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Long-term and Per Rectum Disposition of Clarithromycin in the Desert Tortoise (*Gopherus agassizii*)

Jeffrey Wimsatt,[1,*,†] Alysa Tothill,[2] Cord F Offermann,[1,2] Jenifer G Sheehy,[2] and Charles A Peloquin[3]

The macrolide antibiotic clarithromycin (CLARI) has a wide spectrum of activity and efficacy for *Mycoplasma* species. In addition, CLARI accumulates during re-dosing of Mojave desert tortoises (*Gopherus agassizii*). Here, we characterized plasma concentrations after a single dose, after 3.5 months of dosing, and after per rectum administration; all doses were 15 mg/kg. After a single dose, the median maximal plasma concentration (Cmax) was 1.69 mg/ml and occurred at a median of 6 h after administration, the estimated elimination half-life was 6.9 h, and the median accumulation index was 10%. Plasma concentrations after long-term dosing showed consistent intraturtle concentrations of at least 2 μg/ml, with 1 turtle showing increasing accumulation of CLARI at all 3 time points and the remaining 5 turtles showing increases by 3.5 mo. Compared with expected Cmax values, the median long-term values were approximately 3 times higher than expected in 4 of 6 turtles and approximately 2/3 of that expected in the remaining 2 turtles. Per rectum dosing caused antibiotic retention below target values. Together, these results support accumulation of CLARI after repeated oral dosing and indicate that stable concentrations are reached long-term. Either cystoenteric recycling of CLARI or large intestinal absorption of bypass CLARI may explain the observed cumulative increases. In addition, twice-weekly CLARI maintains target concentrations over time, and per rectum dosing will require higher doses or increased dose frequency to be successful. Based on this work, pharmacokinetic studies in exotic species should include multidose studies to verify initial kinetic estimates from single-dose trends.

Abbreviations: CLARI, clarithromycin; Cmax, maximal concentration median value; URTD, upper respiratory tract disease (refers to *Mycoplasma agassizii* clinical syndrome in tortoises)

Wild and captive tortoises representing at least 5 different genera are susceptible to *Mycoplasma agassizii*,[8,14,15,30,42] the cause of the upper respiratory tract disease (URTD) syndrome,[6,7,34] and similar *Mycoplasma*-associated illnesses[16] of other chelonians and crocodilians.[4,5,10,26,31] After initial infection, URTD presents as a seemingly mild upper respiratory infection; untreated, it typically has a protracted course characterized by serous to mucopurulent oculonasal discharge and edematous swelling of the eyelids.[7,21,24,25] Without intervention, susceptible animals eat less, lose condition, develop progressive wasting, and eventually succumb to opportunistic infections.[21,24] Other diseases with overlapping signs must be ruled out.[16] The disease has had an important role in the decline of native desert (*Gopherus agassizii*) and gopher (*G. polyphemus*) tortoise populations[30,41]and is relatively common in a wide variety of captive tortoises.[41] Antibiotics suggested for therapy include enrofloxacin, gentamicin, tetracyclines, and selected macrolides.[42]

Recent reports describe the use of clarithromycin (CLARI) to successfully treat a box turtle with a novel *Mycoplasma* sp. isolate most closely resembling *M. pulmonis* and *M. agassizii*[16] and a desert tortoise exhibiting URTD.[2] Both animals showed sustained complete reversal of clinical signs during the follow-up period.

Clarithromycin, a C6 OH-substituted, 14-carbon erythromycin derivative, has potent activity against *M. pneumoniae* in humans and is active against a range of bacterial agents, including several *Mycobacterium* species.[20,32] This antibiotic has a broad spectrum of activity and concentrates preferentially in the respiratory epithelium and pulmonary secretions of humans and other species.[12,17,22,35] In comparison to its close relative, erythromycin, CLARI is more stable in response to stomach acidity,[27] and it is better absorbed in the presence of food.[18]

A 14-OH metabolite of CLARI is produced by liver metabolism of the parent compound in nonhuman primates and humans[32] but was not identified in a previous desert tortoise pharmacokinetic study.[45] Even so, the absence of the 14-OH metabolite does not exclude liver enzyme inhibition[47] or accentuated metabolite excretion by other clearance enzymes. In various species, CLARI is excreted unchanged by the kidneys.[27,37,46]

