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Toxicological and Toxicokinetic Analysis of Angiotensin (1–7) in Two Species

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Abstract

The objectives of this study were to determine the potential systemic and local toxicity, as well as evaluate the toxicokinetic (TK) profile of angiotensin (1–7) [A(1–7)] when administered daily via subcutaneous injection for 28 days to Sprague–Dawley rats and Beagle dogs. A(1–7) is a member of the renin–angiotensin system and has undergone clinical evaluation for the treatment of chemotherapy-induced myelosuppression. In this present study, A(1–7) was given at 10 mg/(kg day) for 28 days to rats and canines. At day 27, blood was harvested to evaluate the TK parameters. On day 28, systemic toxicology was evaluated. Following A(1–7) administration for 27 days, no plasma A(1–7) accumulation was detected in canines; however, increased A(1–7) plasma concentrations were detected in rats. Despite the accumulation observed in rats, no detectable toxicity was found following A(1–7) administration for 28 days. The TK analysis of A(1–7) revealed a plasma half-life of 20–30 min in both rats and canines. The time to maximum plasma concentration was found to be 15 and 26.25 min in rats and canines, respectively. This study shows that subcutaneous administration of A(1–7) at 10 mg/(kg day) for 28 days did not lead to any detectable toxicities in either rats or canines.

Keywords

toxicology; pharmacokinetics; LC–MS; peptides; analysis

Introduction

The components of the renin–angiotensin system (RAS) such as angiotensin II (A-II) play an important role in regulating blood pressure and fluid balance in the body.¹ The biological activities attributed to angiotensin peptides have expanded to include accelerated wound healing and hematopoiesis after radiation or chemotherapy-induced myelosuppression.^{2,3} Initially A-II was found to promote wound healing by binding to angiotensin I receptor.^{4,5} This was later followed by evidence supporting that angiotensin (1–7) [A(1–7)] was able to promote wound healing by increasing reepithelialization of wounds and by increasing the proliferation of stem cells at the base of hair follicles.^{3,6,8,10} The biological properties associated with A(1–7) are mediated by binding to the Mas receptor, a G-protein-coupled receptor, which was initially described as a proto-oncogene product.^{11,12} The role of A(1–7)

in wound healing was confirmed when a Mas receptor antagonist was able to delay wound healing in a diabetic mouse model.²

Angiotensin (1–7) is a seven-amino-acid peptide in the RAS that was thought initially to be an inactive metabolite of A-II. However, this heptapeptide was found able to not only just accelerate wound healing, but also have potent hematopoietic activity. Endogenous A(1–7) is metabolically produced by neutral endopeptidase and angiotensin converting enzyme (ACE) 2, depending upon the substrate.⁹ Unlike A-II, A(1–7) does not exhibit hypertensive properties, and thus makes it a promising drug candidate to accelerate hematopoiesis.

Clinical trials to evaluate the safety and optimal dosing for A(1–7) confirmed its ability to stimulate hematopoietic recovery after chemotherapy- or irradiation-induced injuries.^{3,2,7} No identifiable dose-limiting toxicity was established at the dosages used in those studies. In an effort to identify the toxicity profile of A(1–7), a toxicokinetic (TK) analysis was performed in rats and canines. In this study, A(1–7) was administered at 10 mg/(kg day) subcutaneously in rat and canine models for 28 days.

Materials and Methods

Materials

Angiotensin (1–7) (prepared by Good Manufacturing Practices) for animal studies was purchased from Bachem (Torrance, California).

Angiotensin (1–7) and the internal standard Nor-Leu³-A(1–7) for assays were also purchased from Bachem. Solid-phase extraction (SPE) C18 columns (1 mL, 200 mg) were purchased from Honeywell Burdick & Jackson (Morristown, New Jersey, USA). All other chemicals and solvents were high-performance liquid chromatography (HPLC) grade (EMD Chemicals, Darmstadt, Germany), and deionized water was used in all experiments.

