

Characterization of Seasonal Pharmacokinetic Variability in Woodchucks[□]

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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 infection. Under laboratory conditions, woodchucks develop a pseudohibernation condition; 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 seven male and nine female laboratory-maintained woodchucks. These compounds were selected to study changes in oxidative metabolism [antipyrene (AP)], glucuronidation [raltegravir (RTG)], renal clearance [lamivudine (3TC)], and hepatic function [indocyanine green (ICG)]. Seasonal changes in physiologic parameters and PK were determined. Seasonal body weight increases were $\geq 30\%$. 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 pseudohibernation. The lack of seasonal change in ICG exposure suggests there are no significant changes in hepatic function. This information can be used to optimize the scheduling of woodchuck studies to avoid seasonally driven variation in drug PK.

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, who use 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 pseudohibernation condition.

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 as a result of liver diseases associated with chronic hepatitis B (CHB), primarily due to liver diseases such as cirrhosis and hepatocellular carcinoma (World Health Organization) (<https://www.who.int/news-room/fact-sheets/detail/hepatitis-b>). Approved treatment of CHB includes nucleos(t)ide analogs as well as interferon- α . 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 toll-like receptor 7 agonist vesatolimod (GS-9620) and the toll-like receptor 8 agonist selgantolimod (GS-9688) (Menne et al., 2015; Paulsen et al., 2015; Daffis et al., 2017, 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 13-lined ground squirrel (*Citellus tridecemlineatus*) (Hong, 1957).

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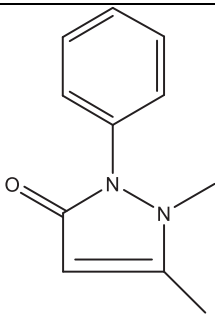
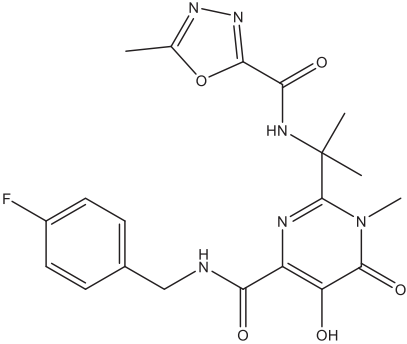
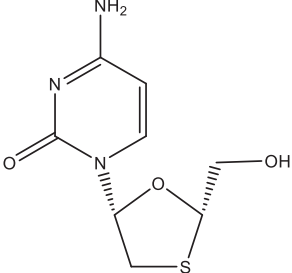
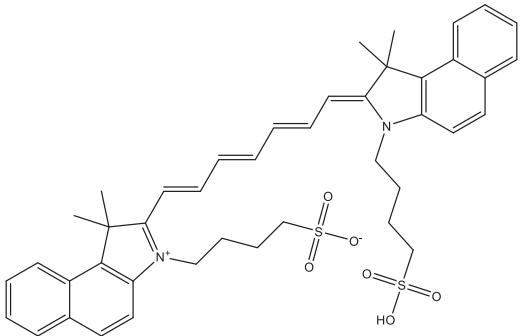
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ABBREVIATIONS: AP, antipyrene; AUC_{0–24h}, area under time-concentration curve from time 0 to 24 hours; AUC_{inf}, area under time-concentration curve from time 0 to infinity; BPM, beats per minute; CHB, chronic hepatitis B; CL, clearance; HBV, hepatitis B virus; ICG, indocyanine green; PK, pharmacokinetics; RTG, raltegravir; $t_{1/2}$, terminal half-life; 3TC, lamivudine; V_{ss}, steady-state volume of distribution; WHV, woodchuck hepatitis virus.

TABLE 1
Summary of structure and disposition pathways of the four probe compounds

Compounds	Structure	Indication	Routes of Clearance in Human
AP		Not applicable	Cytochrome P450-mediated oxidative metabolism by 1A2, 2B6, 2C8, 2C9, 2C18, and 3A4 (Balani et al., 2002)
RTG		Anti-HIV	Phase II glucuronidation by UGT1A1 (Kassahun et al., 2007; Liu et al., 2019)
3TC		Anti-HIV	Active transport and renal clearance by MDR1, MRP1, MRP2, MRP3, MRP4, and BCRP (Johnson et al., 1999)
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).

BCRP, breast cancer resistance protein; HIV, human immunodeficiency virus; MDR1, multidrug resistance mutation 1; MRP, multidrug resistance-associated protein; NTCP, sodium taurocholate cotransporting polypeptide; OATP1B3, organic anion transporter family 1 member B3; UGT1A1, UDP (uridine diphosphate) glucuronosyltransferase family 1 member A1.

In addition, seasonal changes in endocannabinoid concentrations between active and hibernating marmots were observed (Mulawa et al., 2018).

Under laboratory conditions, woodchucks develop a pseudohibernation 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 pseudohibernation 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.

Data describing the potential seasonal changes in metabolism and PK are scarce, especially if animals develop a pseudohibernation condition during winter. This report presents the characterization of seasonal PK changes of four probe compounds dosed intravenously once every

TABLE 2
A snapshot of the seasonal woodchuck PK study design

Dose Frequency	Once Every 4 wk from March 16, 2017, to April 18, 2018
Dose route	Intravenous bolus administration via 2-min 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	Nonfasted
Anesthetic agents	Ketamine/xylazine combination
Number of animals	Seven males, nine females
Sample matrix	Plasma
Time points	5 and 10 min and 1, 2, 4, 8, and 24 h
Physiologic parameters	Body weight, body temperature, and heart rate
Laboratory conditions	Steady room temperature, mimicking external seasonal light cycle

4 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 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).

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 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 (*M. monax*), seven males and nine 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 per milliliter 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

