 2019).

Under wild conditions, seasonal changes in extrinsic factors such as light, temperature and food availability trigger hibernation (Vybíral and Janský, 1997).

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Intrinsically, the existence of the trigger substance, i.e. the so-called “hibernation induction trigger” derived from the blood of hibernating animals including woodchuck, was reported and the effect may be species specific (Vybíral and Janský, 1997). For example, a hibernating specific 88 kilo Dalton protein in the plasma of deeply hibernating woodchucks was described (Horton et al., 1996). Husbandry of woodchucks in a lab facility removed the extrinsic factors such as light, temperature and food availability which can trigger hibernation but unlikely to alter the intrinsic factors such as the trigger substance, which led to a pseudo-hibernation condition.

Under laboratory conditions, the body weight increase from April 12, 2017 to August 14, 2017 was statistically significant for both male and female woodchucks even though the magnitude of change in males was greater than that in females (Fig. 1A). The higher magnitude of change in body weight for males than that for females was likely attributed to the reported 38% higher energy cost for males (Zervanos and Salsbury, 2003). In contrast to wild conditions, no significant changes in body temperature and heart rate were observed throughout the study duration for either male or female woodchucks maintained under lab conditions (Fig. 1B and 1C). The lack of changes in body temperature and heart rate were likely because woodchucks were kept awake and active under lab conditions in contrast to deep hibernation under wild conditions during winter time.

Seasonal plasma $AUC_{0\text{-}24h}$ changes of AP, RTG and 3TC were used as the markers of change in oxidative metabolism, phase II glucuronidation or renal

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clearance in woodchucks, respectively. The significantly higher AP, RTG and 3TC exposures on November 23, 2017 vs May 11, 2017 indicated that oxidative metabolism, phase II glucuronidation and renal clearance in woodchuck were significantly reduced during the pseudo-hibernation. The seasonal sex differences observed at the end of November were mainly because the magnitude of maximum exposure change was less pronounced in females than in males and because the peak exposures were not synchronized between male and female woodchucks.

Seasonal $AUC_{0\text{-}24h}$ of ICG was used as a marker of hepatic function in woodchucks. High variability was observed for ICG $AUC_{0\text{-}24h}$, likely due to a combination of rate of rapid uptake and the precision of time points despite that no protocol violation was reported for sample collection. The lack of change in ICG PK indicated that the hepatic uptake of ICG and blood flow in woodchucks was not significantly altered during the pseudo-hibernation period.

We acknowledge that the lack of direct evidence, i.e. the amount of 3TC recovered in urine, is a limitation when describing the changes in renal clearance. Caution should also be taken when interpreting these results because drugs chosen to be representative of certain drug disposition processes are based on human knowledge and may not be the same in woodchuck. Another limitation was that sex differences in drug metabolizing enzymes or transporters in woodchucks are not known.

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The changes in AUC_{inf} and CL values were consistent with those observed for $AUC_{0\text{-}24h}$. No statistically significant V_{ss} changes were observed in either male or female woodchucks. No consistent trends were observed for C_{max} and $t_{1/2}$.

These observed seasonal changes in PK and metabolism could impact how previously published woodchuck PK data are interpreted and understood. Few single dose woodchuck PK studies reported the study dates. For example, single doses of (-)-P-D-2,6-diaminopurine dioxolane were conducted between October 27 and November 10 which is approximately when indigenous woodchucks would begin winter hibernation (Rajagopalan et al., 1996). Single doses of 1-(2-fluoro-5-methyl-b-L-arabinofuranosyl)uracil were conducted between 17 April and 26 June 1996 (Witcher et al., 1997). However, study dates were not reported in many other woodchuck PK studies, particularly for those efficacy studies required multiple weeks of treatment, for example, 12 weeks treatment of adefovir dipivoxil (Cullen et al., 2001), 4 weeks treatment of tenofovir disoproxil fumarate (Menne et al., 2005), 10 weeks treatment of MIV-210, a prodrug of 3-fluoro-2,3-dideoxyguanosine (Michalak et al., 2009) and 4-8 weeks treatment of VES (Menne et al., 2015). In many of these studies, seasonal PK variability was not taken into consideration. Typically, only PK data from pre-study and/or single doses were reported and these may not be representative of the PK from the efficacy studies. This will make it challenging to compare and reproduce the PK results.

This information can also be used to better plan the long-term woodchuck efficacy studies to not overlap with the pseudo-hibernation period. Treatment

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duration in woodchuck model for HBV typically lasts multiple weeks. If the treatment starts after August or before February, significant change in exposure would be expected given the observed reduction in oxidative metabolism, phase II glucuronidation and renal clearance in woodchucks during pseudo-hibernation. The high seasonal PK variability could lead to significant changes in pharmacologic response and potential toxicity.

In summary, seasonal changes were observed in PK and body weight, but not body temperature and heart rate, in laboratory woodchucks. Significant $AUC_{0\text{-}24h}$ increases for AP, RTG and 3TC indicated decreases in oxidative metabolism, phase II glucuronidation and renal clearance during pseudo-hibernation. The lack of seasonal change in ICG exposure suggested there was no significant change in hepatic function. This information can be used to optimize the scheduling of woodchuck studies to avoid seasonally-driven variation in drug PK, pharmacodynamics and/or toxicity.

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Acknowledgments

We thank Ryan Nguyen and Dr. Robert Strickley for formulation support; Jennifer Tang for editorial assistance using GraphPad Prism; Northeastern Wildlife (Harrison, ID) for conducting the *in vivo* animal PK studies; Charles River Laboratories (Worcester, MA) for bioanalytical support.

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Authorship Contributions

- *Participated in research design: Zheng, Balsitis, Santos, Smith, Subramanian.*
- *Performed data analysis: Zheng, Santos.*
- *Wrote or contributed to the writing of the manuscript: Zheng, Balsitis, Santos, Smith, Subramanian.*

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Footnotes

This research was funded by Gilead Sciences, Inc. All authors are current employees of Gilead Sciences and declaring no competing interest.

[S]This article has supplemental material available at dmd.aspetjournals.org.

