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Oral Repeated Dose Toxicity Studies of Coenzyme Q10 in Beagle Dogs

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Abstract

To support phase III testing of Coenzyme Q10 (CoQ₁₀) in humans, we conducted pharmacokinetic and toxicology studies in beagle dogs. Following single gavage administration of CoQ₁₀ at 600, 1200, 1800 or 2400mg/kg/day no obvious dose response was observed in maximum concentration (C_{max}) or area under the concentration versus time curve (AUC) at the three highest dosages. In a repeated dose study of CoQ₁₀ at 600, 1200, 1800 or 2400mg/kg/day for four weeks, CoQ₁₀ reached steady state in plasma by two weeks at all dosages. C_{max} and AUC increased with increasing dosage of CoQ₁₀. The highest plasma levels were recorded at 1800mg/kg/day. In a 39-week chronic toxicity study of CoQ₁₀ at 1200, 1800mg/kg/day or placebo, CoQ₁₀ reached steady state in plasma by 13 weeks. Behaviors, blood chemistries and detailed histopathology were normal. No deaths occurred. These results support the use of a 2400mg/day dosage of CoQ₁₀ in human clinical trials.

Keywords

Beagle dogs; Coenzyme Q10; neurodegenerative disorders; toxicology; ubiquinone

Introduction

To support the proposals for large phase 3 efficacy and long-term safety studies of coenzyme Q10 (CoQ₁₀) treatment at 2400mg/day in people with Huntington's disease (HD)

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Declaration of Conflicting Interests Dai Watanabe, Mitsuaki Kitano, Kazunori Hosoe and Iwao Funahashi are presently employed by Kaneka Corporation, manufacturers of the CoQ₁₀ product used in these studies. All other authors report no other relevant financial conflicts of interest. The authors alone are responsible for the content and writing of this paper.

and Parkinson's disease (PD), long-term toxicology testing in a non-rodent species was conducted. The study in HD will treat people for five years; the study in PD for 16 months¹. A 39-week non-rodent animal chronic toxicity study was designed to identify any toxic effects of CoQ₁₀ at the maximum feasible dosage. To determine the dosage and formulation of CoQ₁₀ for the 39-week chronic toxicity study, single and repeated dose studies in dogs were first performed.

CoQ₁₀ (2, 3 dimethoxy-5-methyl-6-decaprenylbenzoquinone), also known as ubiquinone (the oxidized form of CoQ₁₀), is a lipid soluble vitamin-like benzoquinone derivative; structurally similar to vitamin K². Found in the inner mitochondrial membrane of most eukaryotic cells, its function is to act as an electron acceptor and donor in the electron transport chain for ATP production, and to buffer free radical damage during oxidative phosphorylation. In many neurodegenerative disorders, including PD and HD, there is strong evidence to suggest that mitochondrial dysfunction may play a role in pathogenesis³ and CoQ₁₀ is neuroprotective in animal models of HD^{4, 5}, PD⁶, Alzheimer's disease⁷ and amyotrophic lateral sclerosis (ALS)⁸. In a number of phase 2 safety and tolerability studies in individuals with inherited defects in CoQ₁₀ production, and in individuals with ALS, PD or HD receiving dosages up to 3600mg/day of CoQ₁₀, no evidence of toxicity has been detected^{9–14}.

Materials and Methods

Overall Design of Toxicology Program

Three studies in dogs were conducted by Bozo Research Center, Inc. (Shizuoka, Japan), a contractor of Kaneka Corporation (Osaka, Japan), in compliance with good laboratory practice regulations for the conduct of non-clinical studies on the safety of drugs, guidelines for toxicity studies by the Ministry of Health and Welfare, Japan, and animal welfare guidelines formulated by the Ministry of Environment, Japan and the Science Council of Japan. Pharmacokinetic investigations were performed in all three studies. Two preliminary dose range finding studies were conducted to select appropriate dosages for the chronic toxicity study in dogs. The definitive chronic toxicity study was designed to test CoQ₁₀ up to a maximum tolerated or a maximum feasible dose. In all studies the oxidized form of CoQ₁₀, ubiquinone, was administered by oral gavage, and herein will be referred to as CoQ₁₀.

