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In vitro and *in vivo* assessment of eprociclovir as antiviral treatment against testudinid herpesvirus 3 in Hermann's tortoise (*Testudo hermanni*)

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ABSTRACT

Tortoises belonging to the Testudinidae family are infected by Testudinid herpesviruses. Testudinid herpesvirus 3 (TeHV-3) is considered the most pathogenic and affects several tortoise species, particularly those from the *Testudo* genus. As most species of this genus are endangered contribute to ecological concerns over this virus. Here, we aimed to explore the rational development of an antiviral treatment against TeHV-3 using Hermann's tortoise (*Testudo hermanni*) as a host model. Ten antiviral compounds were tested in cell culture for their toxicity and their activity against TeHV-3. Eight compounds exhibited different levels of activity against TeHV-3 with either no or only minor cytotoxic effects on cells. Next, eprociclovir (EPV, ciprovir) was selected for further investigations *in vivo*. Its pharmacokinetic properties were investigated after a single sub-cutaneous administration at 5 or 10 mg/kg. Plasma concentrations remained above half maximal effective concentration (EC_{50}) for 2.2 and 4.4 h after administration at 5 and 10 mg/kg, respectively. Finally, EPV toxicity was investigated after administration at the dose of 10 mg/kg, BID for seven consecutive days. As early as one day after initiation of the treatment up to its end, EPV plasma concentration remained under the EC_{50}. Apathy and anorexia developed after 7 days. Biochemical and anatomopathological examinations revealed nephrotoxic effects of EPV. Altogether, these data suggest that EPV is not a suitable molecule for the treatment of TeHV-3. Further studies are required to determine whether the other molecules identified here for their anti-TeHV-3 activity represent potential candidates for the development of efficacious treatments.

There are at least 60 species of tortoises in the Testudinidae family. Herpesviruses have been isolated from most of these species, in both healthy and sick subjects. These herpesviruses have been grouped into four genotypes named as testudinid herpesvirus 1 to 4 (TeHV-1 to TeHV-4) (Bicknese et al., 2010). Among these genotypes, TeHV-3 is the most pathogenic and has been shown to affect several tortoise species, with those from the genus *Testudo* (*e.g.*, *Testudo hermanni*) being the most sensitive to infection (Bicknese et al., 2010; Martel et al., 2009). The fact that these host species are also endangered contributes to ecological concerns over TeHV-3.

The main clinical signs associated with TeHV-3 include nasal discharge, rhinitis, conjunctivitis and diphtheritic plaques in the oral cavity and oesophagus. Weight loss, cachexia, central nervous symptoms and death are associated with advanced stages of the disease. Young tortoises are more susceptible to TeHV-3 disease than adults and can express a mortality rate of up to 100% (Martel et al., 2009). To date, treatments against TeHV-3 infection remain mainly symptomatic and empiric. Etiological treatment based on acyclovir (ACV) (at 80 mg/kg) has been suggested in tortoises (Origgi, 2006; Wright, 2008). However, neither the safety nor the efficacy of this treatment has been tested experimentally in tortoises (Marschang et al., 1997). Here, we initiated the rational development of an antiviral treatment against TeHV-3 using Hermann's tortoises (*Testudo hermanni*) as a host model. First, ten antiviral compounds were tested in cell culture for their toxicity and activity against TeHV-3 replication. *In vitro* antiviral molecule screening was performed in 96-well pates (BD Falcon, Franklin Lakes, USA). Tortoise heart cells (TH-1, subline B1, ATCC CCL-50) were seeded at the density of 1.5×10^4 cells/well (Gandar et al., 2015). After an incubation of 48 h, cells were inoculated with TeHV-3 at a multiplicity of infection of 0.2 PFU/cell and then overlaid with 200 µL per well of Dulbecco's modified Eagle's medium supplemented with 5% (vol/vol) foetal calf serum (Sigma-Aldrich, Overijse, Belgium) and the molecules to be tested (at concentrations of 0, 0.4, 2, 10 and 50 µg/mL). The following antiviral molecules kindly provided by Dr. J. Neyts