Animal Study Design

Sprague–Dawley rats (8–9 weeks old) were used for these studies, where 40 (20 per sex) were assigned to the toxicology portion of the study. These animals received either 10 mg/(kg day) subcutaneously of A(1–7) or vehicle controls only, where 10 animals (females or males) were in each group. An additional, 36 (18 per sex) rats were used in the TK studies. In the TK studies, nine females or males were in each group.

Beagle dogs (10 males and 10 females) were received in good health from Ridgman Farms, Inc., Mount Horeb, Wisconsin. All animals were immunized prior to arrival. The immunizations included for rabies, *Bordetella*, distemper, adenovirus type 2, parvovirus, parainfluenza, and oral papilloma virus. Each animal was uniquely identified with an ear tattoo by the supplier prior to shipment and with a subcutaneous microchip (Bio Medic Data Systems, Seaford, DE, USA) in the right lateral neck area placed during acclimation. The animals were approximately 6–7 months old at receipt. Each animal was examined by a veterinarian upon receipt and weighed on the following day. Animals considered suitable for the study were housed for a 15-day acclimation/pretest period. During this period, each animal was observed twice daily for mortality and changes in general appearance or behavior. Fecal samples were collected prior to the initiation of dose administration and checked for parasites; none were present. These animals received either 10 mg/kg/day of A-(1-7) subcutaneously or vehicle controls only, where eight animals (four females and four males) were there in each group.

Parameters Evaluated

Clinical Observations (Toxicology Animals)

Daily Observations—A clinical observation was performed once daily during pretest, beginning at least 7 days prior to randomization. A clinical examination was performed twice daily, on days of dosing, prior to dosing and approximately 1–2 h after dose. Observations included, but were limited to the following: changes in skin and injection sites (incidence and severity of injection-site observations); fur; eyes and mucous membranes; respiratory, circulatory, autonomic and central nervous system functions; and somatomotor activity and behavior patterns. The absence or presence of findings was recorded for all animals.

Detailed Physical Examinations—A detailed physical examination was conducted at least once during the pretreatment period, at the time of randomization, once weekly during the dosing period, and on the day of the scheduled necropsy.

Dermal Observations—Injection sites were examined for erythema, edema, and other dermal findings on days prior to dosing (all sites) and approximately 1–2 h after dose (only the site dosed that day). Erythema and edema were evaluated based on a four-step grading system of very slight, slight, moderate, and severe.

Body Weights and Food Consumption—Individual body weights and food consumption were recorded weekly during acclimation, at pretest initiation, at randomization, weekly during the dosing period, and just prior to the scheduled necropsy.

Ophthalmic Examination—Ophthalmic examinations were conducted using an indirect ophthalmoscope and slit lamp biomicroscope prior to randomization and at the end of dosing. Prior to examination, animals were treated with an appropriate mydriatic agent.

Electrocardiography and Heart Rates (Canines Only)—Electrocardiograms were recorded using multi-lead electrocardiography equipment with the Ponemah platform from Data Sciences International (St. Paul, MN, USA) during pretest prior to randomization and once near the end of the dosing period. Leads I, II, III, aVR, aVF, aVL and V2, were collected. The DSI Ponemah validated software captures and stores ECGs electronically. Heart rate, QRS duration, P-R, QT, and QTcV (QTc Van de Water's) interval values were generated.

Toxicokinetics (TK Animals)

Intervals and Timepoints—Study days 0 and 27: Samples were collected at predose and at approximately 15, 30, 45, 60, 90, and 120 min after dose. Three rats/sex/group/timepoint were sampled in satellite groups, and no animal was bled more than two times within a 24-h period at the indicated intervals. Blood was collected from the beagles at the same intervals, but not from a satellite group.

Blood Collection—Blood was collected in heparinized tubes containing 10 μ L of 100 $\times$ protease inhibitor cocktail, which contained 45 mg/mL phenanthroline, 70 mg/mL ethylenediaminetetraacetic acid, 1.5 mg pepstatin, and 3.75 mg/mL enalapril. The collected blood was centrifuged at 1250 $\times$ g for 10 min. The plasma was then collected and transferred into bovine serum albumin-coated polypropylene tubes and stored at -70°C until analysis.

