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Pharmacokinetics of a single oral dose of acyclovir and valacyclovir in North American box turtles (*Terrapene* sp.)

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Eastern box turtle (*Terrapene carolina*) populations have significantly declined throughout their range (Currylow *et al.*, 2011). While a combination of factors are likely playing a role in the decline of the box turtle, disease outbreaks have been emerging across the Eastern United States in chelonians and may be important (De Voe *et al.*, 2004; Johnson *et al.*, 2008; Allender *et al.*, 2011).

Herpesviruses and iridoviruses are two common virus families that lead to clinical signs of upper respiratory tract disease in affected chelonians (Harper *et al.*, 1982; Brown *et al.*, 1999; Johnson *et al.*, 2008; Allender *et al.*, 2011). Acyclovir has been used anecdotally in the treatment of both viral agents in chelonians (Marschang *et al.*, 1997; De Voe *et al.*, 2004; Funk & Diethelm, 2006); however, to date, only a single pharmacokinetic study has been performed in a single species (Gaio *et al.*, 2007).

Acyclovir and its pro-drug valacyclovir are guanine analogue antiviral drugs (Elion, 1993). They are activated by phosphorylation of a virus-specific thymidine kinase (TK). Acyclovir uptake has been shown to be enhanced in herpesvirus-infected cells, with a 10- to 30-fold greater affinity for infected cells than uninfected cells (Beutner, 1995). Once incorporated into the viral genome, relatively low levels of the drug are needed to achieve viral inhibition, and adequate intracellular concentrations can be maintained for several hours (Beutner, 1995). Thymidine kinase genes or functional TK enzymes have similarly been identified in iridoviruses (Scholz *et al.*, 1988; Jakob *et al.*, 2001; Coupar *et al.*, 2005; Tsai *et al.*, 2005).

This study evaluated the pharmacokinetics of acyclovir and valacyclovir after a single oral dose in twelve North American box turtles. Animals were housed individually in Vision cages (Model #332; Vision Products Plus Inc., Canoga Park, CA, USA) that maintained a thermal gradient of 72°F to 87°F. A varied diet of fruits and vegetables was provided every other day. All activities were approved by the University of Illinois IACUC (protocol 11003).

A pilot study using oral acyclovir was initially performed at doses of 40 and 80 mg/kg in one animal each. After the results demonstrated low maximum observed plasma concentration (C_{max}) at the doses used, a decision was made to evaluate the pharmacokinetics of oral valacyclovir at 20 and 40 mg/kg in one animal each. Based on these results, eight animals were administered a single oral dose of 40 mg/kg valacyclovir. Venipuncture was performed via the subcarapacial sinus for all phases of the study. For the final study, blood (up to 0.3 mL) was collected in a 3-mL syringe with a 22-ga needle at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 120 h following oral valacyclovir. Samples were placed in a lithium heparin microtainer (Becton Dickinson, Franklin Lakes, NJ, USA), centrifuged immediately, the plasma placed in a separate cryovial, and stored at −20 °C. Samples were transported on dry ice to the Pharmacology Laboratory at the University of Tennessee for analysis.

Plasma samples were analyzed using a reverse-phase high-performance liquid chromatography method (HPLC). The system consisted of a 2695 separations module, a 2475 fluorescence detector, and a computer equipped with Empower software (Waters, Milford, MA, USA). Valacyclovir and acyclovir were extracted from plasma samples using 1 cc HLB solid-phase extraction (SPE) cartridges. The compounds were separated on an Atlantis T3 (4.6 × 100 mm, 5 μm) column with a guard column. The mobile phase was a mixture of (A) 10 mM ammonium phosphate pH 2.9 and (B) acetonitrile (97:3). The flow rate was 1.2 mL/min, and the column temperature was ambient. Fluorescence was measured at an excitation of 260 nm and an emission of 375 nm with a gain of ×100.

Standard curves for plasma analysis were prepared by fortifying untreated, pooled turtle plasma with valacyclovir and acyclovir to produce a linear concentration range of 10–1500 ng/mL. Average recovery for both drugs was 87%, while intra- and inter-assay variabilities were <10%. The lower limit of quantification was 10 ng/mL.