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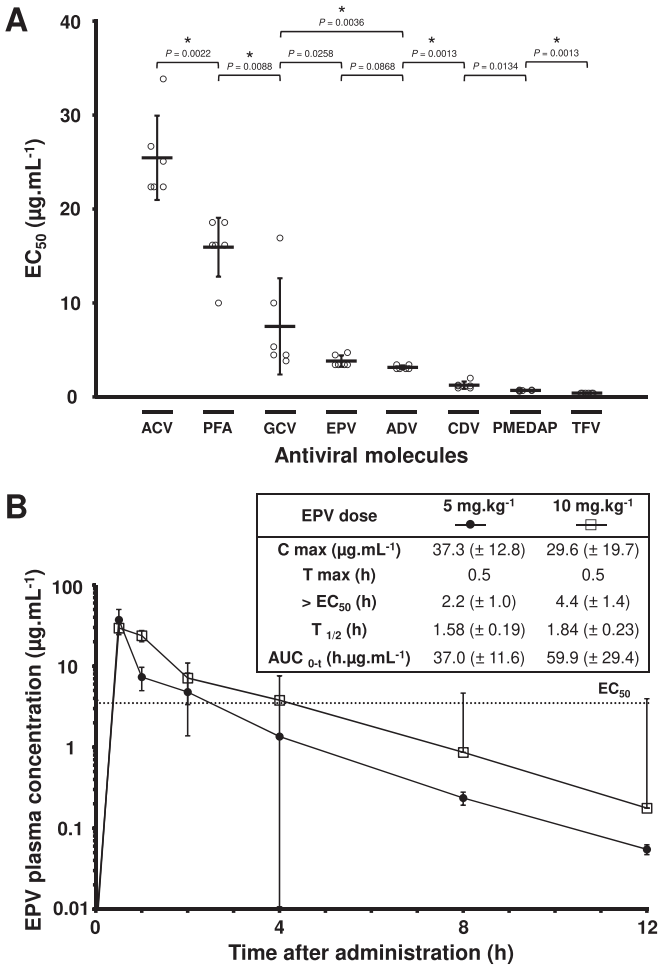

Fig. 1. (A) EC_{50} (µg/mL) of eight antiviral molecules tested against TeHV-3 in TH-1 cell cultures. ACV: acyclovir; PFA: phosphonoformic acid; GCV: gancyclovir; EPV: eprociclovir; ADV: adefovir; CDV: cidofovir; PMEDAP: 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; TFV: tenofovir. Data represent the analysis of replicates (n = 6). The mean of the 6 replicates ± SD is presented for each drug. (B) Pharmacokinetic and acute toxicity evaluation of EPV following a single subcutaneous administrations in *Testudo hermanni*. Mean plasma concentration (µg/ml ± SD) after a single subcutaneous administration at 5 mg/kg (•, n = 3) and 10 mg/kg (□, n = 3) of EPV. Maximal concentration (Cmax), time-to-maximum (Tmax), time above EC_{50} (> EC_{50}), half-life ($T_{1/2}$) and area under the curve (AUC) were determined for each concentration. Horizontal dashed line represents the *in vitro* EC_{50} value of EPV.