Plasma Extraction for Analysis—To 100 μ L of plasma sample, 50 μ L of internal standard and 100 μ L 500 ng/mL of Nor-Leu³-A(1–7) were added. The samples were

diluted with deionized water to a total volume of 650 μL , mixed thoroughly, and centrifuged at $5000 \times g$ for 10 min. The clarified supernatant was applied onto C18 (Honeywell Burdick & Jackson) SPE columns (100 mg) that were preconditioned with 2 mL of methanol followed by 2 mL of deionized water. The sample was then washed with 500 μL of deionized water, and A(1–7) and Nor–Leu³–A(1–7) were eluted from the column using 3 mL of methanol with 5% formic acid. The eluent was evaporated to dryness under a steady stream of filtered air. The residue was reconstituted with 200 μL , 80% methanol with 0.1% formic acid in deionized water, and the entire sample was centrifuged at 13,000 rpm for 10 min to remove any remaining debris.

Liquid Chromatography–Tandem Mass Spectrometry Analysis—To quantify the A(1–7) concentration in plasma, 30 μL of the reconstituted sample was injected into an Agilent 1100 HPLC system (Agilent, Santa Clara, USA) linked to an Applied Biosystems Sciex API 3000 mass spectrometer (Applied Biosystems Sciex, Carlsbad, CA, USA) equipped with an electrospray ionization (ESI) source. The analytes were separated on a C18 Hypersil column ($50 \times 2.1 \text{ mm}^2$, 5 μm ; Thermo Scientific). The mobile phase consisted of methanol and deionized water with 0.1% formic acid at a flow rate of 0.25 mL/min. The mobile phase gradient program was initially set at 45% methanol and 55% deionized water with 0.1% formic acid for 4 min, and increased to 85% methanol for 6 min. The mobile phase then returned immediately to 45% methanol for the remaining 10 min.

The positive ESI mass spectrometric conditions were as follows: dwell time, 250 ms; ion-spray voltage, 2.5 kV; temperature, 400°C; turbo ion-spray gas, 6000 L/min. Nitrogen was used as the collision gas, utilized with the collision energies set at 45–53 eV. The analytes were quantified by using the multiple reaction monitoring (MRM) mode, where m/z transition ions of $457.3 \rightarrow 110.3$ and $450.5 \rightarrow 110.2$ were used for doubly charged Nor–Leu³–A(1–7) and A(1–7), respectively. The calibration curves were linear over a range of 10–1000 ng/mL, with the lowest level of detection at 10 ng/mL.

Plasma Analysis and TK Analysis—Plasma TK parameters were calculated from individual rat plasma concentration values for each dosage level and interval. The TK parameters that were evaluated included maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), area under the plasma concentration versus time curve (AUC), dose proportionality, and half-life. TK parameters and descriptive statistics (mean, standard deviation) were calculated using noncompartmental pharmacokinetics.

Dose proportionality was assessed using power analysis. Graphs were prepared, where necessary, to illustrate salient features of the TK data in GraphPad Prism, version 5.0d (La Jolla, CA, USA).

Clinical Pathology—Clinical pathologic parameters were evaluated on all animals prior to their scheduled necropsy. Blood samples were used for the analysis of routine hematology, coagulation, and serum chemistry parameters. Urine was collected overnight using metabolism cages.

Anatomic Pathology—A complete necropsy was conducted on all animals at the scheduled necropsy. Necropsy included examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. At the time of necropsy, all standard tissues and organs were collected.

Organs Weights—The adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid with parathyroids, and uterus were collected and weighed at necropsy.

Microscopic Examination—Microscopic evaluation included all tissues (including injections sites) collected.