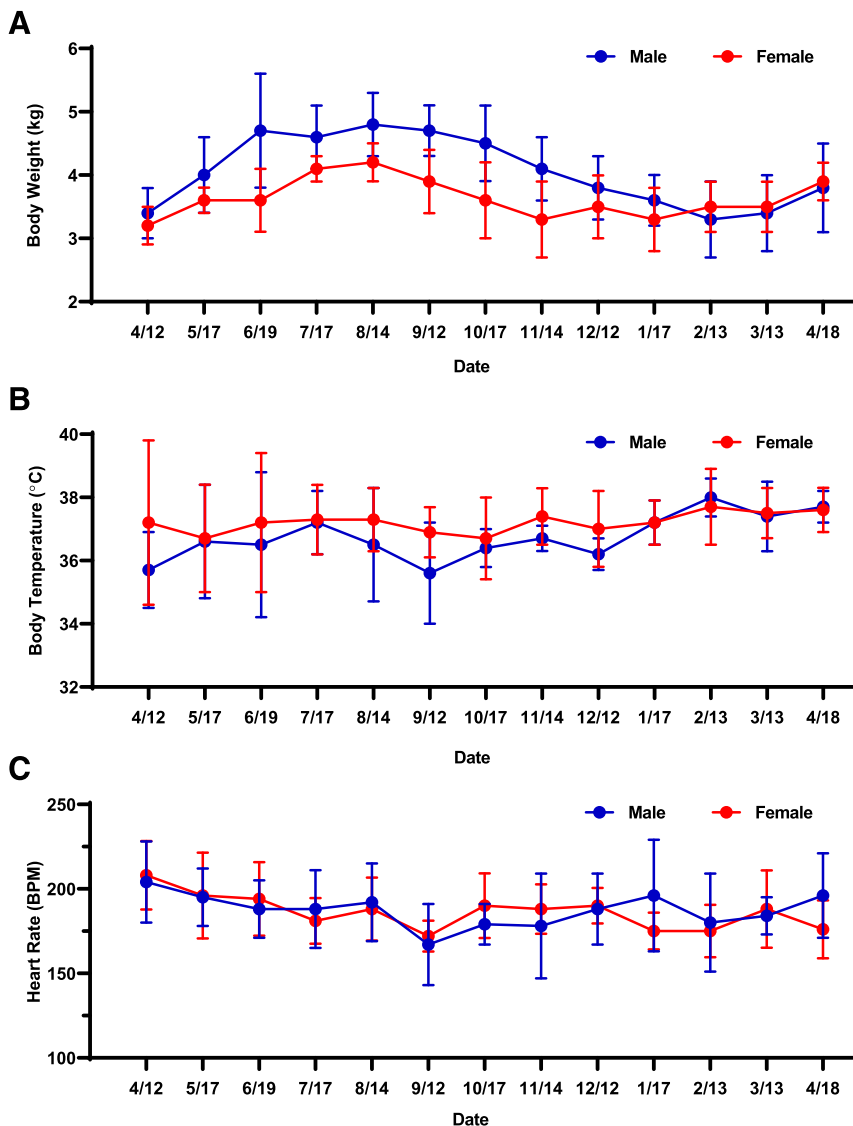


Fig. 1. Seasonal changes of physiologic parameters from April 12, 2017, to April 18, 2018: (A) body weight; (B) body temperature; (C) heart rate. Dates were plotted from April 12, 2017, to April 18, 2018. The body weight increases from April 12, 2017, to August 14, 2017, were statistically significant 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 \pm S.D., $n = 6$ or 7 for male, $n = 7$ or 9 for female; three woodchucks died during the study).

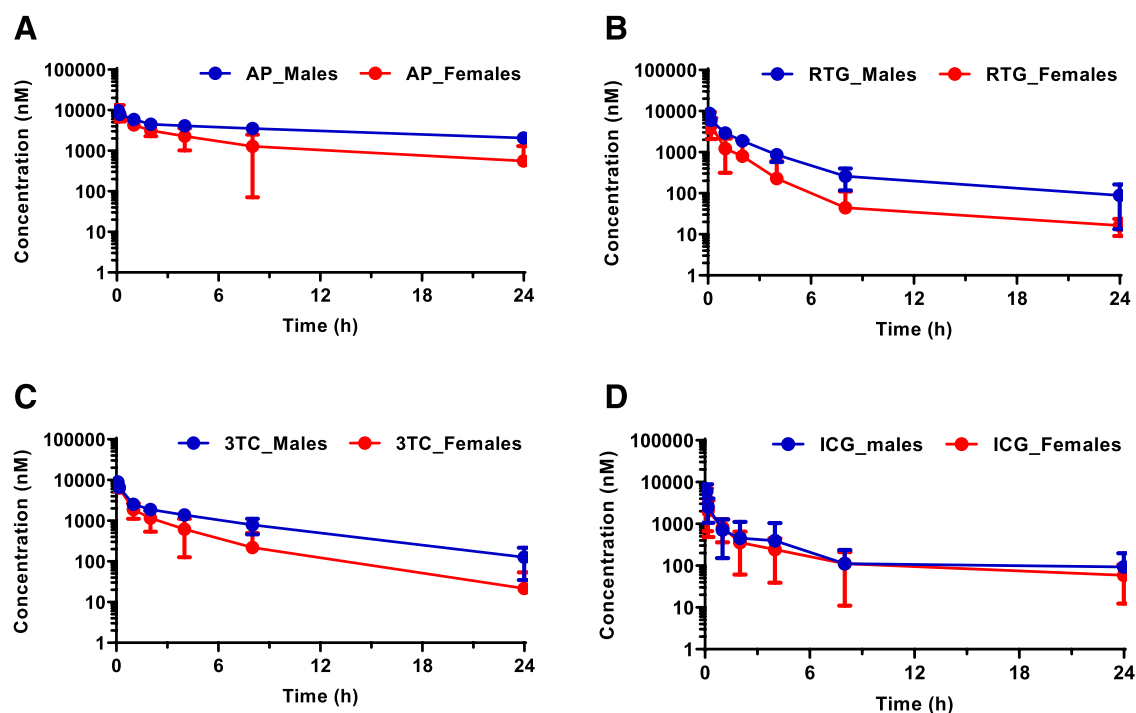


Fig. 2. Representative plasma concentration–time profiles after an intravenous bolus injection to woodchucks on November 23, 2017: (A) AP; (B) RTG; (C) 3TC; (D) ICG. Statistically significant seasonal sex differences were observed for AP ($P = 0.0011$), RTG ($P = 0.011$), and 3TC ($P = 0.012$) but not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

shavings, maintained under daily 12-hour light/dark cycles, and fed with rabbit chow [Purina; 16% crude protein ($\geq 18\%$ for pups and pregnant animals), 2% crude fat, $\sim 15\%$ crude fiber, vitamin A (4500 IU), 1% calcium, and 0.5% phosphate]. Animals were not fasted prior to treatment.

Seasonal Single-Dose PK Studies in Woodchucks. The animal protocol and all procedures involving woodchucks were reviewed and approved by the Northeastern Wildlife institutional animal care and use committee 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 prestudy was conducted on March 16, 2017, to ensure animal safety and to safeguard any potential adverse event due to a four-drug cocktail coadministration. In that study, five separate vials of formulated solution were provided, including four vials containing the individual compounds formulated separately and a fifth vial with all four compounds coformulated into a single formulation. The four compounds, AP, RTG, 3TC, and ICG, were either individually formulated or coformulated 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 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 2–8°C, and protected from light. Animals were divided into five groups of $n = 3$ for each. ICG, RTG, 3TC, AP, and the four-compound coformulation were administered to woodchucks in groups 1–5, 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 coformulated cocktail of all four compounds consisted 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 2–8°C, and protected from light. One vial was shipped to Northeastern Wildlife, Inc., for each monthly dosing.

The monthly dosing was scheduled to occur once every 4 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. K₂EDTA was used for PK and hematology sample collection. No anticoagulant was used for samples collected for clinical chemistry. Roughly 1.0 ml of blood was collected for complete blood count and hematology. PK samples were collected at 5 and 10 minutes and 1, 2, 4, 8, and 24 hours 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, complete blood count, 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 samples were not collected, to minimize disruption of the animals.