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Figure Legends

Fig. 1. Seasonal changes of physiological parameters from April 12, 2017 to April 18, 2018: A) Body weight; B) Body temperature; C) Heart rate. Dates were plotted from April 12, 2017 to April 18, 2018. The body weight increases from April 12, 2017 to August 14, 2017 were statistically significant for both male ($p < 0.0001$) and female ($p < 0.0001$) woodchucks even though the magnitude of change in males was greater than that in females. No significant changes in body temperature and heart rate were observed throughout the study duration for either male or female woodchucks. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean ± SD, n = 6 or 7 for male, n = 7 or 9 for female. Three woodchucks died during the study).

Fig. 2. Representative plasma concentration-time profiles following an IV bolus injection to woodchucks on November 23, 2017: A) AP; B) RTG; C) 3TC; D) ICG. Statistically significant seasonal sex differences were observed for AP ($p = 0.0011$), RTG ($p = 0.011$) and 3TC ($p = 0.012$) but not for ICG ($p = 0.88$) at the end of November. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean ± SD, n = 6 for male, n = 7 for female).

Fig. 3. Individual and mean seasonal $AUC_{0\text{-}24h}$ changes of AP: A) Male; B) Female. Dates were plotted from April 13, 2017 to March 18, 2018. The $AUC_{0\text{-}24h}$ of AP remained relatively unchanged from April to August, then gradually increased from August to the end of November, reaching the maximum by the end of November and subsequently returned to baseline in March of the following year. A similar trend was observed in female woodchucks, but the peak exposure

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was reached at the end of October. The exposure increases from May to November in both male and female woodchucks were statistically significant. The magnitude of exposure change was less pronounced in females than in males. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method. Three woodchucks died during the study.

Fig. 4. Individual and mean seasonal $AUC_{0\text{-}24h}$ changes of RTG: A) Male; B) Female. Dates were plotted from April 13, 2017 to March 18, 2018. The $AUC_{0\text{-}24h}$ of RTG remained relatively unchanged from April to August, then gradually increased from August to the end of November, reaching the maximum by the end of November and subsequently returned to baseline in March of the following year. A similar trend was observed in female woodchucks, but the peak exposure was reached at the end of October. The exposure increases from May to November in both male and female woodchucks were statistically significant. The magnitude of exposure change was less pronounced in females than in males. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method. Three woodchucks died during the study.

Fig. 5. Individual and mean seasonal $AUC_{0\text{-}24h}$ changes of 3TC: A) Male; B) Female. Dates were plotted from April 13, 2017 to March 18, 2018. The $AUC_{0\text{-}24h}$ of 3TC remained relatively unchanged from April to August, then gradually increased from August to the end of November, reaching the maximum by the end of November and subsequently returned to baseline in March of the following year. A similar trend was observed in female woodchucks, but the peak exposure was reached at the end of October. The exposure increases from May to

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November in both male and female woodchucks were statistically significant. The magnitude of exposure change was less pronounced in females than in males. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method. Three woodchucks died during the study.

Fig. 6. Individual and mean seasonal $AUC_{0\text{-}24h}$ changes of ICG: A) Male; B) Female. Dates were plotted from April 13, 2017 to March 18, 2018. No significant ICG $AUC_{0\text{-}24h}$ changes were observed in either male or female woodchucks. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method. Three woodchucks died during the study.

Fig. 7. Representative seasonal $AUC_{0\text{-}24h}$ comparison between May 11 and November 23, 2017 in males: A) AP; B) RTG; C) 3TC; D) ICG. The exposure increases were statistically significant for AP, RTG and 3TC but not for ICG. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean ± SD, n = 6 or 7 for male, n = 7 or 9 for female. Three woodchucks died during the study).

Fig. 8. Representative seasonal $AUC_{0\text{-}24h}$ comparison between May 11 and November 23, 2017 in females: A) AP; B) RTG; C) 3TC; D) ICG. The exposure increases were statistically significant for AP, RTG and 3TC but not for ICG. Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean ± SD, n = 6 or 7 for male, n = 7 or 9 for female. Three woodchucks died during the study).

Fig. 9. Comparison of representative other seasonal PK parameters (AUC_{inf}, C_{max}, $t_{1/2}$, V_{ss} and CL) between May 11 and November 23, 2017 in: A) male; B)

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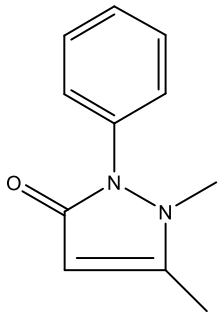
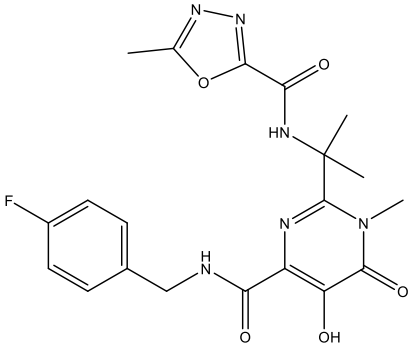
female. Data in green were from May 11, 2017 and data in red were from Nov 23, 2017. AUC_{inf} and CL values were plotted in log scale while other PK parameters were plotted in linear scale. Symbols: ∆, AP; X, RTG; ○, 3TC; □, ICG. In both male and female woodchucks, AUC_{inf} increases were statistically significant for AP ($p < 0.0001$ for male and $p = 0.026$ for female), RTG ($P < 0.0001$ for both male and female) and 3TC ($p < 0.0001$ for male and $p = 0.041$ for female) but not for ICG ($p = 0.69$ for male and $p = 0.77$ for female). No statistically significant V_{ss} changes ($p \geq 0.22$) were observed for any of the four compounds tested in either male or female woodchucks. In both male and female woodchucks, CL decreases were statistically significant for AP ($p < 0.0001$ for male and $p = 0.0047$ for female), RTG ($p < 0.0001$ for both male and female) and 3TC ($p < 0.0001$ for male and $p = 0.016$ for female) but not for ICG ($p = 0.29$ for male and $p = 0.48$ for female). In male, increases of C_{max} were statistically significant for AP ($p = 0.0017$), RTG ($p < 0.0001$) and 3TC ($p < 0.0001$) but not for ICG ($p = 0.68$). In female, C_{max} increases were statistically significant for ICG ($p = 0.037$) but not for AP ($p = 0.14$), RTG ($p = 0.28$) or 3TC ($p = 0.36$). In male, increases of $t_{1/2}$ were statistically significant for AP ($p < 0.0001$) and 3TC ($p = 0.0003$) but not for RTG ($P = 0.061$) or ICG ($p = 0.48$). In female, $t_{1/2}$ increases were statistically significant for AP ($p = 0.039$) but not for RTG ($p = 0.30$), 3TC ($p = 0.14$) or ICG ($p = 0.70$). Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean ± SD, n = 6 or 7 for male, n = 7 or 9 for female. Three woodchucks died during the study).