Although an injectable form of CLARI is available in Europe, only the oral suspension is marketed in the United States. As some chelonians are difficult to treat with oral medications, per rectum administration of CLARI could present a convenient alternative. In addition, desert tortoises (like other terrestrial tortoises) have a large bladder and retain water that can later be used by reabsorption when the need arises.[13] This readily utilizable fluid reservoir may be essential to Mojave desert tortoises to maintain a positive energy balance.[36] A previous preliminary multi-dose study in desert tortoises[45] revealed that CLARI accumulated in desert tortoises regardless of gender or age. Possible explanations for accumulation include extended

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[1]Department of Clinical Sciences, Colorado State University, Fort Collins, CO; [2]Kyle Veterinary Clinic, Kyle TX; [3]Infectious Disease Pharmacokinetics Laboratory, National Jewish Medical and Research Center, Denver, CO
Research site: Foothill's Campus Tortoise Colony, Colorado State University, Fort Collins, CO
[†]Current address: Center for Comparative Medicine and Department of Biology, University of Virginia, Charlottesville, VA
[*]Corresponding author. Email: jhw5b@virginia.edu

enteric uptake, urine-to-cloaca reflux, and tissue retention of the parent compound.

The 3 objectives of the present study were to: 1) confirm the plasma concentration versus time profile of CLARI over 14 d after a single dose; 2) determine long-term plasma concentrations when CLARI is administered over 3.5 mo, as might occur during therapy; and 3) evaluate per rectum administration of CLARI as an alternative method of antibiotic delivery and as a potential site of antibiotic recovery or urine recycling, leading to accumulation.

Materials and Methods

Animals and reagents. All procedures were approved by the institutional animal care and use committee. Desert tortoises used in these studies were federally listed as threatened and were donated as confirmed URTD-positive animals; this group was important to use in this study as they represented a potential target group for CLARI therapy. All study animals were wild-captured (from the area around St George, UT) and seropositive for *M. agassizii* (that is, enzyme immunoassay antibody-positive, Biotechnology for the Ecological, Evolutionary, and Conservation Sciences program, University of Florida, Gainesville, FL) in addition to showing the typical cyclic recurrence of classic URTD signs. *Mycoplasma* PCR was used to confirm the enzyme immunoassay results, indicating the colony animals had active *Mycoplasma* infections.[2] In light of their chronic infections and in the absence of a confirmed treatment that would definitively eliminate *Mycoplasma* infection, these colony animals could not be released to the wild and were maintained as a teaching and research colony under permit from the US Fish and Wildlife Service.

Animals were maintained as previously described,[45] at 30.0 to 33.3 °C (86 to 92 °F) within 2-m^3 dirt (clean fill from a fallow field)-floored enclosures fitted with 250-W infrared heat lamps suspended 0.5 m above the floor. This arrangement allowed the tortoises to regulate their own body temperatures. Water was provided continuously in low 45-cm^2 'soak pans' and food consisted of grass hay supplemented weekly with rinsed leafy produce (approximately 0.2 kg/kg body weight).

Tortoises were fitted with cardiac access ports for plasma sampling as previously described,[44] 2 to 3 mo prior to study. Animals had stable or gaining body weights, normal activity, normal stool production, and good appetites at the time of study. Intermittently, the tortoises exhibited URTD signs, as is typical of this chronic disease. Animals housed in this way, even when infected with *Mycoplasma*, become more active in mid-January with increasing seasonal light, exhibit courtship behaviors in the spring (typically late February onward), and have accelerated eating patterns and faster gastrointestinal transit times. From August through September, a second smaller surge in courtship behavior occurs before eating and activity slows in anticipation of winter. The first study started in mid-February on increasing photoperiod, was followed directly by the second study, and involved 6 animals. The third study started in early June, and used another group of 7 animals. Because of the vast storage capacity of the desert tortoise bladder, the animals were assumed to be well-hydrated during the studies in response to weekly provisioning of water in low soak tubs, and their hydration status mirrored that of a previous study.[45]

Oral CLARI suspension (50 mg/ml, Biaxin oral suspension, Abbott Labs, Abbott Park, IL) was reconstituted from powder according to the manufacturer's instructions immediately before each trial, and a new aliquot of powder was reconstituted every 14 d as needed. In all cases, the dose delivered was 15 mg/kg.[45] Oral dosing was accomplished quickly while holding each tortoise by its shell, extending the neck, and passing a premeasured large rodent metal gavage tube gently down the esophagus to the level of the stomach. All dosing was done between 0900 and 1000, except during the long-term dosing study, when the second dose each week was delivered at 2100 to 2200 (dosing every 3.5 d).