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 they were shipped to Charles River Laboratories (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 Tables 1 and 2. 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 hours (AUC_{0-24h}), area under the plasma concentration–time curve from time 0 to infinity (AUC_{inf}), C_{max} , terminal half-life ($t_{1/2}$), steady-state volume of distribution (V_{ss}), and clearance (CL), were determined by noncompartmental analysis using Phoenix WinNonlin 6.4 (Pharsight Corporation, Princeton, NJ).

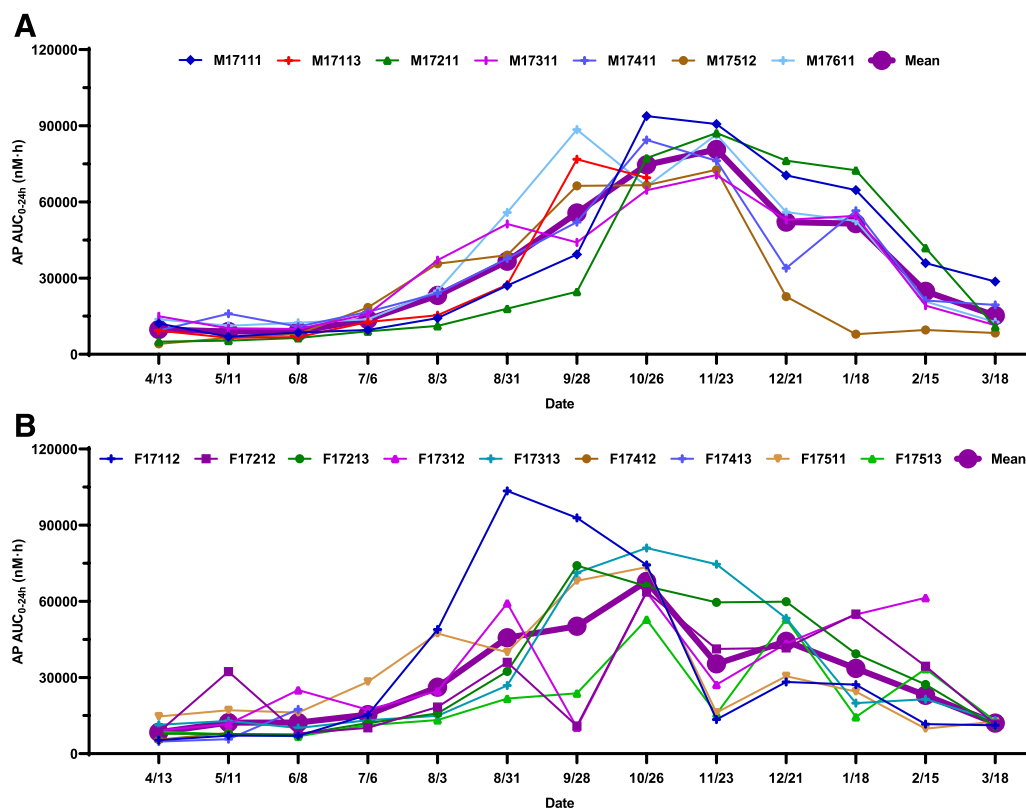


Fig. 3. Individual and mean seasonal AUC_{0-24h} changes of AP: (A) male; (B) female. Dates were plotted from April 13, 2017, to March 18, 2018. The AUC_{0-24h} of AP remained relatively unchanged from April to August and then gradually increased from August to the end of November, reaching the maximum by the end of November and subsequently returning 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.

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 S.D. for both populations. P values less than 0.05 were considered statistically significant. At least two values were required to calculate the mean. At least three values were required to report S.D.

Results

Woodchuck Baseline Characteristics. The mean body weight was 3.3 (S.D. 0.4) kg (range 2.6–3.9 kg). The mean (S.D.) body temperature was 37.7°C (0.9) (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 γ -glutamyl transferase <10 international units per liter at the time of study initiation and were in good overall health.

Seasonal Changes of Physiologic 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 woodchucks 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 ($42\% \pm 12\%$) was greater than that in females ($30\% \pm 18\%$). No significant changes (<10%) in body temperature and heart rate were observed throughout the study duration for either male or female woodchucks maintained under laboratory conditions (Fig. 1, B and C).

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

The prestudy results (Supplemental Table 3) did not show any significant drug–drug interactions when comparing the corresponding AUC_{0-24h} values of AP, RTG, 3TC, and ICG after the intravenous administration of individual formulation versus four-drug coformulation.

The AUC_{0-24h} of AP remained relatively unchanged from April to August and 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

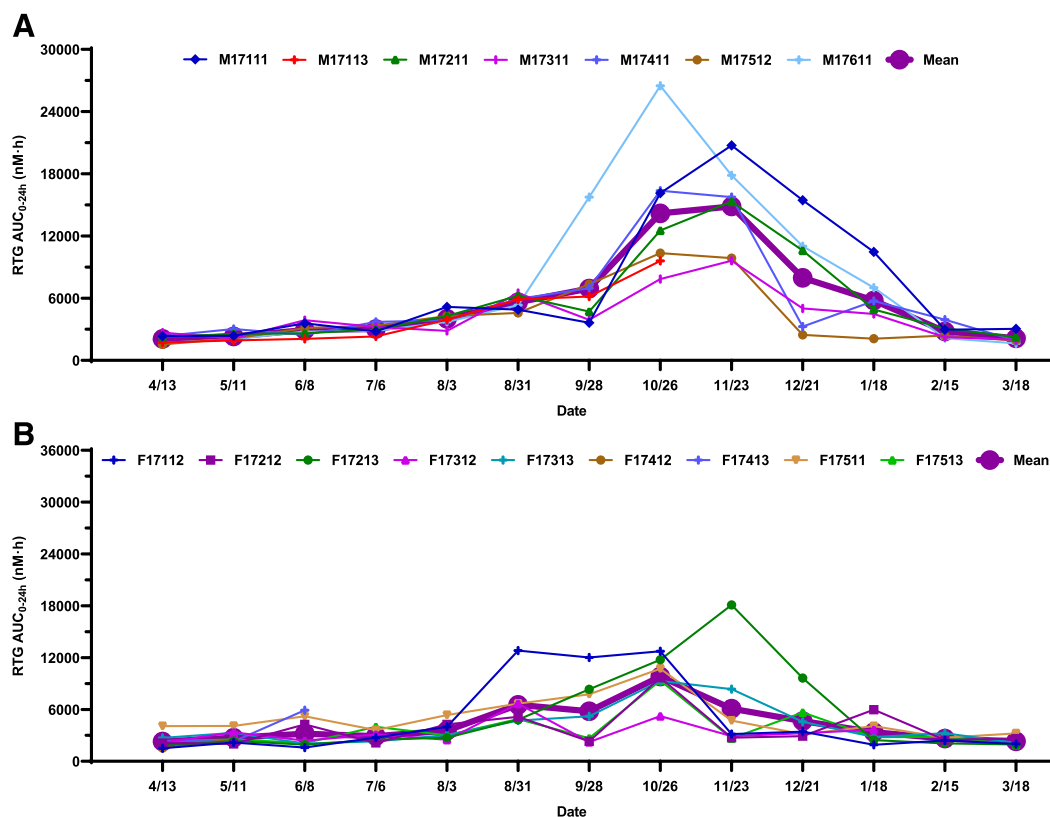


Fig. 4. Individual and mean seasonal AUC_{0-24h} changes of RTG: (A) male; (B) female. Dates were plotted from April 13, 2017, to March 18, 2018. The AUC_{0-24h} of RTG remained relatively unchanged from April to August and then gradually increased from August to the end of November, reaching the maximum by the end of November and subsequently returning 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. 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) 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 Supplemental Fig. 1A).