Tables

TABLE 1

Summary of structure and disposition pathways of the four probe compounds

Compounds	Structure	Indication	Routes of Clearance in Human
Antipyrine (AP)		Not applicable	CYP-mediated oxidative metabolism by 1A2, 2B6, 2C8, 2C9, 2C18 and 3A4 (Balani et al., 2002)
Raltegravir (RTG)		Anti-HIV	Phase II glucuronidation by UGT1A1 (Kassahun et al., 2007; Liu et al., 2019)

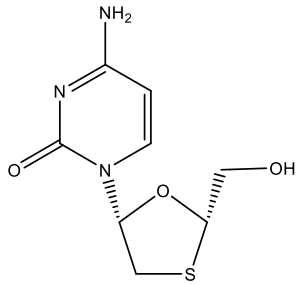
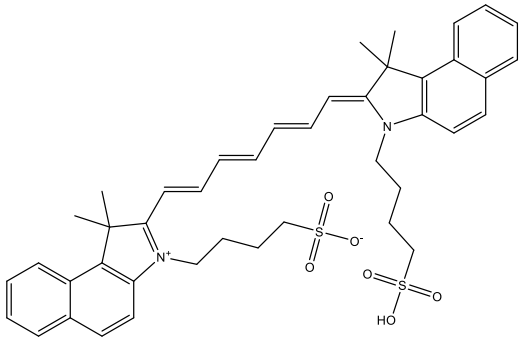
Lamivudine (3TC)		Anti-HIV	Active transport and renal clearance by MDR1, MRP1, MRP2, MRP3, MRP4 and BCRP (Johnson et al., 1999)
Indocyanine Green (ICG)		Medical diagnostics	Transported by OATP1B3 and NTCP (de Graaf et al., 2011). Clearance route for ICG is unclear (Huang and Vore, 2001; Hwang et al., 2017).

TABLE 2

A snapshot of the seasonal woodchuck PK study design

Dose Frequency	Once every four weeks from March 16, 2017 to March 13, 2018
Dose Route	Intravenous bolus administration via 2-minute slow push
Dose Volume, Concentration, Amount	1 mL/kg, 0.5 mg/mL, cassette dose at 0.5 mg/kg for each compound
Formulation Vehicle	10% ethanol, 10% polyethylene glycol 300 and 80% water
Fasted/Fed	Non-fasted
Anesthetic agents	ketamine/xylazine combination
Number of Animals	7 Males, 9 Females
Sample Matrix	Plasma
Time Points	5 min, 10 min, 1 h, 2 h, 4 h, 8 h, 24 h
Physiological Parameters	Body weight, body temperature and heart rate

Lab Conditions	Steady room temperature, mimicking external seasonal light cycle

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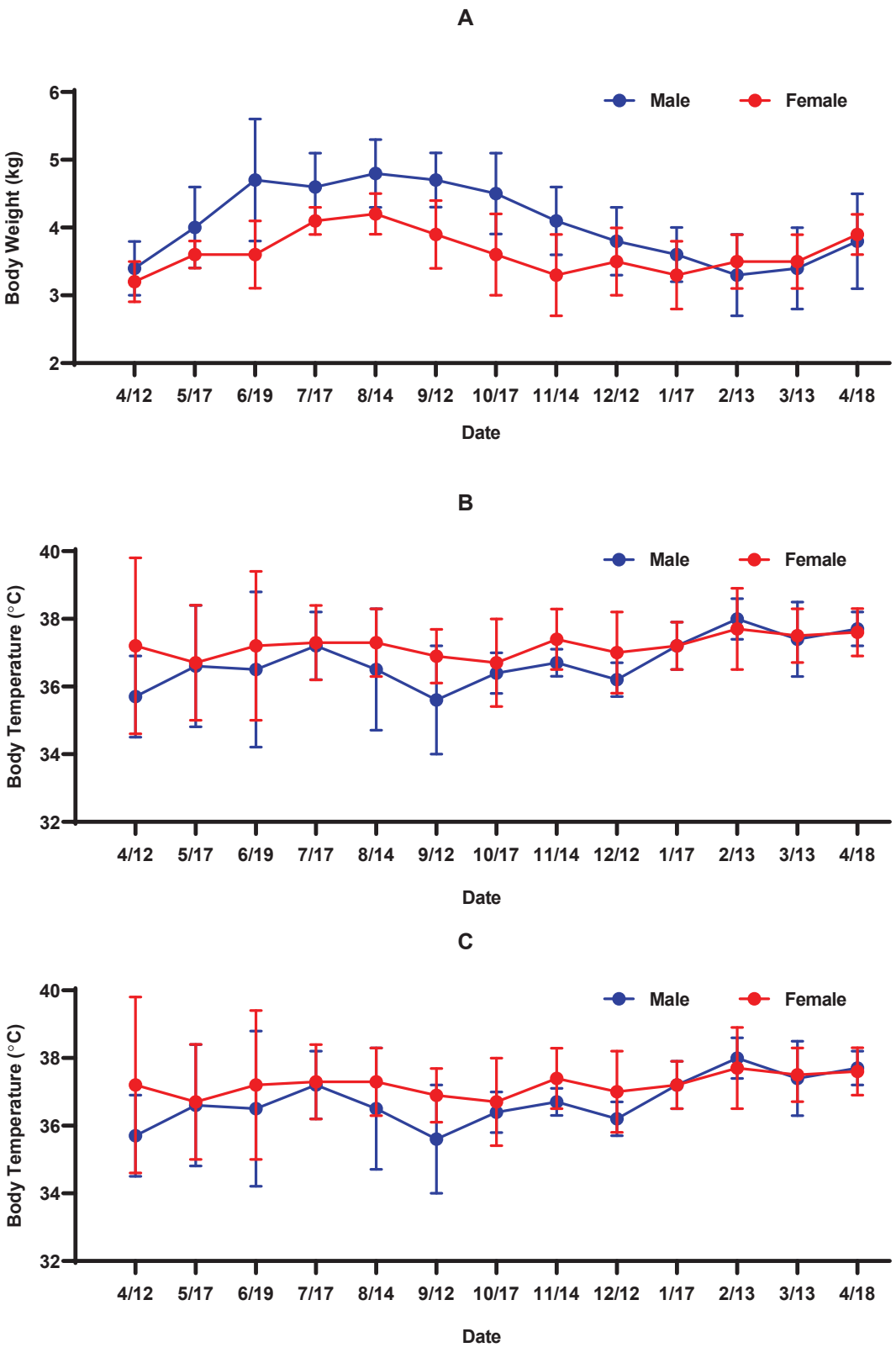