The purpose of the first study was to examine the total plasma concentration profile after single administration of CoQ₁₀ by gavage in eight beagle dogs, at one of the following dosages: 600, 1200, 1800 or 2400mg/kg/day (two dogs per dosage group). The results were used to simulate total plasma CoQ₁₀ concentration curves to predict plasma concentration, if CoQ₁₀ were repeatedly administered daily. Using these simulated curves the appropriate treatment period length for the repeated dose study was predicted. The purpose of the second study was to examine the steady state plasma total CoQ₁₀ concentration profile for four weeks and to select the dosages for the 39-week chronic toxicity study. This study involved a repeated dose of CoQ₁₀ in a total of eight male beagle dogs at one of the following dosages: 600, 1200, 1800 or 2400 mg/kg/day (two dogs per dosage group). The highest dose that can be administered to dogs at one time is 1200mg/kg. For ease of administration, animals in the 1800 or 2400mg/kg/day groups received CoQ₁₀ by gavage in two equally divided doses daily, approximately six hours apart. The purpose of the third study was to determine any toxic effects of CoQ₁₀ at the maximum feasible dosage over a 39-week period. This study involved a chronic, repeated dose of one of two dosages, low and high, of CoQ₁₀ in a total of 24 beagle dogs. The low dosage level for the 39-week chronic toxicity study was chosen as 1200mg/kg, along with a high dosage level of 1800mg/kg/day, and a placebo group (four dogs of each sex per dosage group). For ease of administration the

1800mg/kg/day dosage was given by gavage in two equally divided doses per day, six hours apart.

Materials

CoQ₁₀ was used in all of the studies described below, and was both manufactured and supplied by Kaneka Corporation (Osaka, Japan). In the single and repeated dose studies the purity of CoQ₁₀ was 99.7% and in the chronic toxicity study 100%. This is the same product as is being used in the clinical trials in individuals with HD and PD.

Animals

All three studies used the Hazelton Research Animal (HRA) strain of beagle dog (Covance Research Products Inc., VA, USA). Both the single dose and repeated dose studies used eight male dogs, aged between 10 to 12 months and weighing between 8 and 11 kg at initiation of treatment. The chronic toxicity study used equal numbers of male and female dogs all (24 in total) at seven months of age at initiation of treatment. The males weighed between 7 and 10 kg and the females between 6 and 8 kg at initiation of treatment. Animals were housed in individual metallic cages (W 900 × D 850 × H 750mm, Shinetsu-Kanaami Corporation, Niigata, Japan). Environmental conditions for the animal rooms were maintained at a temperature of 22 ± 4°C, a relative humidity of 55 ± 15%, air ventilation at 13 to 15 times per hours and lighting for 12 hours a day (07.00 to 19.00). Animals were given 300g/day of a pelleted diet DS-A (Oriental Yeast Co., Ltd. Tokyo, Japan), at set times, except when they were fasted. Quarantine and acclimation periods were one to two weeks long, during which time animals were weighed weekly and checked daily for their appetite and general condition, including nutritional and fecal. Animals were allocated to dosage group by a computer generated assignment system based on stratified weight to achieve identical group weight averages and avoiding assigning litter mates to the same group.

Dose Preparation

Dose calculations in all three studies were based upon the most recent body weights of each animal. Test article was prepared by filling gelatin capsules (½ ounce, Torpac Inc., New Jersey, USA) with CoQ₁₀. Animals in the control group received placebo in the form of empty gelatin capsules. CoQ₁₀ preparations were mixed once or twice a week, with capsules kept in cases arranged by animal number and stored either frozen (at -20°C or at -35°C) for the preliminary studies or refrigerated (4°C) for the chronic toxicity study. All capsules were used within seven days of preparation. Stability of CoQ₁₀ filled capsules was verified at the end of each study using ubiquinone as a reference standard and CoQ₇ as an internal standard.