Cumulative blood sampling never exceeded 1% of body weight. Each blood sample was collected in lithium heparin, and processed as previously described.[45]

Study 1: 14-d single-dose antibiotic profile. During the study, 0.5-ml blood samples were taken. Blood sampling started immediately before administration of the CLARI dose. Blood sampling continued at 4, 8, and 12 h after dosing and daily until 14 d after treatment.

Study 2: Long-term dosing. During the first study, CLARI washout was complete and below the detection threshold by 14 d in all animals studied. Because the trend in levels after chronic administration was the primary outcome of the long-term dosing study, the same tortoises were used, and dosing for the second study was started immediately after an initial blood sample was drawn (14 d after the single dose was administered for the first study). For the second study, the tortoises were gavaged with CLARI twice weekly (3.5 d apart) for 6 mo. Samples were collected for CLARI assay at 0.5, 2.5, and 3.5 mo during the continuous dosing period.

Study 3: Per rectum administration. In a separate group of animals, after an initial blood sample was drawn, antibiotic was delivered directly into the rectum by using a prefilled lightly lubricated Brunswick feeding tube (9 French; outer diameter, 3 mm) to a premeasured craniad depth corresponding to 2 mid-dorsal carapace scutes in distance from its midsaggital caudal margin. Dosing was repeated at 12, 24, and 36 h after the first dose and daily thereafter for a total of 8 d. In some cases, initial tube placement stimulated defecation and necessitated tube replacement before antibiotic administration. Blood samples were collected before administration. Additional samples were collected at 4, 8, 12, 24, 36, 48, 60, and 72 h and at 14 and 15 d (336 and 360 h, respectively) after dosing started.

Assay for CLARI. A validated HPLC technique for measuring CLARI was adapted for the assay of desert tortoise samples as previously described.[45] Briefly, erythromycin B was added before extraction as an internal standard. After extraction, 20-µl samples of plasma were injected into an isocratic reverse-phase HPLC system (model 8875, Spectra Physics, San Jose, CA; gradient controller: model 680, Waters, Milford, MA; electrochemical detector: ESA Coulchem II, Bedford, MA), and data handling and analysis was provided by using the Dynamax (Rainin, Woburn, MA) system. Peak amplitude ratios of CLARI:erythromycin B were used to construct a weighted standard curve. Sample ratios were compared with standard ratios to obtain CLARI concentrations over a range of 0.2 to 10 µg/ml. The 14-OH CLARI range was 0.18 to 4.42 µg/ml. The standard curve coefficient of variation was > 99.8% at all times. Extraction efficiencies calculated per tube ranged from 98.3% to 103.3%. The intraassay coefficient of variation for CLARI and 14-OH CLARI was 1.9%, and the interassay coefficient of variation was 3.7%. The low standard for the assays had a concentration of 0.2 µg/ml for CLARI, although smaller concentrations could be detected and were quantified down to 0.15 µg/ml. Lower values were defined as trace amounts.

Data analysis. Data were analyzed by using WinNonlin version 4 (Pharsight, Cary, NC). The data were explored graphically and, where appropriate, by using noncompartmental analysis. The accumulation index was defined as:

$$1 / [\, 1 - e\text{^}(-\lambda z \times T),$$

where λz was the elimination rate constant from noncompartmental analysis, and T was the dosing interval. Accumulation also was simulated by using noncompartmental superposition with the data from the single-dose study; dosing intervals (T) of 24 and 72 h were simulated.