Fig. 1

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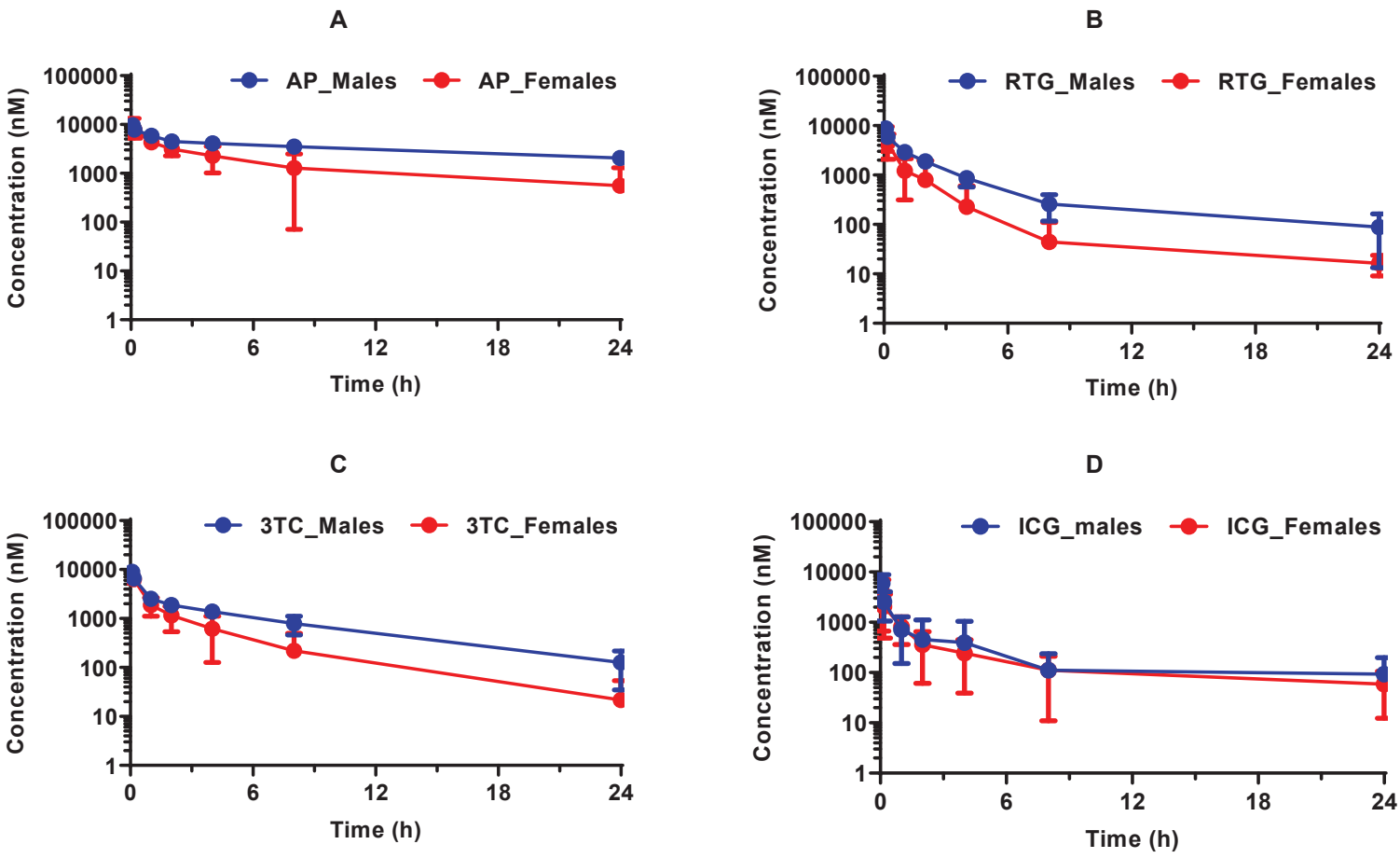