Dose Administration - Single and Repeated Dose Studies

Both the single and repeated dose studies used dosages at 600, 1200, 1800 and 2400mg/kg/day. Animals in the low (600) and low-middle (1200) dose groups received gelatin capsules once daily. To maintain animal welfare, animals in the high-middle (1800) and high dose (2400) groups received capsules twice daily, once in the morning and then approximately six hours later, because it was technically difficult to give CoQ₁₀ in a single dose by gavage at dosages greater than 1200mg/kg/day. Animals in the repeated dose study received CoQ₁₀ for a total of four weeks.

Dose Administration - Chronic Toxicity Study

Animals in the chronic toxicity study were given gelatin capsules by gavage for a total of 39 weeks. Males and females were equally divided into three groups, receiving one of two dosages of CoQ₁₀, either 1200 or 1800mg/kg/day, or placebo (empty gelatin capsules).

Those in the 1200mg/kg/day group received capsules once daily. Animals in the 1800mg/kg/day group received capsules twice daily, 900mg/kg once in the morning and then approximately six hours later, and those in the placebo group, empty gelatin capsules twice daily. Divided doses were given where it was technically difficult to give the test article in a single dose by gavage.

Clinical Observation and Examination

Animals in all three studies were examined by a veterinarian and observed for morbidity, by visual inspection and palpation, and mortality. Body weight was monitored in the morning and if on a dosing day, before first dosing. Body weights were not intended to be used in the evaluation of CoQ₁₀ toxicity but as a means by which to calculate the correct dose for administration. One-day food consumption was calculated as the difference between the amount of feed supplied and that left uneaten. Daily food consumption (weekly mean value) was calculated from the cumulative food consumption in a week.

Clinical Observation - Single Dose Study

Animals were observed for clinical signs according to the following schedule: on the day of administration (Day 0) prior to dosing, immediately after dosing, 1, 2, 3, 4, 5, 6, 7, and 8 hours after dosing, and once daily during the observation period (Days 1 to 3). Body weight was measured one week prior to administration (Day -7), on the day before administration (Day -1), and on the day of administration (Day 0) prior to dosing. Food consumption was measured daily from one week prior to administration until the end of the study.

Clinical Observation - Repeated Dose Study

Animals were observed for clinical signs according to the following schedule: once daily during the week prior to administration (Day -7 to Day -1), two to three times daily during the administration period (Days 1 to 28), and once daily after the end of the administration period for three days (Days 29 to 31). Body weight was measured once during the week prior to administration, once on the starting day of administration and once weekly throughout the administration period. Food consumption was measured daily from one week prior to administration until the end of the study.

Clinical Observation - Chronic Toxicity Study

Animals were observed for clinical signs according to the following schedule: once daily during the week prior to administration (Day -7 to Day -1), and two to three times daily during the administration period (Day 1 to Day 273). Body weight was measured once during the week prior to administration, once on the starting day of administration, once weekly throughout the administration period, and once on the day of necropsy (Day 274), prior to the procedure. Food consumption was measured daily from one week prior to administration until the end of the study.

Pharmacokinetic Analysis

To determine CoQ₁₀ concentration in plasma 1.5mL blood samples were collected from the cephalic vein of all animals at each sampling time point. As the bioavailability of CoQ₁₀ is enhanced by food, samples were taken without prior overnight fasting of animals. Samples were collected in tubes containing heparin sodium (Venoject II, VP-H052K, Termo Corporation Ltd., Tokyo, Japan), cooled in ice, centrifuged (approximately 1,600 × g for 10 minutes) to harvest plasma (0.5mL) and stored frozen at -20°C until analysis. Plasma ubiquinone was then identified by a high pressure liquid chromatography (HPLC) method, modified and validated for support of this study¹⁵. Pharmacokinetic analysis of the data included, if appropriate, determinations of maximum concentration (C_{max}) (micrograms/

mL), time to reach the maximum concentration ($T_{\max}$) (hours), area under the concentration versus time curve (AUC_{0-72h}) (microgram hours/mL), and the elimination half-life ($T_{1/2}$) (hours).