The trends of seasonal exposure changes for both RTG and 3TC in male (Fig. 4A; Fig. 5A) and female (Fig. 4B; Fig. 5B) woodchucks were very similar to those corresponding changes for AP (Fig. 3, A and B). Statistically significant increases of RTG and 3TC exposures from May 11 to November 23 were observed in both male (Fig. 7, B and C) and female (Fig. 8, B and C) woodchucks. No significant sex differences in AUC_{0-24h} 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. 2, B and C, Supplemental Fig. 1, B and C). No significant ICG AUC_{0-24h} changes were observed in either male (Fig. 6A; Fig. 7D) or female (Fig. 6B; Fig. 8D) woodchucks, and no sex difference was observed (Fig. 2D and Supplemental Fig. 1D) 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 ($P \geq 0.22$) 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 males, 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 females, 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 males, 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 females, $t_{1/2}$ increases were statistically significant for AP ($P = 0.039$) but not for RTG ($P = 0.30$), 3TC ($P = 0.14$)s or ICG ($P = 0.70$). Increased $t_{1/2}$ led to a higher percentage of extrapolated AUC_{inf} values, which were used to calculate CL values. As such, seasonal AUC_{0-24h} values were selected as the most representative PK parameters for discussion.

Discussion

Under wild conditions, it is well known that hibernating animals need to store energy by gaining weight before the winter and slow

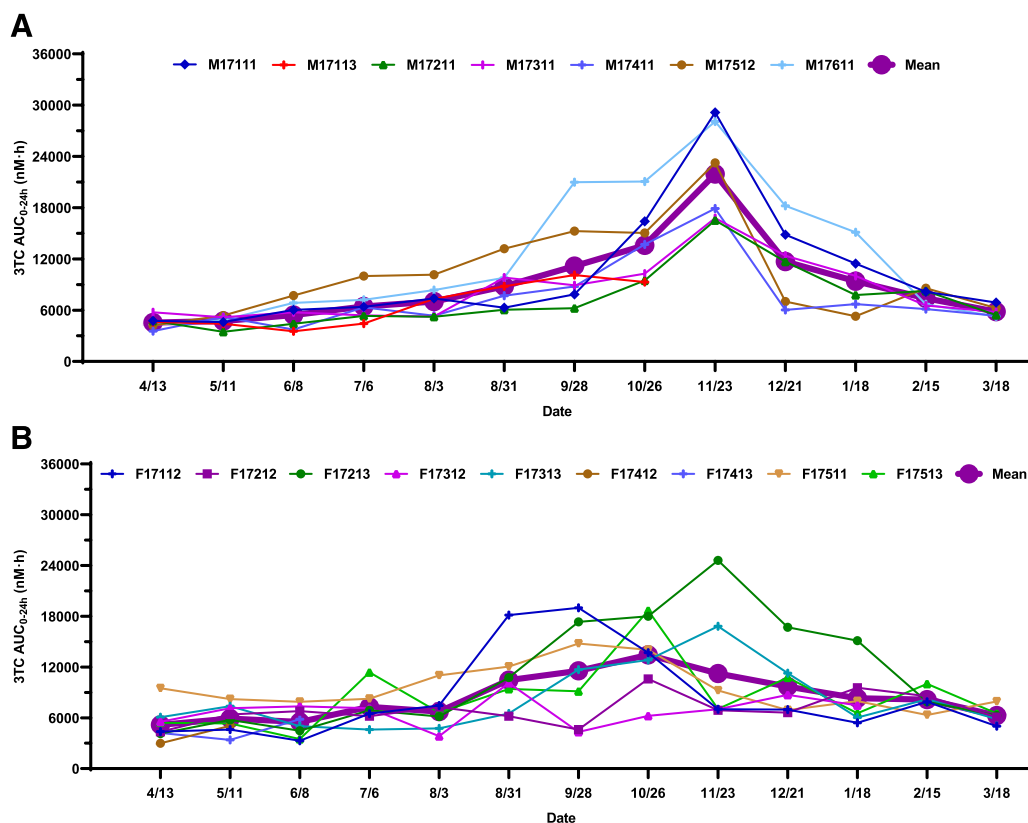


Fig. 5. Individual and mean seasonal AUC_{0-24h} changes of 3TC: (A) male; (B) female. Dates were plotted from April 13, 2017, to March 18, 2018. The AUC_{0-24h} of 3TC remained relatively unchanged from April to August and then gradually increased from August to the end of November, reaching the maximum by the end of November and subsequently returning 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.

their metabolism, lower body temperature, and 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 13-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 (Czeraniak, 2001), and hibernating animals such as woodchuck were not typically used as animal models to explore sex differences in toxicology studies (Curry, 2001).

The eastern woodchuck is a hibernating species that is being extensively used in a laboratory setting 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, 2020), and treatment of WHV-associated hepatocellular carcinoma (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 (Vybiral and Janský, 1997). 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 (Vybiral and Janský, 1997). For example, a hibernating-specific 88 kDa protein in the plasma of deeply hibernating

woodchucks was described (Horton et al., 1996). Husbandry of woodchucks in a laboratory facility removed the extrinsic factors such as light, temperature, and food availability that can trigger hibernation but is unlikely to alter the intrinsic factors, such as the trigger substance, that led to a pseudohibernation 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 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 laboratory conditions (Fig. 1, B and C). The lack of change in body temperature and heart rate was likely due to woodchucks being kept awake and active under laboratory conditions in contrast to deep hibernation under wild conditions during winter time.