(Katholieke Universiteit Leuven, Belgium) were tested: pencyclovir (PCV), 9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine (PMPDAP), ACV, phosphonoformic acid (PFA), gancyclovir (GCV), eprociclovir (EPV, previously known as ciprovir), adefovir (ADV), cidofovir (CDV), 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) and tenofovir (TFV). The proportion of cytopathogenic effect on total surface of each infected well was estimated 7 days post infection by macroscopic examination of the cell monolayer subdivided in 6 equivalent parts. Effective concentration (EC_{50}) was calculated for each molecule using the logarithmic interpolation method (Jin-Li et al., 2015) based on 6 replicates. Statistical analyses of EC_{50} data were performed by using the Wilcoxon nonparametric test. Value of $P < .05$ and $P < .01$ (*) were considered significant. None of the compounds revealed cell death at the doses tested (data not shown). However, abnormal aspects of TH-1 cells consisting of cytoplasm densification and shape modifications were observed for ADV, CDV, PMEDAP and TFV at a concentration of 50 µg/mL suggesting a mild cytotoxicity. PCV and PMPDAP did not inhibit TeHV-3 at the concentrations tested (data not shown), and so were not included in Fig. 1A. The calculated EC_{50} of each efficacious molecules were as follows (in increasing order of efficacy, mean ± SD): ACV = 25.5 (± 4.5); PFA = 15.9 (± 3.5); GCV = 7.5 (± 5.1); EPV = 3.9 (± 0.7); ADV = 3.2 (± 0.2); CDV = 1.3 (± 0.4); PMEDAP = 0.6 (± 0.1); TFV = 0.7 (± 0.1) µg/mL. Mean EC_{50} values were all statistically different from each other ($P < .05$, Fig. 1A) with exception of EPV and ADV data ($P = .0868$). EPV induced a complete inhibition of viral replication at 10.0 (± 0.7) µg/mL.

Based on the *in vitro* results above (both toxicity on cells and inhibition of TeHV-3 replication), EPV was selected for further *in vivo* investigations. All *in vivo* protocols described below were approved by the Ethic Committee of the University of Liège (protocol n°1235). Five-year old Hermann's tortoises (*T. hermanni*) (mean weight of 298.0 g, ± 24.3) were kept individually as described elsewhere (Gandar et al., 2015). Relative humidity was maintained at 60–70%. Lighting was controlled automatically on standard 12 h light and 12 h dark circadian cycles, with an UVb light switched on during the 12 h light period. The temperature of an infrared basking spot was regulated at 29 °C during the light period and 24 °C during the dark period, respectively. A temperature gradient of approximately 6 °C was present in the terrariums, with the basking spot being the warmest place. Fresh water and vegetables were provided daily. Clinical examinations of tortoises immediately prior to the experiments revealed that they were clinically healthy. Experiments were preceded by an acclimatization period of 2 weeks. To investigate the pharmacokinetic properties of EPV in tortoises, a single administration of EPV at the dose of 5 (3 subjects) or 10 mg/kg (3 subjects) was performed by subcutaneous injections in the forelimbs. One subject was injected with 0.9% NaCl as negative control. Blood samples were collected from the jugular vein at 0, 0.5, 1, 2, 4, 8, 12, 24 and 48 h post injection. EPV plasma concentrations were determined by high-performance liquid chromatography with the modified pre-column derivatization method as previously described (Iwayama et al., 1999). To investigate possible acute toxicity of the molecule, complete blood counts and serum biochemical exams were performed just before administration of EPV and 48 h post injection. At the end of the experiment, all animals were euthanized and necropsied according to conventional techniques and guidelines (Terrell and Stacy, 2007). Brain, spleen, lungs, kidneys, liver, and a portion of small intestine were fixed in neutral buffered 10% formalin for histological examination. EPV plasma concentrations were measurable in all treated tortoises (Fig. 1B). Non-compartmental PK analysis were performed using Phoenix WinNonlin® 8.0 (Certara, Princeton, USA) on individual values. Theoretical sampling times were used for calculation of PK parameters. Statistical analyses of time above EC_{50} were performed with a Wilcoxon test. Cmax and half-life time ($T_{1/2}$) means of the two doses were compared using a Student's *t*-test. *P* values under 0.05 were considered to be statistically significant. For both dosages, Tmax was reached after 30 min and $T_{1/2}$ was estimated at 1.58 (± 0.19) and 1.84 (± 0.23) hours at the dose of 5 and 10 mg/kg, respectively. No significant difference was detected between the $T_{1/2}$ of the two doses. However, a significant difference ($P = .0467$) was noticed between durations above the EC_{50} with 2.2 (± 1.0) and 4.4 (± 1.4) hours at 5 and 10 mg/kg, respectively. Haematology and biochemistry values observed before and 48 h post injection were compared for each animal. An increase in creatine phosphokinase (CPK) was observed. The value observed 48 h after injection ranged from 15 to 156 fold the value observed before the injection. Red blood cell count, white blood cell count, total plasma proteins, albumin, globulins, calcium, phosphate aspartate transaminase (AST) and uric acid (UA) were not affected and were in the normal range for the species (data not shown) (Andreani et al., 2014). No gross lesions were noted at necropsy examination, also histology of collected organs did not reveal lesions suggesting absence of acute toxicity.