Statistical Methods—All analyses for toxicological analysis were two-tailed for significance levels of 5% and 1%. All statistical tests were performed using GraphPad Prism 5.0. Body weights, body-weight changes, food consumption, organ weights (absolute and relative), and clinical pathology values were subjected to a parametric two-sample *t*-test (Snedecor and Cochran, 1980) to determine intergroup difference.

Results

Toxicology Studies

There were no test article-related effects on survival, clinical observations, dermal observations, body weights, food consumption, clinical pathology, macroscopic observations, or organ weights in either Sprague–Dawley rats or Beagle dogs. Some reactions were observed at the local injection site. In dogs, microscopic findings of a slightly increased severity and/or incidence of inflammation in the subcutis at the injection sites, although considered test article-related, were not considered to be adverse. In rats, there were no clear differences at the injection sites between the control and test article-treated groups. Most of the hemorrhage, cellular infiltrates, and inflammation were seen between the panniculus carnosus muscle (associated with the skin/deep subcutaneous tissue) and the deeper skeletal muscle associated with the regions of the injection sites.

In rats, effects of treatment with A(1–7) on organ weights consisted of higher absolute and relative (to brain and body weight) thyroid/parathyroid weight in females; these differences were statistically significant ($p < 0.05$). A(1–7) microscopic findings were limited to the thyroid follicular epithelial cells in the treated males. Minimal cytoplasmic vacuolation was seen in one out of 10 control-group males and six out of 10 males in the 10 mg/(kg day) group. There was some variation within the control-group males as to what constituted the “normal” morphology of the thyroid follicular cells; about half had no appreciable vacuolation and a near equal number had vacuolation that was multifocally distributed. One control-group male and six of the 10 mg/(kg day) group males had diffuse and prominent cytoplasmic vacuolation that was considered “atypical” and was graded as a minimal finding. A similar distinction was not seen in the female rats. Therefore, a microscopic correlation for the higher thyroid/parathyroid gland weights seen in the test article-treated group females was not detected.

Once-daily subcutaneous administration of A(1–7) to male and female Beagle dogs or Sprague–Dawley rats for 28 consecutive days was well tolerated at 10 mg/(kg day) and resulted in no test article-related effects on survival, clinical observations, dermal observations, body weights, food consumption, clinical pathology, macroscopic observations, or organ weights. Microscopic findings of inflammation in the subcutis were noted at the injection sites, and although test article-related, were not considered adverse. No histological toxicities were detected from the target organs analyzed.

Canine TK Study

The plasma concentration of A(1–7) was determined using a validated liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay, where the lower limit of quantitation (LLQ) was 10 ng/mL. This assay detects the 2⁺ charged A(1–7) and its corresponding transition ion using MRM. The concentration of A(1–7) detected in dog plasma is summarized in Figure 1, and concentration versus time curves for individual canines are shown in Figure 2. In canines, A(1–7) TK profiles on days 0 and 27 were very

similar. Individual noncompartmental toxicokinetics were performed where the geometric mean for each parameter is summarized in Table 1. The half-life of A(1–7) on days 0 and 27 were similar (30.86 ± 12.59 min and 27.70 ± 4.02 min for days 0 and 27, respectively). As expected, the elimination constant (K_{el}) was 0.03 min^{-1} for both days 0 and 27. When the AUC for A(1–7) was evaluated for days 0 and 27, a reduction of 15% was seen on day 27 as compared with day 0.

Rat TK Study

A similar serial TK study was performed in rats. Different from the study performed using canines, serial blood samples could not be collected in the same rat due to the limited total blood-sample volume. Thus individual noncompartmental TK analyses could not be performed. A consolidated analysis was performed using the mean plasma A(1–7) concentration in six rats per timepoint (Figure 3). The consolidated toxicokinetics are summarized in Table 2 with mean A(1–7) concentrations at various timepoints. The corresponding toxicokinetics were calculated, where the elimination and half-life was found to be similar between days 0 and 27. A notable TK parameter difference was seen with C_{max} and AUC between the two timepoints. The C_{max} for A(1–7) in rats were 26 and 257 ng/mL for days 0 and 27, respectively, which is approximately a 10-fold increase on day 27 as compared with day 0. Correspondingly, the AUC for A(1–7) on days 0 and 27 were 949 and 6069 ng min/mL, respectively, which is approximately a sixfold increase on day 27 as compared with that of day 0. In addition, samples from animals receiving diluent only as controls were also collected. Predose samples were analyzed, as well as samples from the C_{max} timepoint groups (15 min for rats and 30 min for canines). In these samples, plasma A(1–7) concentrations were either below the LLQ or no peaks were detected.