Fig. 2

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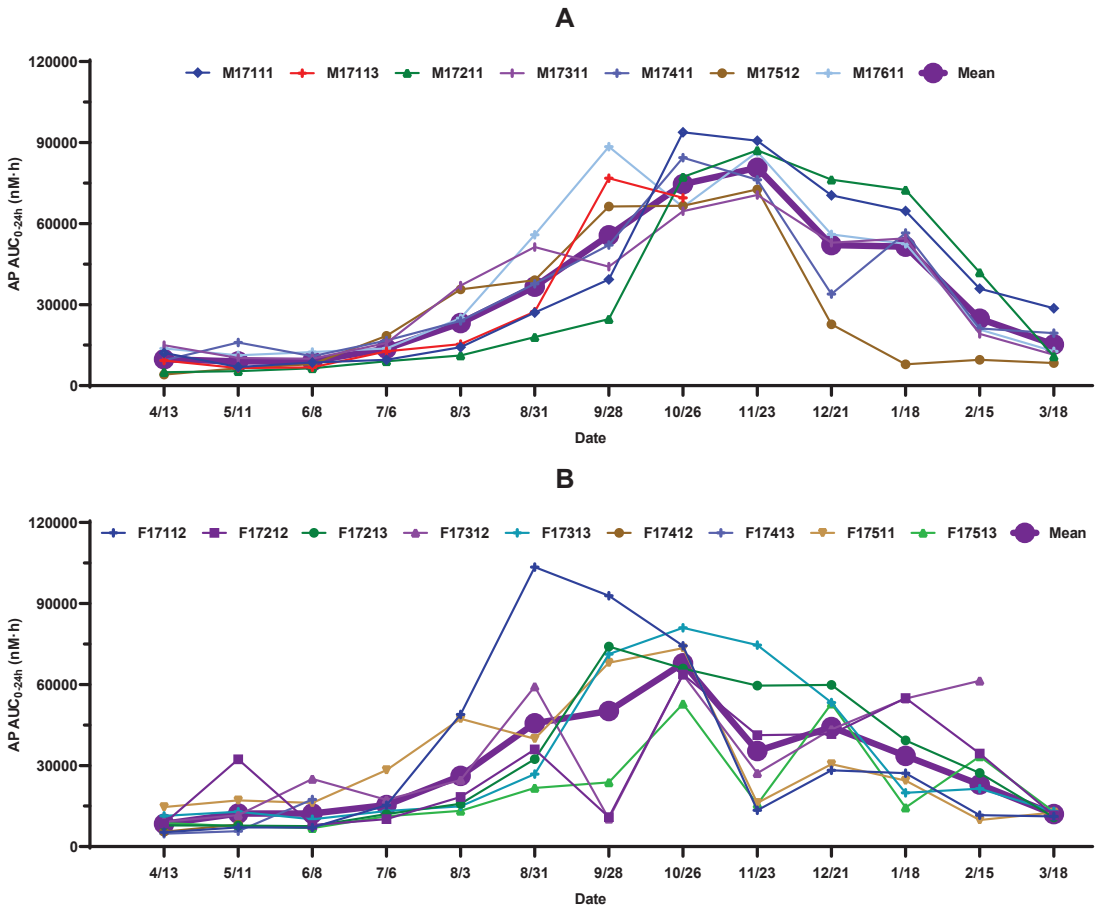

Fig. 3

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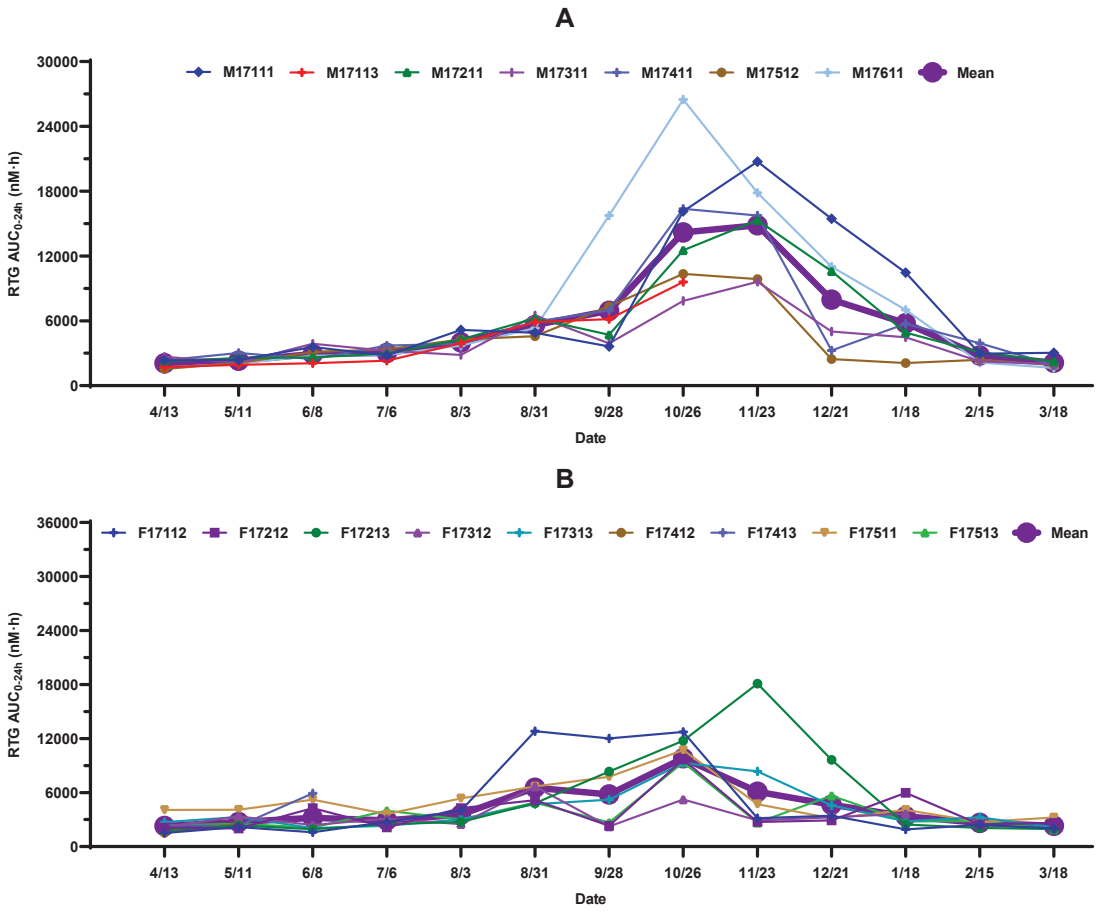