Next, we investigated the toxicity of an EPV regime consisting of a daily subcutaneous injection at the dose of 10 mg/kg (3 subjects) for

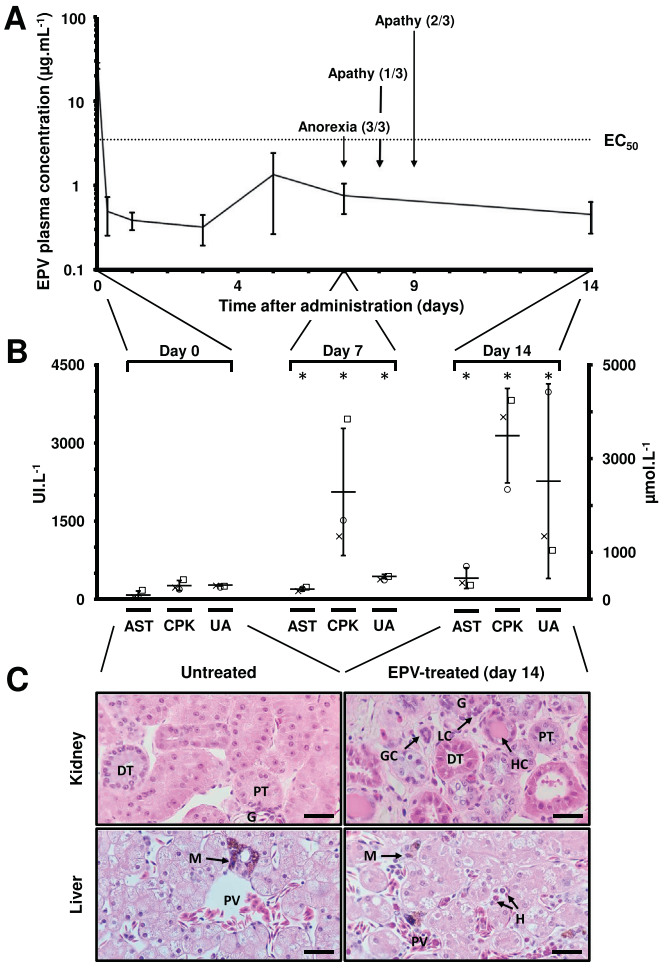

Fig. 2. Chronic toxicity evaluation of EPV following repeated subcutaneous administrations in *Testudo hermanni* (n = 3). (A) Mean plasmatic concentration (µg/ml ± SD) after subcutaneous administration of EPV, BID, for 7 days at the dose of 10 mg/kg. Horizontal dashed line represents the *in vitro* EC_{50} value of EPV. (B) Aspartate transaminase (AST; $UI.L^{-1}$, left Y axis), creatine phosphokinase (CPK; $\mu mol.L^{-1}$, right Y axis) and uric acid (UA; µmol.L-1, right Y axis) plasmatic concentrations (mean ± SD) were determined at 0, 7 and 14 days post administration of EPV. The symbols in the graph represent each of the three individuals at each time point, *i.e.* □ = subject 1, × = subject 2 and ○ = subject 3. Significant differences between normal references ranges of the species are marked (*). (C) Histopathological lesions of kidney and liver observed in EPV-treated tortoises. PT, proximal tubule; DT, distal tubule; G, glomerulus; GC, giant cell; HC: hyaline cast; LC: lymphocyte; M, melanomacrophage; PV: portal vein; and H: heterophil. Haematoxylin eosin stain, bars = 50 µm.