Pharmacokinetic parameters for valacyclovir and acyclovir were calculated using WinNonlin 5.2 (Pharsight Corp., Mountain View, CA, USA) (Table 1, Fig. 1). No valacyclovir was detected in any sample, and all analysis consisted solely based on plasma acyclovir. Values for plasma half-life ($t½$), C_{max}, time to maximum plasma concentration (T_{max}), and area under the plasma concentration–time curve ($AUC_{0-\infty}$) from time 0 to infinity were calculated from noncompartmental analysis. The AUC and AUMC were calculated using the log-linear trapezoidal rule. Mean residence time (MRT) was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$. The compartmental pharmacokinetic model was a one-compartment model with first-order elimination and weighted 1/Yhat*Yhat, and the model was used to simulate the concentration–time profile for several dosage regimens. In this model, it is assumed that K01>>>K10 or that there is no 'flip-flop' effect caused by slow absorption. The best model included an absorption term and biexponential decay. Parameters of this average model were V_F (mL/kg),_K_{01} (1/h), and K_{10} (1/h), which were 29.3971 ± 13.6595, 0.2241 ± 0.1718, and 0.0456 ± 0.0083, respectively, and were used to generate Fig. 2.

Table 1. Pharmacokinetic parameters (mean ± SD) of acyclovir in the plasma of eight box turtles after a single oral dose (40 mg/kg) of valacyclovir

Pharmacokinetic parameter	Acyclovir
$t_{½}$* (h)	15.2 ± 2.3
T_{max} (h)	13.0 ± 7.0
C_{max} (μg/mL)	1.94 ± 0.81
$AUC_{0-\infty}$ (h·μg/mL)	42.0 ± 15.3
$AUMC_{0-\infty}$ (h·h·μg/mL)	958.9 ± 307.5
$MRT_{0-\infty}$(h)	23.1 ± 3.4
V_F (mL/kg)	29.3971 ± 13.6595
K_{01} (1/h)	0.2241 ± 0.1718
K_{10} (1/h)	0.0456 ± 0.0083
Cl_F (mL·h/kg)	1116 ± 370
K_{01} $T_{½}$ (h)	3.1 ± 1.0
K_{10} $T_{½}$ (h)	17.2 ± 3.6

*Harmonic mean.

Valacyclovir, an esterified version of acyclovir, is rapidly converted to acyclovir after absorption and has greater oral bioavailability than acyclovir (Garre *et al.*, 2008). The oral bioavailability of valacyclovir is 3–5 times greater in humans, eight times greater in horses, and 2–3 times greater in cats than oral acyclovir itself, respectively (Nasisse *et al.*, 1997; Garre *et al.*, 2007; Maxwell *et al.*, 2008). In our pilot study evaluating 80 mg/kg oral acyclovir, our C_{max} was 2200 ng/mL. This was within one standard deviation of our 40 mg/kg valacyclovir dose C_{max}. While bioavailability was not measured in this study or the previous study in marginated tortoises, this pilot data indicate that, despite a lower dose of valacyclovir, it reached the same concentration as the higher acyclovir dose in box turtles.

The half-life of acyclovir after a single oral dose of valacyclovir in box turtles was 14.6 h. This is longer than the half-life of acyclovir in humans (3.1 h), cats (3.1 h), and horses (5.05 h) given oral valacyclovir and marginated tortoises (*Testudo marginata*) (8.8 h) given oral acyclovir (Beutner, 1995; Owens *et al.*, 1996; Wilkins *et al.*, 2005; Gaio *et al.*, 2007; Garre *et al.*, 2007). The results demonstrate a slower elimination in box turtles, which could reduce dosing intervals.