Fig. 4

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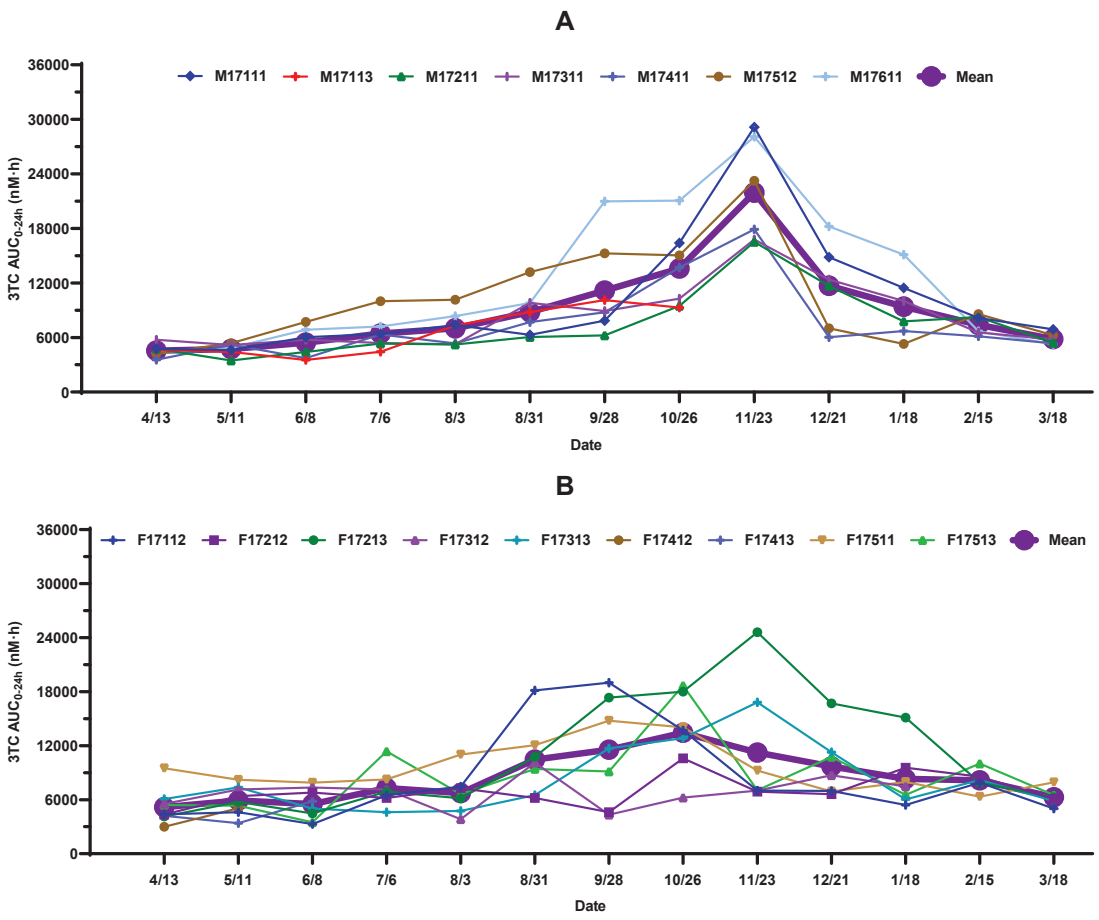

Fig. 5

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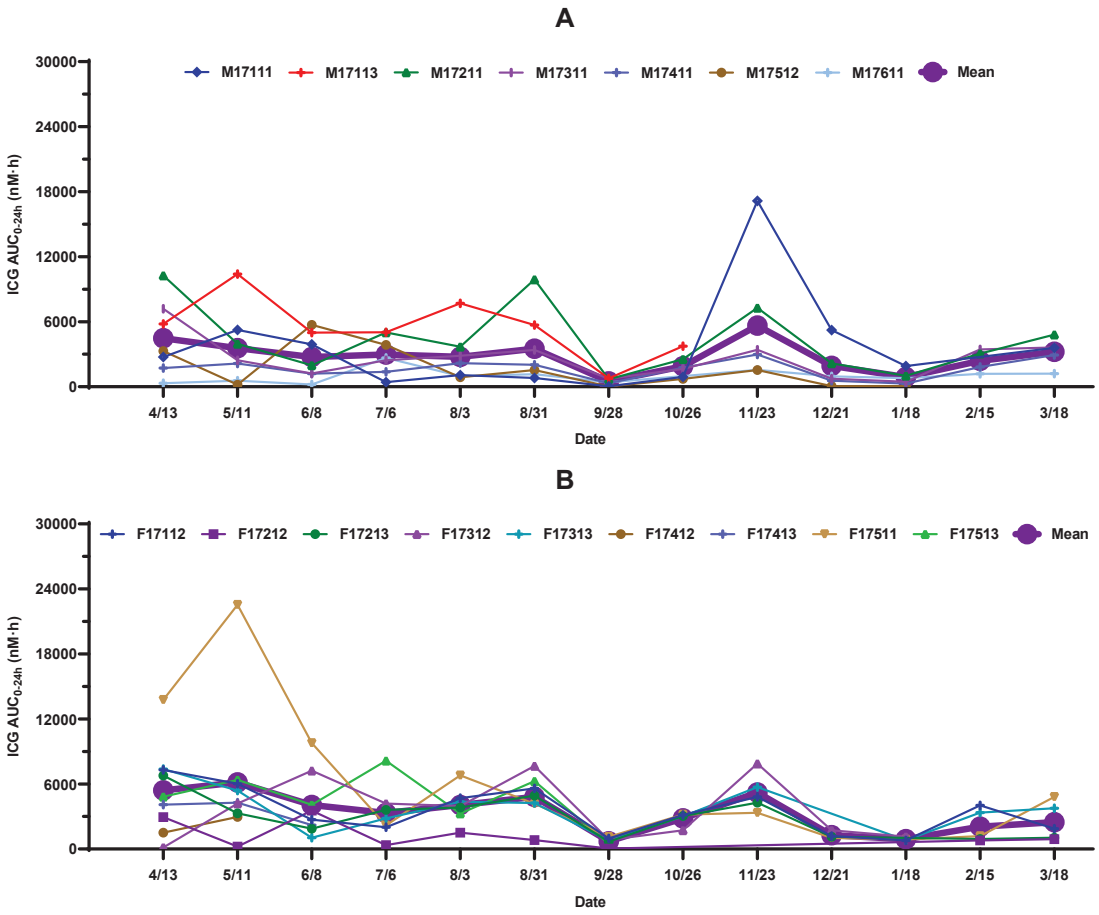