Results

Study 1: 14-d single-dose antibiotic profile. Plasma concentrations were reasonably consistent across tortoises B, E, F, and G (Figure 1). Tortoise D had an unexplained low value at 12 h, with apparent rebound at 24 h. In tortoise C, plasma concentrations of CLARI at 24 and 48 h and later declined gradually from 12-h values; the last 2 time points were excluded in the estimation of the elimination half-life for this tortoise. The single-dose data yielded a median Cmax of 1.69 µg/ml, occurring at an estimated median Tmax of 6 h, between the 4- and 8-h samples. Quantifiable concentrations were present until 48 h in 2 tortoises, and beyond 48 h in an additional animal. Simulating 24-h dosing, the median accumulation index was 10%, whereas simulating 72-h dosing, it was negligible.

Study 2: Long-term dosing. CLARI did not cause adverse effects in tortoises that received 6 mo of oral administration. Stool consistency, species-typical behaviors, and activity were normal. Moreover, CLARI administration for 6 mo led to complete remission of clinical signs and improved body conditions during the 9 mo that followed. After this period, the animals were housed in pens adjacent to the main colony of infected animals to determine whether treated tortoises were immune against renewed disease development. However beginning 3 mo after rehousing, the long-term CLARI-treated animals started to exhibit URTD signs again.

In response to twice-weekly oral dosing, CLARI concentrations collected immediately prior to dosing at 0.5, 2.5, and 3.5 mo ranged from 1.02 to 11.17 (median, 3.32) µg /ml to 2.37 to 6.67 (median, 3.93) µg /ml and 1.91 to 9.48 (median, 4.79) µg /ml, respectively (Figure 2). The 14-OH metabolite was never detected. Plasma CLARI concentrations remained at 2 µg/ml or higher for all tortoises and were typically well above this cut-off. Several of the values determined were higher than is typically seen in humans receiving multiple standard doses and higher than expected in light of single-dose predictions and noncompartmental superposition.[1,29] Therefore, chronic dosing of tortoises with CLARI leads to accumulation of the drug within the plasma space that was not anticipated based on single-dose study predictions.

Study 3: Per rectum administration. Tortoises showed per rectum absorption of CLARI. Plasma concentrations of CLARI after per rectum dosing typically were low, with only 1 animal exhibiting plasma levels exceeding 1 µg/ml (Figure 3). No tortoise achieved plasma concentrations in the target range (equal to or greater than 2 µg /ml). Tortoises 5 and 6 had only 1 detectable CLARI value each (0.29 and 0.22 µg/ml, respectively); their data were not included in Figure 3.

Discussion

Prolonged sampling of desert tortoises after a single oral dose of CLARI was performed to characterize the pattern of the drug's concentration in plasma over an extended period. A previous study of gastrointestinal motility in this colony suggests that under optimal conditions, complete gastrointestinal transit times are as short as 12 d, but often a few days longer

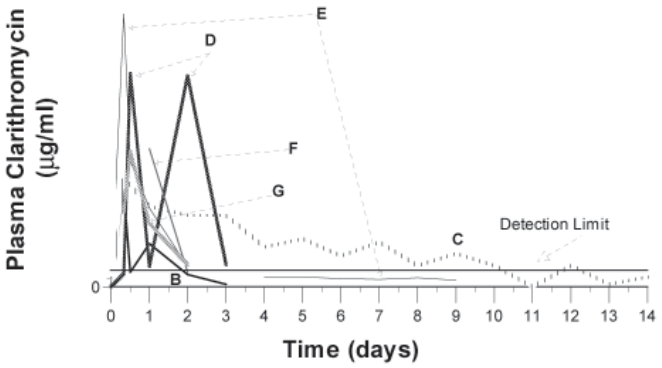

Figure 1. Plasma CLARI concentrations in 6 tortoises (designated B through G) after a single oral dose of 15 mg/kg. Although plasma concentrations declined as expected overall, the shape of individual curves varied considerably, suggesting either a delay in absorption or tissue drug retention followed by slow release into the blood.