Acyclovir has been proposed for treatment of herpesvirus in several species, and for iridovirus in chelonians (Beutner, 1995; Gaio *et al.*, 2007). For herpesvirus in humans and horses, inhibitory concentrations need to be maintained above 0.45 and 3 μg/mL, respectively (Gaio *et al.*, 2007). However, *in vitro*

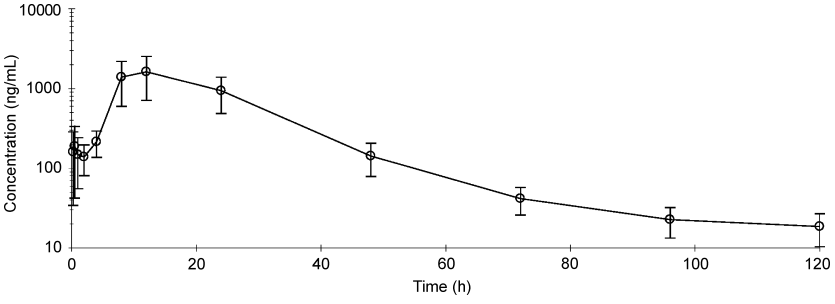

Fig. 1. Mean ± SD acyclovir plasma concentrations (μg/mL) following oral dose of valacyclovir (40 mg/kg) to eight box turtles.

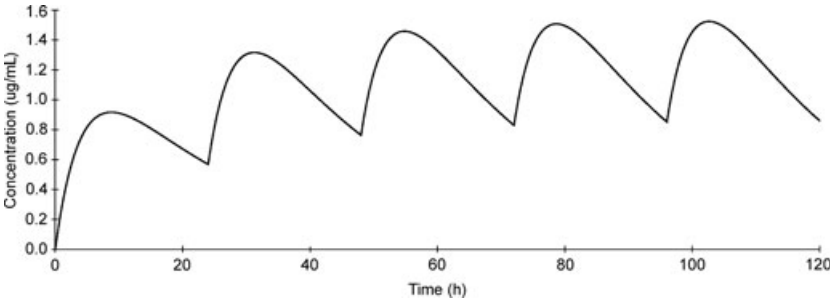

Fig. 2. Simulated average pharmacokinetic profile of plasma acyclovir concentrations (μg/mL) after oral dose of valacyclovir at 40 mg/mL every 24 h.

concentrations that completely inhibit tortoise herpesvirus were above 50 μg/mL (Marschang *et al.*, 1997). Additionally, *in vitro* studies against an iridovirus indicated only a dose-dependent partial inhibition at 25 μg/mL acyclovir concentration (Johnson, 2006). There have been no *in vivo* studies evaluating efficacy or inhibitory concentrations in chelonians against either virus. The C_{max} of valacyclovir determined in this study was 1.94 μg/mL, which is far below the partial *in vitro* inhibitory concentration of the chelonian studies. However, the C_{max} observed in this study is higher than the concentration considered effective for herpesvirus infection in humans (0.45 μg/mL), but lower than those shown to be effective in feline herpesvirus infection (18 ug/mL) (Beutner, 1995; Owens *et al.*, 1996). Future studies should further evaluate *in vivo* efficacy against iridoviruses to determine whether higher concentrations of drug are reached in infected cells, as has been demonstrated with herpesviruses in other species.

Toxicity was not specifically evaluated in this study; however, anorexia was seen in two animals, and prolonged lethargy was seen in a turtle administered valacyclovir. This study was performed in October, and it is possible that these problems were physiologic responses to the time of year. In cats, nephrotoxicity and bone marrow suppression were seen with repeated dosing of acyclovir and valacyclovir (Owens *et al.*, 1996).

In summary, valacyclovir appears to reach therapeutic plasma concentrations in box turtles based on *in vivo* doses effective for herpesvirus infections in humans (0.45 μg/mL), but not *in vitro* studies for herpesvirus or iridovirus in chelonians. However, its increased affinity for infected cells may allow intracellular concentrations to reach therapeutic levels for these pathogens when plasma concentrations do not. A simulated oral dose of 40 mg/kg every 24 h is predicted to maintain concentrations above 0.45 μg/mL in the average animal. Some limitations of our predictions include the assumption of linear pharmacokinetics within the range of simulated concentrations, the unknown effect of inter-individual pharmacokinetic variability in the population of turtles (temperature, season), the lack of bioavailability data, and the application of a human therapeutic window to turtles. Therefore, although our selected dosage regimen is a reasonable starting point, pharmacodynamic and repeat dosing pharmacokinetic studies are needed to further characterize optimal dosing of acyclovir in turtles.

In conclusion, this study provides valuable insight into the treatment of clinically important diseases in this species. Furthermore, it provides a scientific rationale for the development of other dose regimes for related species.

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