Fig. 6

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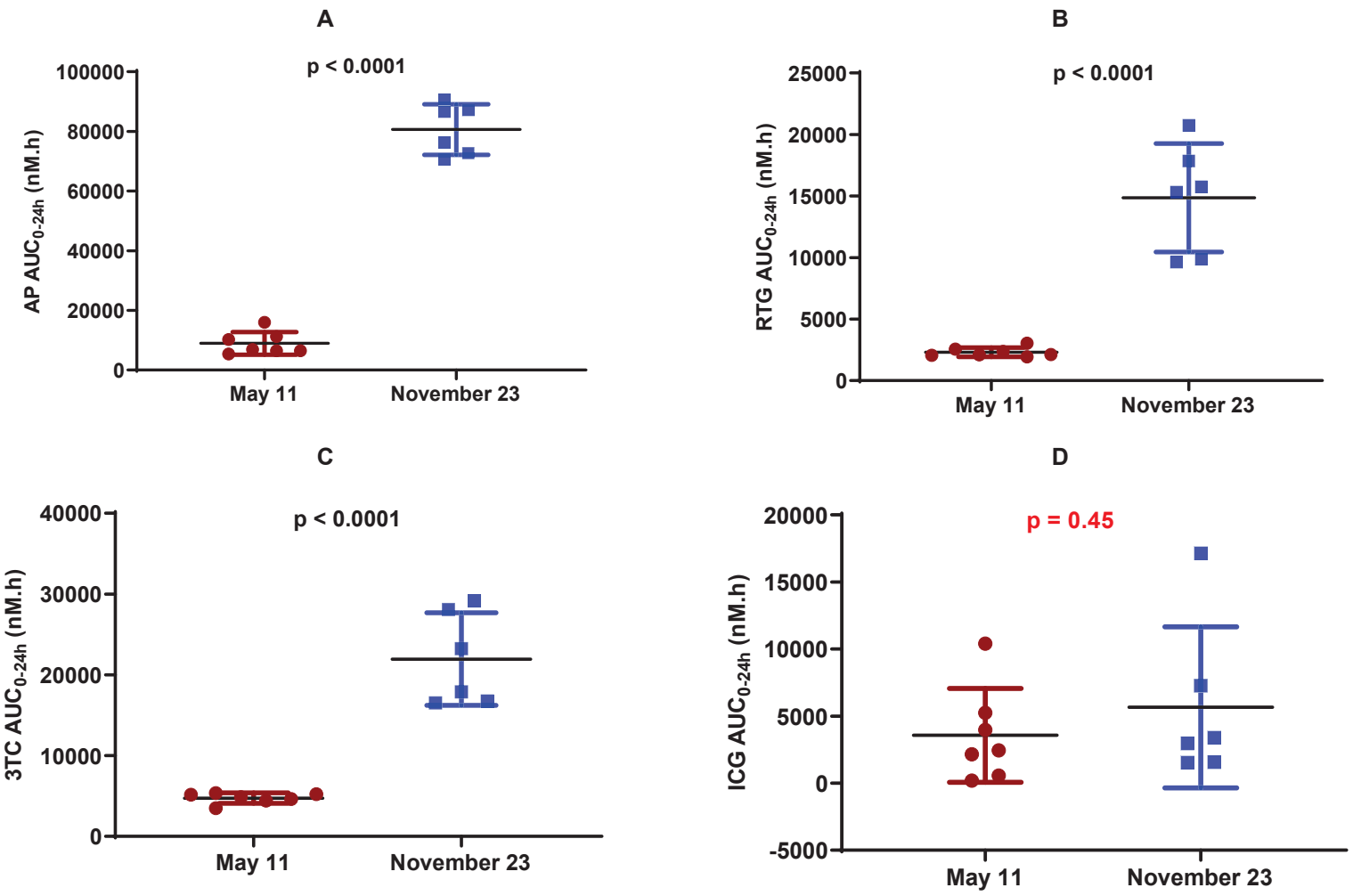

Fig. 7

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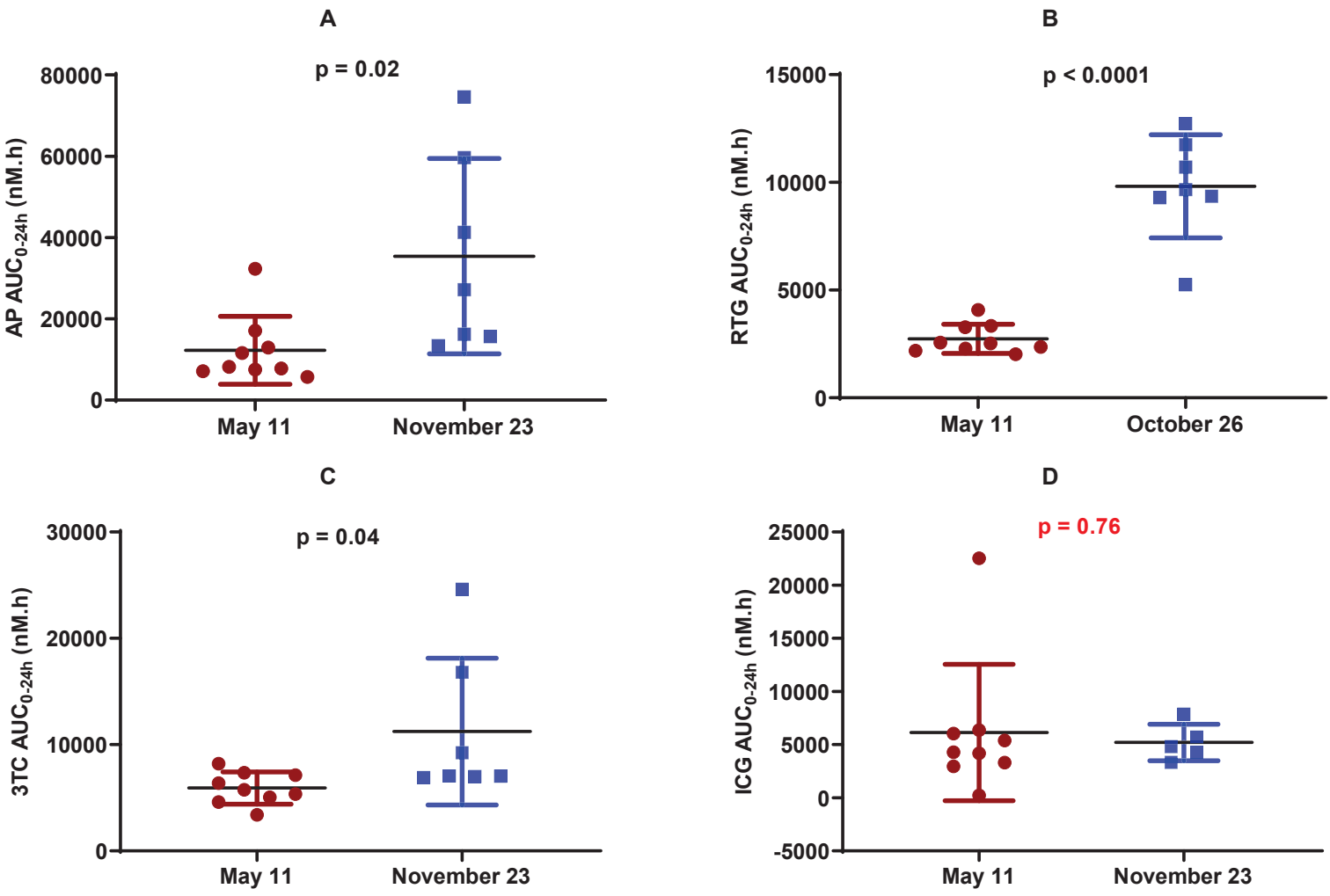