Discussion

The TK analysis of A(1–7) in canines showed no accumulation where the AUC was decreased by 15% after 27 days of administration. On day 0 of this study, the mean T_{max} was 26.25 ± 10.61 min following subcutaneous injection of A(1–7), and the C_{max} was 1153 ± 489 ng/mL. The mean plasma half-life was determined to be 30.86 ± 12.59 min, with a corresponding K_{el} of $0.03 \pm 0.01 \text{ min}^{-1}$. When a noncompartmental analysis was performed, the mean AUC of A(1–7) was $54,862 \pm 12,251$ ng min/mL ($914 \pm 204 \mu\text{g h/L}$).

When serial TK analysis was performed in canines treated for 27 days, the T_{max} was 18.75 ± 10.61 min following subcutaneous administration of A(1–7), where the C_{max} was 953 ± 377 ng/mL. There were no significant changes in the elimination half-life after 27 days, where the mean half-life was determined to be 27.70 ± 4.02 min. The AUC of A(1–7) was $47,380 \pm 12,605$ ng min/mL on day 27, which was significantly lower than the AUC on day 0 ($p < 0.05$).

When 10 mg/(kg day) of A(1–7) was administered subcutaneously to rats, a significant change in the AUC was noted on day 27. The AUC for A(1–7) on days 0 and 27 were 950.5 and 6069.2 ng/mL, respectively, which represents an approximately sixfold increase from day 0 ($p < 0.05$). In addition, the C_{max} on day 27 was approximately 10-fold higher as compared with that on day 0 ($p < 0.05$).

In samples from animals receiving only vehicle, either no peaks were detected or the peak levels were below the LLQ (<10 ng/mL). In canine samples, there were two animals that had quantifiable peaks in the predose (day 0) group. None of the samples collected at 30 min following administration of vehicle had a detectable peak. In rat samples, multiple samples had detectable peaks, but all were below the level of detection.

The pharmacokinetics of A(1–7) was also evaluated recently by Petty et al.¹³ in 18 patients with advanced cancer. Pharmacokinetics in their study was similar to that was seen in the present study, where the reported A(1–7) plasma half-life was comparable and some accumulation was seen at higher dosing range. However, the A(1–7) plasma $T_{\max}$ reported by Petty et al.¹³ was about 1 h, approximately twice as long as the $T_{\max}$ we observed in this study. Importantly, the first sampling time following A(1–7) dosing in the Petty et al.¹³ study was also 1 h, whereas the first time-point following dosing in present study was 15 min, which could account for the difference seen. Also, pharmacokinetics of the same drug in various species is very different, and although Petty et al.¹³ evaluated patients with advanced cancer, our study evaluated rats and canines without an underlying and active disease state. In addition, the maximum dose used in the Petty et al.¹³ study was more than 14 times lower than the dose used in the present study. Lastly, in this study, we used a validated LC–MS method where the lower level of sensitivity was 10 ng/mL, whereas Petty et al.¹³ used an enzyme-linked immunosorbent assay.

The toxicity of A(1–7) has been previously evaluated in humans at a significantly lower dosage. Rodgers et al.¹⁸ showed that at 100 $\mu\text{g}/(\text{kg day})$ for at least 10 days, A(1–7) attributable toxicities were mild with no dose-limiting toxicities. In a similar dose escalation study, the safety of A(1–7) was also demonstrated, where two patients developed dose-limiting toxicities at 700 $\mu\text{g}/(\text{kg day})$ dosage levels.¹³ In this study, no detectable toxicities were revealed when A(1–7) was employed at 10 mg/(kg day).