In the single and repeated dose studies, pharmacokinetic parameters were calculated using the one-compartment model of WinNonlin (version 5.0.1, Pharsight Corporation). In the chronic toxicity study the mean and standard deviation (S.D.) of these parameters was calculated for each group (placebo, low dose and high dose) at each time point. On Day 1 and at Week 39 $C_{\max}$ and $T_{\max}$ were determined from the measured values, and the AUC_{0-72h} was calculated by the trapezoidal method¹⁶.

Pharmacokinetic Sampling Schedule

In the single dose study plasma samples for pharmacokinetic analysis were collected pre-dose, and then at 2, 4, 8, 10, 24, 34, 48, 58 and 72 hours after dosing. In the repeated dose study plasma samples were collected at the following time points: pre-dose and at 4, 8 and 24 hours after first capsule administration on Days 1, 7, 21 and 24, and pre-dose and at 4, 8, 24, 34, 48, 58 and 72 hours after first capsule administration on Day 28. In the chronic toxicity study plasma samples were collected on Days 1, and 273 (week 39), at the following time points: pre-dose and 1, 4, 8 and 24 hours after first capsule administration of the day (i.e. five sampling time points). On Day 91 (week 13), and 182 (week 26) sampling time points were pre-dose, and at 4 and 24 hours after first capsule administration of the day.

Standard Toxicological Examinations - Chronic Toxicity Study

Additional monitoring performed in the chronic toxicity study consisted of standard toxicological examinations conducted in accordance with guidelines formulated by the Ministry of Health and Welfare, Japan¹⁷⁻¹⁹. These standard examinations included electrocardiographic (ECG) and ophthalmologic examinations, and standard laboratory tests for safety including urinalysis, hematological and blood chemistry including liver enzyme analysis (Supplemental Tables 1, 2 and 3). All were performed once in the week prior to administration and three times during the administration period (weeks 13, 26 and 39) prior to the first dosing of the day. Continuously fresh urine was collected for about four hours prior to the first dosing, while the animals were deprived of food and water. Cumulative urine was collected for approximately 16 hours, with food and water being provided ad libitum. Blood samples for hematology and chemistry were collected from the cephalic vein of all animals after an overnight fast and before dosing. For hematological analysis blood was collected in tubes containing EDTA-2K (SB-41: Sysmex Corporation, Hyogo, Japan). For coagulation analysis blood was collected in tubes containing 3.8% sodium citrate, and for chemistry analysis blood was collected in tubes containing heparin sodium (Ajinomoto Pharma Co., Ltd., Japan).

Anatomical Pathology

At the end of the chronic toxicity study all animals were sacrificed and a necropsy performed. On the day of final administration, all surviving animals were fasted for at least 16 hours. On the day of necropsy (Day 274) animals were anesthetized with pentobarbital sodium solution (20–30mg/kg, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) and pathological necropsy was performed as soon as possible after they expired.

For all animals, brain (the cerebrum, cerebellum and medulla oblongata), pituitary, thyroid, adrenal glands, thymus, spleen, heart, lungs, submandibular glands, liver with gallbladder, pancreas, kidneys, testes/ovaries, epididymis, and prostate/uterus were weighed. Paired organs were weighed separately. Organ weight / body weight ratio was calculated based on an animal's terminal weight. Forty four organs/tissues (male dogs), and 44 organs/tissues

(female dogs) were fixed in phosphate buffered 10v/v% formalin. Eyes and optic nerves were fixed in phosphate buffered 3v/v% glutaraldehyde / 2.5v/v% formalin. Testes and epididymis were fixed in Bouin's solution. All organs were preserved in phosphate buffered 10v/v% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and examined histopathologically.

Statistical Analyses - Single and Repeated Dose Studies

Statistical analysis was not performed in either preliminary study due to the small sample sizes. Evaluation was done on an individual basis in these studies.