Fig. 8

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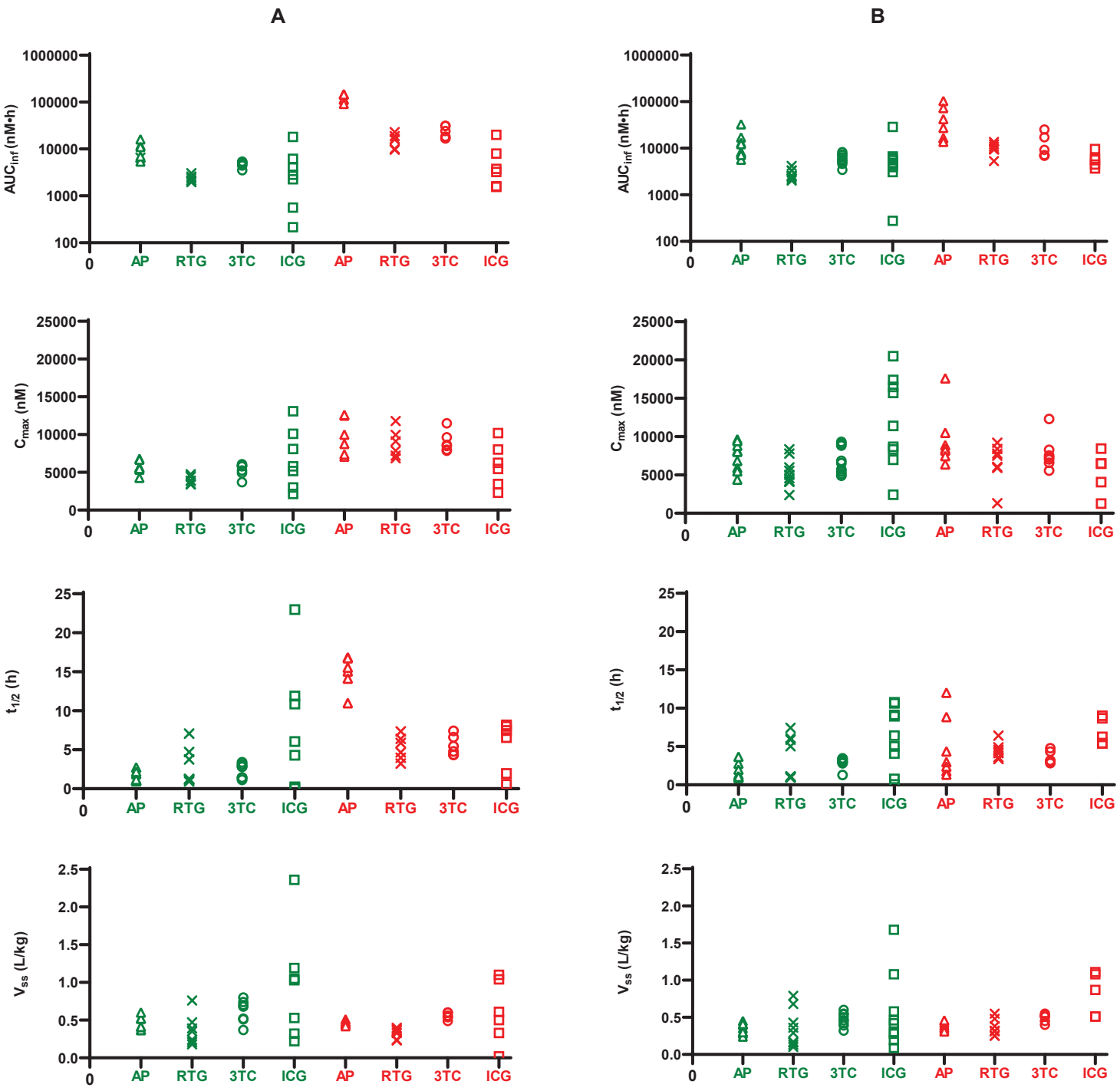

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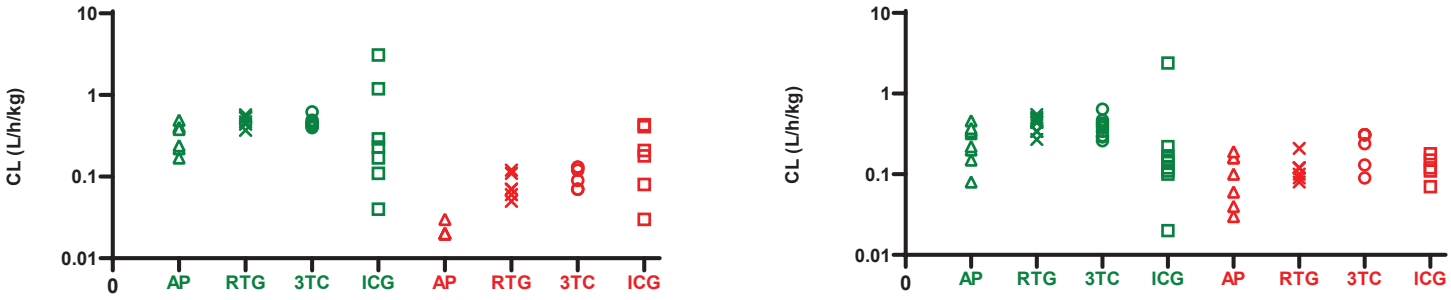

Fig. 9