Seasonal plasma AUC_{0-24h} changes of AP, RTG, and 3TC were used as the markers of change in oxidative metabolism, phase II glucuronidation, or renal clearance in woodchucks, respectively. The significantly higher AP, RTG, and 3TC exposures on November 23, 2017, versus May 11, 2017, indicated that oxidative metabolism, phase II glucuronidation, and renal clearance in woodchuck were significantly reduced during the pseudohibernation. The seasonal sex differences observed at the end of November were mainly due to the magnitude of

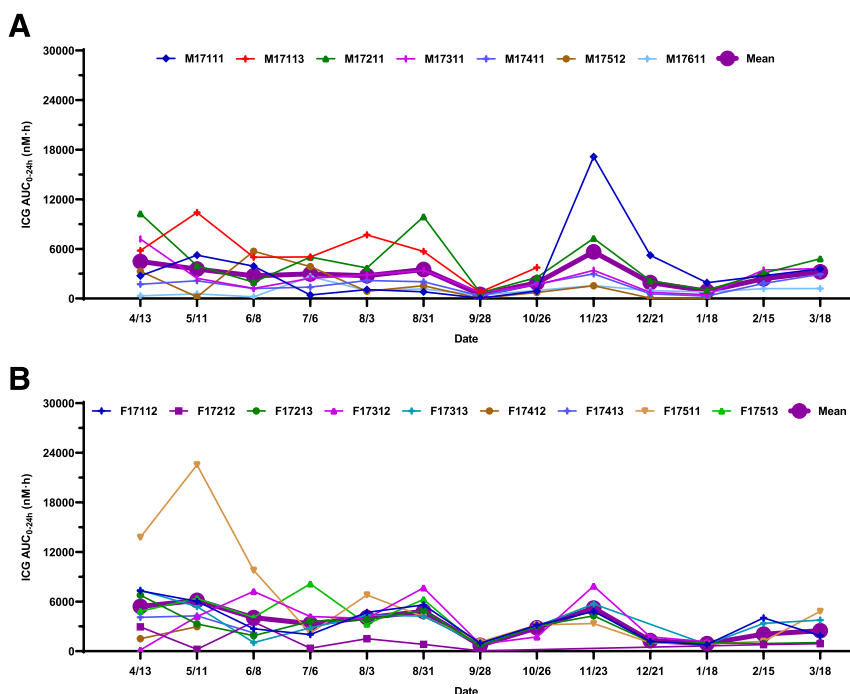


Fig. 6. Individual and mean seasonal AUC_{0-24h} changes of ICG: (A) male; (B) female. Dates were plotted from April 13, 2017, to March 18, 2018. No significant ICG AUC_{0-24h} changes were observed in either male or female woodchucks. Statistical analysis was performed using an unpaired, two-tailed,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

maximum exposure change being less pronounced in females than in males and because the peak exposures were not synchronized between male and female woodchucks.

Seasonal AUC_{0-24h} of ICG was used as a marker of hepatic function in woodchucks. High variability was observed for ICG AUC_{0-24h} , likely due to a combination of rate of rapid uptake and the

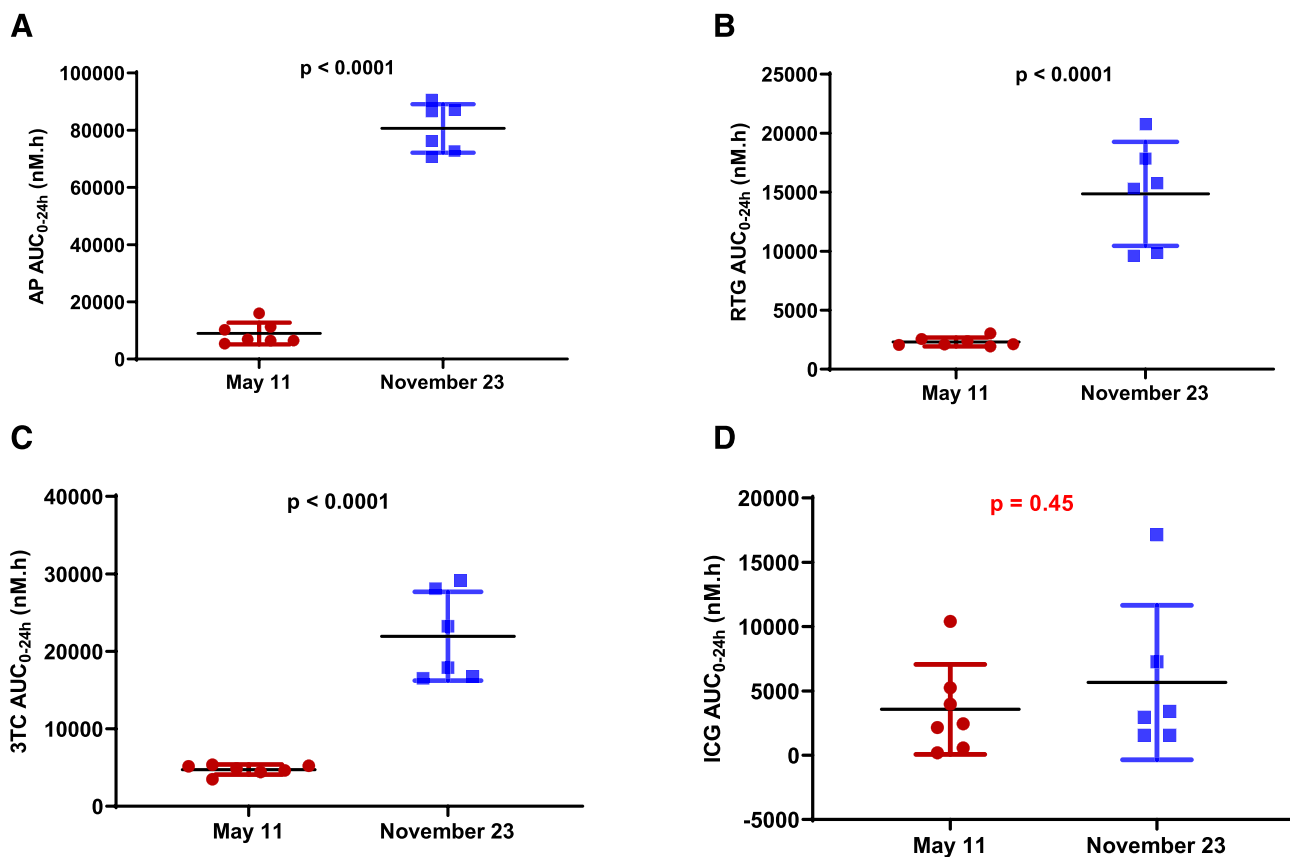


Fig. 7. Representative seasonal AUC_{0-24h} comparison between May 11 and November 23, 2017, in males: (A) AP; (B) RTG; (C) 3TC; (D) ICG. The exposure increases were statistically significant for AP, RTG, and 3TC but not for ICG. Statistical analysis was performed using an unpaired, two-tailed, parametric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