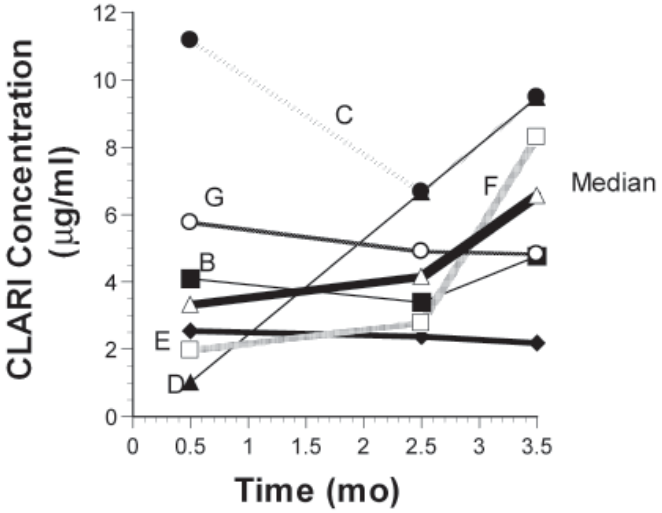

Figure 2. Blood samples were collected at 3 time points during 6 mo of antibiotic administration of twice-weekly oral CLARI at 15 mg/kg. Over the period of administration, plasma concentrations either reached plateau or increased. The median values increased as shown, reflecting a generally increasing trend. Values at 0.5, 2.5, and 3.5 mo ranged from 1.02 to 11.17, 2.37 to 6.67, and 2.28 to 9.48 µg/ml plasma, respectively (n = 6, designated B through G).

early in the year.[43] After a single dose, plasma concentrations of CLARI in most tortoises dropped below the assay detection cut-off by 3 d after dosing; in 1 animal, CLARI concentrations declined steadily until they were below the limit of detection on day 10. On the basis of a comparable motility study, small intestinal transit normally is completed well before 3 d,[38] and absorption profiles are consist with the small intestine as the primary site of antibiotic uptake. Therefore, either protracted lower or large intestinal absorption or cystoenteric reflux contributed to values detected beyond this time point. Tissue retention of CLARI, especially by lung tissue, has been reported,[17] and similar retention in tortoises could enlarge existing compartments, delaying clearance. Because tortoises eat a higher fiber roughage diet than do humans, some delay in clearance can be expected, as the antibiotic is retained in undigested material in the intestine after oral dosing. Horses are another roughage-eating species that similarly perform hindgut fermentation; however, they do not show delayed CLARI clearance.[17] The blood levels of CLARI noted in tortoises after oral dosing exhibit the early extremes in peak and trough excursions seen in mam-

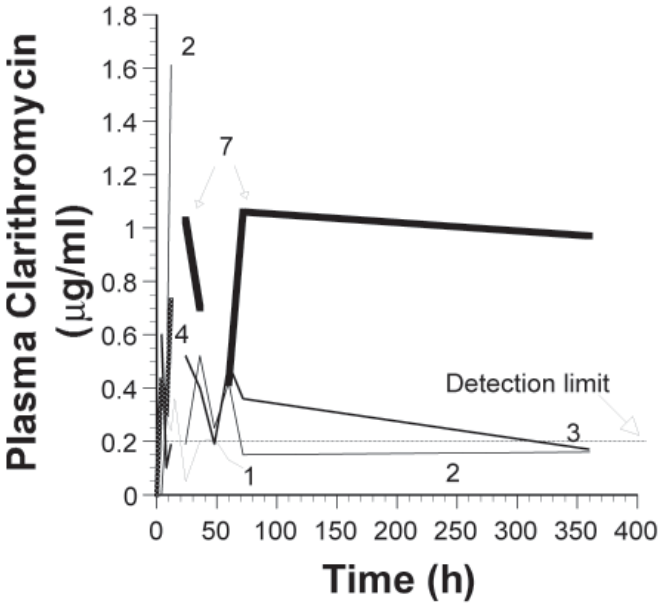

Figure 3. Plasma concentrations of CLARI based on per rectum dosing of 7 tortoises. Five tortoises are shown after treatment with 15 mg/kg every 12 h for the first 3 doses (to 36 h), followed by the same dose delivered daily until day 8. In 2 animals (tortoises 5 and 6), concentrations of CLARI rose above the limit of detection at only 1 point each, so their data were not included in the graph.

mals.[9] Feeding different diets with varied transit times during CLARI oral dosing might help to establish whether this delay in clearance is related to diet and the effect that dietary form can have on transit times.