seven days (mean weight of 302.3 g, ± 25.5). One subject was injected with 0.9% NaCl as negative control. EPV plasma concentrations were determined at the following time points: 1, 2, 3, 5, 7 and 14 days. Complete blood counts and serum biochemical exams were performed at day 0, 7 and 14 to investigate possible chronic toxicity of the molecule. EPV plasma concentrations remained under EC_{50} after day 1 (Fig. 2A). No accumulation effect of the molecule was observed. In addition, tortoises became anorectic and apathetic between days 7 and 9 after initiation of the treatment. Consistent with the results of the first experiment, an increase in CPK was observed in each tortoise at day 7 and 14. A similar increase was observed in the negative control indicating that this parameter is likely influenced by the manipulations performed (injection and/or blood collection). In all EPV treated animals, AST and UA values were increased in comparison with the normal values for this species (Fig. 2B) (Andreani et al., 2014). Other parameters were in the normal ranges. At necropsy, kidney pallor (light tan) in all treated animals was noted. Histologic examination revealed a loss of structure of the renal parenchyma with a heterophilic and lymphocytic infiltration compatible with an acute interstitial nephritis (Fig. 2C). The presence of hyaline casts in proximal and distal tubule lumens with dilation of some distal tubules was also noticed. In the liver parenchyma of treated tortoises, a heterophilic infiltration compatible with a non-specific inflammation process was observed. Histological examination of other organs did not reveal any abnormalities.

Etiological treatment of TeHV-3 infection with ACV (at 80 mg/kg) has been suggested in tortoises (Origgi, 2006; Wright, 2008). However, neither the safety nor the efficacy of this treatment has been tested experimentally in tortoises (Marschang et al., 1997). Our EC_{50} results of ACV (Fig. 1A) demonstrated that this molecule has a reduced activity against TeHV-3 compared to other herpesviruses (Neyts et al., 1998). These data suggest that ACV is unlikely to represent an efficacious treatment against TeHV-3. Based on our *in vitro* results, EPV, ADV, CDV, PMEDAP and TFV appear all as potential candidates for TeHV-3 treatment. Unfortunately, no studies are available on these molecules in reptiles, similarly their toxicity and their pharmacokinetics in tortoises are unknown. However, ADV, CDV and TFV are well known to be nephrotoxic in long-term treatment in humans (Broekema and Dikkers, 2008; Gara et al., 2012; Lacy et al., 1998; Tourret et al., 2013; Vittecoq et al., 1997). Also, PMEDAP has been revealed to be highly toxic in pigeons (unpublished data). In the present study, we observed that ADV, CDV, PMEDAP and TFV affect the morphology of TH-1 culture cells suggesting toxicity. Based on its safety and efficacy profile, we selected EPV for *in vivo* study in tortoises.

The half-life of EPV in tortoises is shorter than that of ACV in turtles (*Terrapene carolina*) (Allender et al., 2013). However, it is important to note that EPV is a nucleoside analogue which accumulates in the cytoplasm of cells after its conversion into a triphosphate active form (Jordheim et al., 2013). Consequently, plasma concentrations and half-life time determination could give an underestimation of the intracellular half-life of the molecule. However, the results obtained in the present study in relation to EPV plasma concentration (Fig. 1. B) and its toxicity (Fig. 2 B and C) do not support its use for the development of a safe and efficacious treatment against TeHV-3 under these conditions.

In conclusion, the present study seeds the basis for the rational development of an antiviral treatment against TeHV-3, which is contributing to the population decline of endangered species of tortoises. Furthermore, it also highlights the importance of taking into consideration the physiology of this particular type of host, which is very different from that of the species in which candidate antiviral molecules have been previously tested.

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