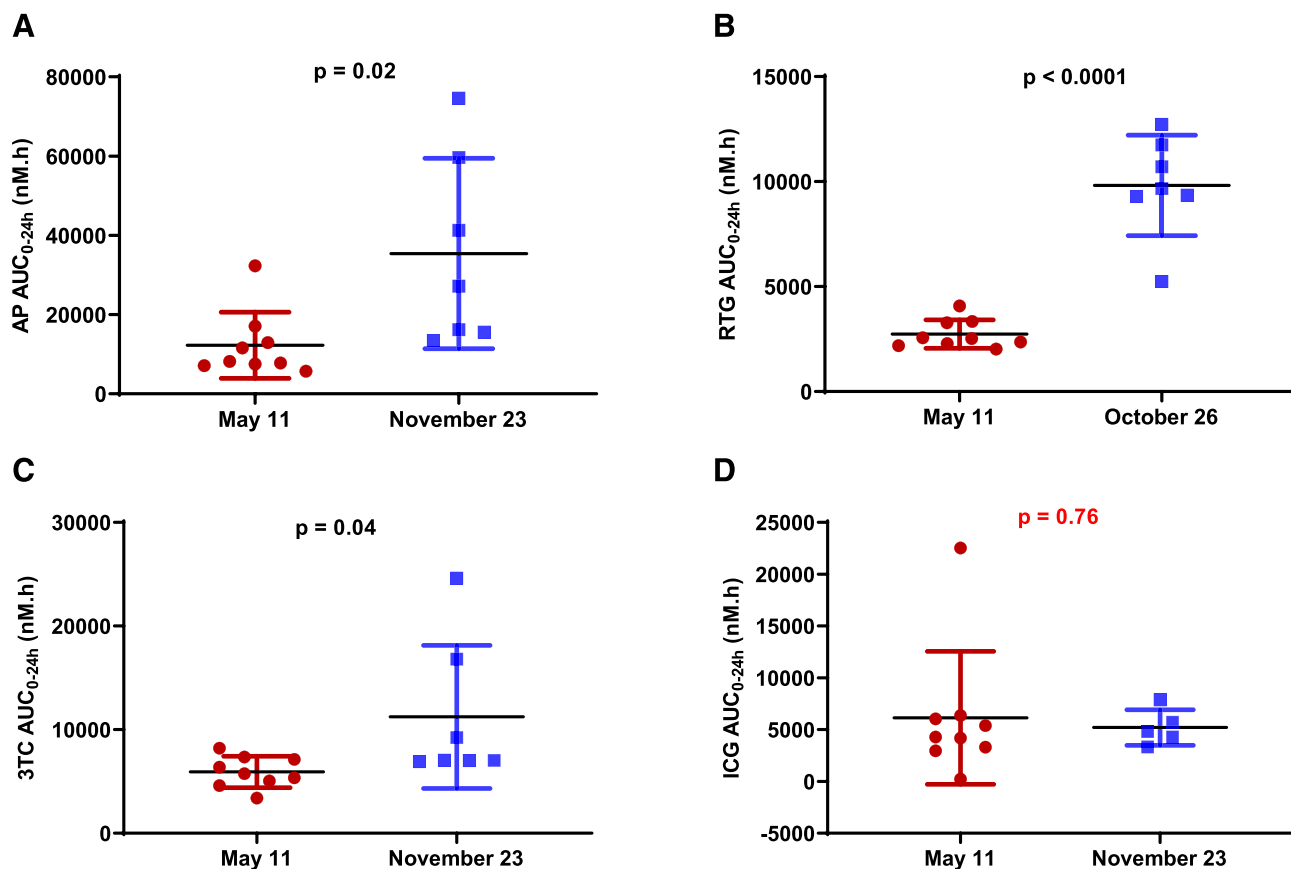


Fig. 8. Representative seasonal AUC_{0-24h} comparison between May 11 and November 23, 2017, in females: (A) AP; (B) RTG; (C) 3TC; (D) ICG. The exposure increases were statistically significant for AP, RTG, and 3TC but not for ICG. Statistical analysis was performed using an unpaired, two-tailed, parametric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

precision of time points, even though 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 pseudohibernation 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.

The changes in AUC_{inf} and CL values were consistent with those observed for AUC_{0-24h} . 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 June 26, 1996 (Witcher et al., 1997). However, study dates were not reported in many other woodchuck PK studies, particularly for those efficacy studies requiring multiple weeks of

treatment—for example, 12-week treatment of adefovir dipivoxil (Cullen et al., 2001); 4-week treatment of tenofovir disoproxil fumarate (Menne et al., 2005); 10-week treatment of MIV-210, a prodrug of 3-fluoro-2,3-dideoxyguanosine (Michalak et al., 2009); and 4- to 8-week treatment of vesatolimod (Menne et al., 2015). In many of these studies, seasonal PK variability was not taken into consideration. Typically, only PK data from prestudy 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 pseudohibernation period. Treatment duration in a 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 pseudohibernation. 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_{0-24h} increases for AP, RTG, and 3TC indicated decreases in oxidative metabolism, phase II glucuronidation, and renal clearance during pseudohibernation. 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