Another reason for establishing the time frame for CLARI persistence in tortoises was to determine whether the modeled kinetic profile followed the pattern of drug accumulation observed previously in a preliminary study.[45] The results of the present study confirmed that CLARI accumulates, as has been described for carbenicillin in 2 *Testudo* species.[28] The CLARI accumulation dissipated after 24 to 72 h in most tortoises, suggesting that delayed absorption might contribute to drug accumulation and a steady state level. A previous study found that gastrointestinal transit times were influenced by pen location for this tortoise colony.[43] As a result, all the animals studied were housed in the same enclosure to control for this potential influence. However, delayed large intestinal transit could not be distinguished from cystoenteric reflux and recycling in this series, and the per-rectum dosing study showed that rectal (large intestine) absorption of CLARI does occur. In addition, because CLARI likely collects in the bladder, direct absorption through the bladder wall is another possibility.

Compared with mammals, reptiles often require protracted antibiotic treatment periods. In particular, CLARI treatment for URTD in tortoises may require prolonged administration over weeks to months at increased doses to effect a complete remission of signs.[16] Oral drug administration requires either pharyngostomy tube placement or regular head restraint; the latter in particular can be stressful to the animal if applied over long periods. Thiabendazole serum levels were significant and the treatment clinically effective after per rectum dosing.[3] In the present study, plasma levels were above the limit of detection in several animals and exceeded 1 μg/ml in a single animal. Therefore, the distal large intestine of tortoises has some absorptive capacity, and large intestinal absorption might contribute to increased oral availability or to cystoenteric recycling, either of which could lead to CLARI accumulation over time. The disposition of radiolabeled CLARI has been studied in the rat,[27] and a similar study in tortoises likely would reveal the probable mechanism(s) for CLARI accumulation. Defecation of rectally instilled drug would decrease detected levels, a possibility that may have gone unnoted and could explain some of the variability in blood levels detected in different animals.

Overall, the results of the current studies suggest that CLARI oral administration to tortoises leads to accumulation of the drug in the blood stream and that large intestinal absorption or recycling may contribute to this accumulation. Long-term dosing is safe and mirrors the experience in humans, in whom the incidence of severe side effects is low.[19] At the dose used (15 mg/kg), twice-weekly dosing can maintain the target levels previously recommended for the treatment of mycoplasmosis;[45] this dose was verified here during long-term administration in chronically *Mycoplasma*-infected tortoises.

Clarithromycin has a broad spectrum of activity.[39] Our original goal in studying CLARI was to reach blood values considerably greater than 1 μg/ml, which correspond to the high levels seen in bronchial lavage samples[11,13] and alveolar tissue[33] from human volunteers. Our suspicion was that although the minimal inhibitory concentration of CLARI for *M. pneumoniae* was quite low,[23] still higher levels might be required for *M. agassizii* control due to animal debility. Previous experience in our colony with enrofloxacin and tylosin and gentamicin eyedrops suggested that the treatment of *M. agassizii* infected tortoises led to the frequent recurrence of clinical signs within 2-4 weeks of stopping treatment.

On the basis of clinical experience and repeated studies in tortoises, CLARI appears relatively nontoxic, even in moderately URDS-debilitated animals. Higher or more frequent doses might be of value for optimal clinical outcomes in severe cases. To reach target levels, per rectum dosing will require doses considerably higher than those attempted here and may not be of practical value; greater variability in plasma levels is expected per rectum than by other routes of delivery. Further studies are required to characterize the complete disposition of CLARI in terrestrial chelonians after oral and parenteral administration and particularly to determine airway concentrations needed to treat mycoplasmosis. Given the preliminary studies presented here and clinical experiences with this drug for the treatment of URTD, CLARI shows considerable promise in treating this chronic progressive disease, providing that sufficiently high tissue levels can be sustained in animals that are not too debilitated. The evidence that CLARI accumulates in tortoises should serve as a cautionary tale, in that single dose studies, although widely reported in the literature for exotic species, may be insufficient to provide therapeutic guidelines for new drugs.

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