There are several potential reasons for the differences seen in $C_{\max}$ and AUC following 27 days of A(1–7) administration in rats and canines in this study. A(1–7) is metabolized by ACE.¹⁴ This enzyme is expressed in a number of tissues and the expression and activity of the enzyme is modulated by A(1–7).^{15,16} Inhibition of ACE activity by A(1–7) and its metabolites may affect the $C_{\max}$ and AUC, as the drug is being adsorbed and eliminated from the plasma, but would not lead to an accumulation of A(1–7) from the prior injection due to several half lives for the drug between injections. Further, changes in ACE expression by A(1–7) may modulate these pharmacokinetic parameters. Administration of A(1–7) also modulates expression of its receptor, Mas.¹⁶ Modification of tissue levels of this receptor may change the distribution of A(1–7) after repeated injection of high doses.

Further studies have shown differences between canines and rats in ACE distribution and inhibitory capacity. For example, one past study has demonstrated differences in A-II receptor and ACE expression in human, canine, and rat mitral valves.¹⁹ In another study of the ACE inhibitor, enalapril, there are interspecies differences between canines and rats with regards to the ability of the drug to inhibit ACE.¹⁷

Because RAS system expression is variable and often tissue-specific, increased or decreased expression of this receptor or enzyme could result in altered plasma levels of this heptapeptide. An alteration in ACE levels over time could result in changes in A(1–7) plasma levels in different species. Although the tissue disposition of A(1–7) was not evaluated in this study, increased or decreased sequestering of A(1–7) into various tissues in rats or canines could also explain the differences seen in plasma A(1–7) $C_{\max}$ and the AUC between these two species.

In summary, daily administration of 10mg/(kg day) A(1–7) did not result in significant toxicological effects. TK evaluation showed a distinction in AUC between days 1 and 27 in both rats and canines.

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Figure 1. Time versus concentration curve of A(1-7) determined in canines, wherein the closed circles represent animals sampled on day 0 and the open squares represent animals that were administered A(1-7) subcutaneously for 4 weeks.

Figure 2.

Time versus A(1-7) concentration curve in rats, where the closed circles represent day 0, whereas the open squares represents day 27.

Figure 3. Concentration versus time for each individual canine. Plasma A(1-7) concentration on day 0 is represented by the closed circles and A(1-7) concentration on day 27 is represented by the open squares.

Table 1

Pharmacokinetic Analysis from Day 0 and Day 27 in Canines

Day 0	Average ± SD	Median
C_{max} (ng/mL)	1153 ± 489	1097
T_{max} (min)	26.25 ± 10.61	30
Half-life (min)	30.86 ± 12.59	28.50
K_e (min ⁻¹)	0.03 ± 0.01	0.03
AUC (ng min/mL)	54862 ± 12251	54491
Day 27	Average ± SD	Median
C_{max} (ng/mL)	953 ± 377	841
T_{max} (min)	18.75 ± 10.61	15.00
Half-life (min)	27.70 ± 4.02	27.16
K_e (min ⁻¹)	0.03 ± 0.00	0.03
AUC (ng min/mL)	47380 ± 12605	44936

Table 2

Rat Plasma A(1-7) Concentration Versus Time

Time (min)	Day 0		Day 27	
	Concentration (ng/mL)		Conc (ng/mL)	
0	1.72		1.707	
15	25.55		257.133	
30	11.67		98.117	
45	9.05		7.833	
60	5.93		18.955	
90	2.91		5.180	
120	1.53		1.887	
Pharmacokinetic parameters				
$C_{\max}$ (ng/mL)	Day 0		Day 27	
	25.6		257.1	
$T_{\max}$ (min)	15.0		15.0	
Half-life (min)	27.2		16.0	
K_{el} (min^{-1})	0.025		0.043	
AUC (ng min/mL)	950.5		6069.2	