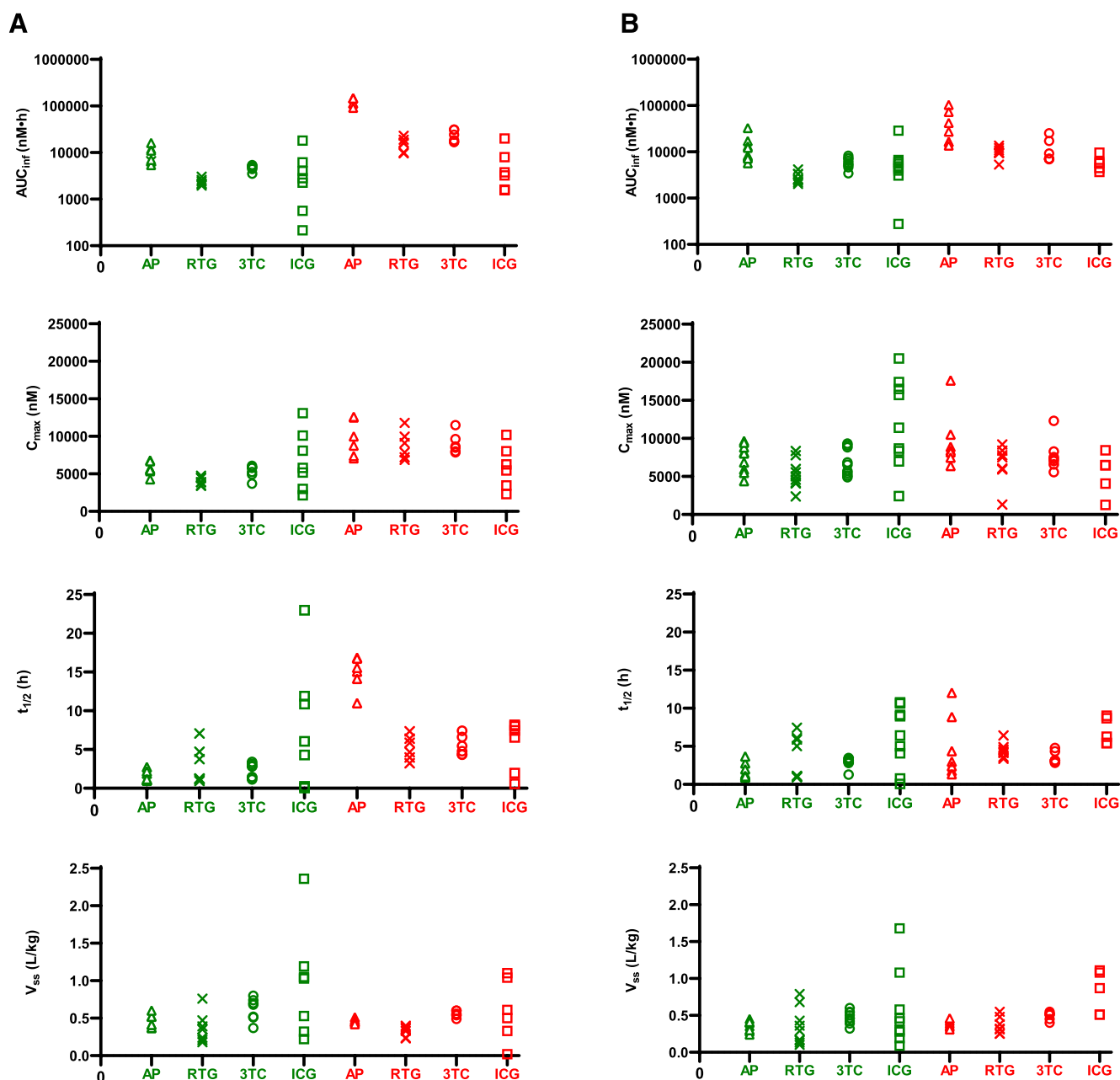


Fig. 9. Comparison of representative other seasonal PK parameters (AUC_{inf} , C_{max} , $t_{1/2}$, V_{ss} , and CL) between May 11 and November 23, 2017, in (A) males and (B) females. Data in green were from May 11, 2017, and data in red were from Nov 23, 2017. AUC_{inf} and CL values were plotted in log scale, whereas other PK parameters were plotted in linear scale. Symbols: Δ , AP; X, RTG; \circ , 3TC; \square , 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 males, 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 females, 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 males, 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 females, $t_{1/2}$ 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 \pm S.D., $n = 6$ or 7 for male, $n = 7$ or 9 for female; three woodchucks died during the study).

seasonally driven variation in drug PK, pharmacodynamics, and/or toxicity.

Acknowledgments

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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.

Note Added in Proof—It was discovered Figure 1B was accidentally duplicated as 1C in the Fast Forward version of the article published September 5, 2020. Figure 1C has been corrected.

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Supplemental Data to:

**Characterization of Seasonal Pharmacokinetic Variability in
Woodchucks**

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Drug Metabolism and Disposition

ABBREVIATIONS: 3TC, lamivudine; AP, antipyrene; AUC_{0-24h} , 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; CL, clearance; C_{max} , maximum concentration; E_h , hepatic extraction; h, hour; HPLC, high-performance liquid chromatography; HR, heart rate; ICG, indocyanine green; kg, kilogram; LBF, liver blood flow; min, minute; mL, milliliter; PK, pharmacokinetics; RTG, raltegravir; SD, standard deviation; $t_{1/2}$, terminal half-life; V_{ss} , steady-state volume of distribution.

Supplemental Materials and Methods

Bioanalytical method for seasonal PK study

A protein precipitation procedure was used for sample processing. Specifically, 300 μL of 100 ng/mL caffeine, glyburide, carbamazepine and 50 nM 5-IT (5-(2-aminopropyl)indole) in methanol was added to a 50 μL aliquot of each plasma sample with exception of the matrix blanks. Caffeine, glyburide, carbamazepine and 5-IT were used as the internal standard for antipyrine (AP), raltegravir (RTG), lamivudine (3TC) and indocyanine green (ICG), respectively. The matrix blank samples received 300 μL of methanol only. The precipitated proteins were removed by centrifugation and supernatant was transferred into three sets of clean 96 deep-well plates. These transfers are:

Transfer 1: 50 μL of supernatant was transferred into clean 96 deep-well plates. Plates were evaporated to dryness under a constant flow of nitrogen and reconstituted with 100 μL of 95: 5: 0.1 water: acetonitrile: formic acid;

Transfer 2: 50 μL of supernatant was transferred into a clean 96 deep-well plates. A 50 μL aliquot of water was added to each sample;

Transfer 3: 100 μL of supernatant was transferred into a clean 96 deep-well plates. Chromatography was accomplished on a MacMod ACE C18 column, 50 X 2.1 mm, 3 μm (MAC-MOD, Chadds Ford, PA), maintained at ambient temperature. Mobile phase A (MPA) was 0.1% formic acid and 99.9% water, and mobile phase B (MPB) was 0.1% formic acid and 99.9% acetonitrile for AP, RTG and 3TC. The MPA was 10 mM ammonium acetate in 95:4:1 water: acetonitrile: formic acid, and the MPB was 10 mM ammonium acetate in 95:4:1 acetonitrile: water: formic acid for ICG.

An Agilent 1200 series high pressure binary pump (G1312) was used for elution and separation. A CTC PAL autosampler was used as part of a Cohesive (Thermo Scientific San Jose, CA) LX-2 multiplexed system. The liquid chromatograph gradients for AP, RTG, 3TC and ICG are shown in Supplemental Table S1. An API-5500 triple stage quadrupole (TSQ) mass spectrometer (Applied Biosystems, Foster City, CA) was equipped with an electrospray ionization interface and operated in positive ionization mode for quantitation with collision induced dissociation activation method. Mass spectra were acquired in multiple reaction monitoring (MRM) mode. The MRM channels for the test compounds and the internal standards are listed in Supplemental Table S2. The parameters for the TSQ mass spectrometer used for the analysis are: spray voltage: +5.5 kV; capillary temperature: 550°C; sheath gas: 50 (arbitrary unit); auxiliary gas: 50 (arbitrary unit). An aliquot of 3-12 µL was injected into the API-5500 liquid chromatography–tandem mass spectrometry system. Representative lower limits of quantitation were 5 nM for AP and 2 nM for RTG, 3TC, ICG in plasma from March 18, 2018. The corresponding upper limits of quantitation were 5000 nM for AP, RTG, 3TC and 10000 nM for ICG. R² values were great than 0.99 by either linear or quadratic curve fitting with weighting index of 1/x². Retention time of AP, RTG, 3TC and ICG ranged from 1.55 to 1.62 min.

Supplemental Results

Seasonal PK study clinical observations

A female woodchuck, F17413, was observed with losing weight and foul odor on June 15, 2017. Mass was felt in abdomen and surgical anastomosis was done

on June 22, 2017. This animal died on June 24, 2017 from complication of blood loss following chronic state and surgery. Another female woodchuck, F17412, was found dead on June 26, 2017. Necropsy of F17412 showed thrombus in right jugular vein, right atrium, and right pulmonary artery. A male woodchuck, M17113, was found dead on December 8, 2017 due to hemorrhagic pneumonia. No other death or any additional clinical observation was reported.

Legends for Supplemental Tables

Table S1. HPLC elution programs for AP, RTG, 3TC and ICG

Table S2. MRM channels for the test compounds and the internal standards

Table S3. PK parameters of AP, RTG, 3TC and ICG in woodchucks from pre-study on March 16, 2017 (data from mixed sex with mean \pm SD, n = 3)

Legends for Supplemental Figure

Figure S1. Seasonal sex difference on November 23, 2017: A) AP; B) RTG; C) 3TC; D) ICG. Statistically significant sex difference was observed for AP ($p = 0.0011$), RTG ($p = 0.011$) and 3TC ($p = 0.012$) but not for ICG ($p = 0.88$).

Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean \pm SD, $n = 6$ for male and $n = 7$ for female).

SUPPLEMENTAL TABLE S1

HPLC elution programs for AP, RTG, 3TC and ICG

Time (second)	Step Comment	Flow Rate (mL/min)	AP		RTG		3TC		ICG	
			MPA (%)	MPB (%)	MPA (%)	MPB (%)	MPA (%)	MPB (%)	MPA (%)	MPB (%)
0 - 30	Step	0.700	90	10	70	30	100	0	55	45
30 - 150	Ramp	0.700	70	30	50	50	75	25	35	65
150 - 155	Ramp	0.700	5	95	5	95	5	95	5	95
155 - 180	Step	0.700	5	95	5	95	5	95	5	95
180 - 240	Step	0.700	90	10	70	30	100	0	55	45

SUPPLEMENTAL TABLE S2

MRM channels for the test compounds and the internal standards

Analyte	Compound ID	Parent Mass (m/z)	Product Mass (m/z)	Collision Energy (V)
Test compound	AP	189.1	77.2	30
Test compound	RTG	445.2	361.2	24
Test compound	3TC	230.0	112.1	20
Test compound	ICG	753.4	330.1	50
Internal Standard	Caffeine	195.2	138.0	37
Internal Standard	Glyburide	494.0	369.0	15
Internal Standard	Carbamazepine	237.0	194.0	13
Internal Standard	5-IT	393.1	260.9	25

SUPPLEMENTAL TABLE S3

PK parameters of AP, RTG, 3TC and ICG in woodchucks from pre-study on March 16, 2017 (data from mixed sex with mean \pm SD, n = 3)

Group ID	PK Parameters	AP		RTG		3TC		ICG		
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	
individual formulation	AUC _{0-24h} (nM•h)	33222	7671	3046	606	6815	1160	1621	1331	
	AUC _{inf} (nM•h)	35100	7731	3059	608	6850	1141	1899	1678	
	C _{max} (nM)	7340	788	4407	655	5770	436	3870	2115	
	t _{1/2} (h)	5.70	0.72	2.12	1.54	2.46	0.99	6.63	5.78	
	V _{ss} (L/kg)	0.74	0.49	0.25	0.06	0.54	0.11	1.52	0.98	
	CL	L/h/kg	0.08	0.02	0.38	0.07	0.32	0.06	0.96	1.18
		E _h *	1.9%	0.5%	9.2%	1.6%	7.9%	1.4%	23%	29%

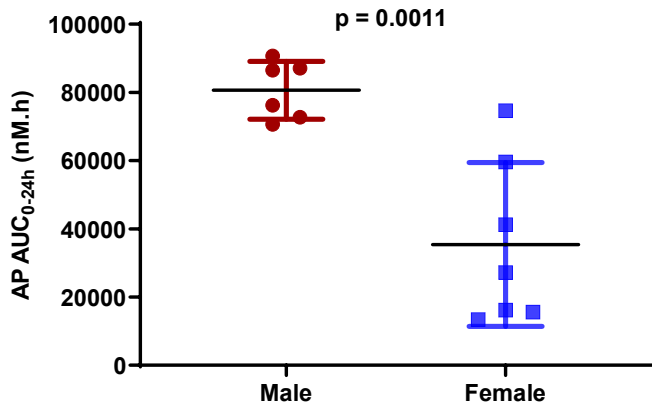
coformulation	AUC _{0-24h} (nM•h)	25814	3313	3272	355	7932	1510	3262	1622
	AUC _{inf} (nM•h)	26088	3420	3282	353	7944	1511	3871	1947
	C _{max} (nM)	5593	76	4423	220	6987	709	7300	497
	t _{1/2} (h)	3.79	0.36	1.11	0.10	2.80	0.08	11.2	5.9
	V _{ss} (L/kg)	0.48	0.02	0.21	0.03	0.45	0.06	1.57	0.86
	CL	L/h/kg	0.10	0.01	0.34	0.04	0.28	0.05	0.22
E _h *		2.5%	0.4%	8.4%	0.9%	6.8%	1.3%	5.5%	3.9%
<p>* The liver blood flow (LBF) in woodchucks was not published. We estimated the woodchuck LBF to be 4.1 L/h/kg based on allometric scaling. This value was used to calculate the hepatic extraction (E_h = CL/LBF).</p>									

Supplemental Figure

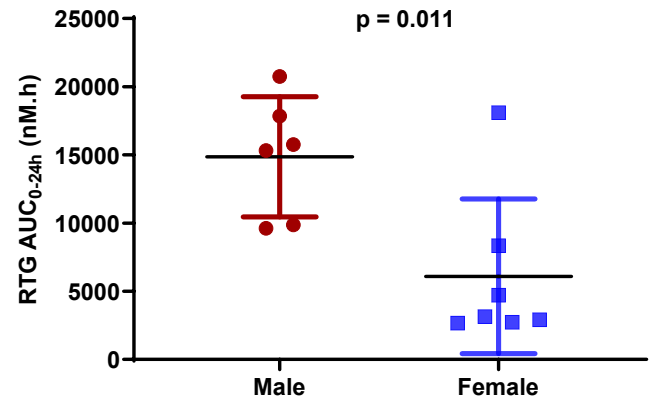
Fig. S1. Seasonal sex difference on November 23, 2017: A) AP; B) RTG; C) 3TC; D) ICG. Statistically significant sex difference was observed for AP ($p = 0.0011$), RTG ($p = 0.011$) and 3TC ($p = 0.012$) but not for ICG ($p = 0.88$).

Statistical analysis was performed using an unpaired, two-tailed, parametric t-test method (mean \pm SD, $n = 6$ for male and $n = 7$ for female).

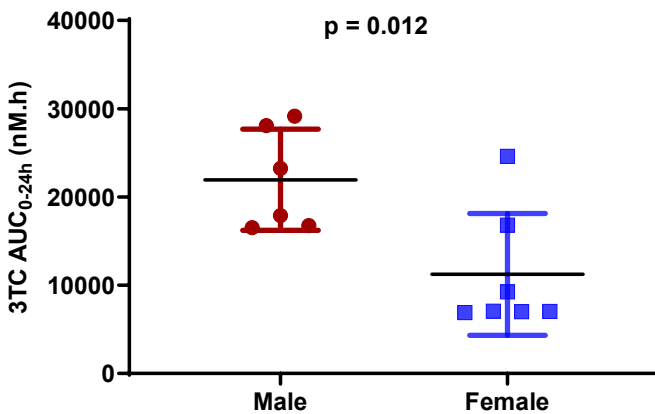
A



B



C



D