Statistical Analysis - Chronic Toxicity Study

All data collected [body weight, food consumption, pharmacokinetic analysis, ECG (except electric axis), urinalysis (urine volume, osmolality and urine electrolytes), hematology, blood chemistry and organ weight] were recorded numerically. The numerical data were used to calculate the mean $\pm$ S.D. for each group (placebo, low dose and high dose) at each time point and then statistically analyzed using multiple comparisons. Bartlett's test (analysis of variance - ANOVA) was initially performed to test for variance homogeneity²⁰. If the ANOVA was significant Dunnett's multiple comparison *t*-test was used to determine pair wise comparisons between the control group, (placebo) and the treatment groups (low dose and high dose)²¹. If samples were from heterogeneous populations, i.e. with unequal variances a Dunnett-type mean rank test was performed relative to the control group. In each of the statistical tests mentioned above, a two-tailed test was used and *p* values of <0.05 were considered statistically significant.

Results

Single Dose Study - Pharmacokinetic Analysis

Prior to the single dosing, plasma total CoQ₁₀ concentrations ranged from 0.3 μ g/mL to 0.8 μ g/mL. After dosing, plasma total CoQ₁₀ concentration increased in all animals, excluding one at 600mg/kg. The reason for this is unclear as a dosing error was unlikely and unchanged capsule, indicating lack of dissolution in the stomach or intestine, was not observed in the stools of this animal. The concentration of total CoQ₁₀ in plasma reached *C*_{max} between 8 and 24 hours and thereafter decreased, but higher than baseline levels were still detected in plasma 72 hours after dosing in all animals. There were no apparent dose responses in the *C*_{max} and AUC_{0-72h} in the 1200, 1800 and 2400mg/kg/day groups (Figure 1), suggesting non-linear kinetics and saturated gastrointestinal absorption. Based on the plasma concentrations measured, total CoQ₁₀ concentration over time curves were simulated using the one-compartment model of WinNonlin (version 5.0.1, Pharsight Corporation) for a repeated dosing schedule at all dosages levels. The simulated curves predicted plasma CoQ₁₀ should reach steady state after three or four weeks. It was therefore judged that the appropriate treatment period for the repeated dose study should be four weeks.

Single Dose Study - Clinical Observations and Examinations

Stool containing test article-like material was observed only once in the time period between six hours after dosing on Day 0 and the following day, Day 1, in all animals excluding one in the 1800 mg/kg group. Body weights of the animals on the day of administration ranged from 8.5 to 10.3 kg. The orange color of stools was thought to be due to the presence of the orange colored CoQ₁₀ test article and was not considered to be a toxic response. This was noted by observation only and not by any further evaluation. No significant changes were noted in food consumption in any animal at any dose.

Repeated Dose Study - Pharmacokinetic Analysis

Prior to the first dosing of the day, plasma total CoQ₁₀ concentration was detected in all animals on Days 1, 7, 14, 21, and 28. Steady state was reached after two weeks in all dosage groups (Table 4). The pharmacokinetic parameters C_{max} and AUC_{0-72h} on Day 28 increased with increasing dosage of CoQ₁₀, between 600 and 1800mg/kg/day. No further increase was seen with the 2400mg/kg/day dosage (Figure 2 and Supplemental Table 5). The highest plasma total CoQ₁₀ levels were therefore recorded at 1800mg/kg/day, with no apparent toxic changes observed in this group. A dose of 1800mg/kg/day (900 mg/kg/day twice daily) was therefore considered to be the highest appropriate level for the chronic toxicity study. Levels of CoQ₁₀ higher than those at baseline were still detected in plasma 72 hours after dosing in all animals. To check data variability plasma CoQ₁₀ concentrations were compared between animals within respective dosage groups using non-parametric ANOVA (Kruskal-Wallis)²². All p values for H (Kruskal-Wallis) were estimated by a Chi-Square test (χ^2) with three degrees of freedom (D.F.). The following non-significant p -values were obtained: Day 1, $\chi^2 = 5.17$, with 3 D.F, $p = 0.160$, Day 7, $\chi^2 = 5.50$, with 3 D.F, $p = 0.139$, Day 14, $\chi^2 = 5.17$, with 3 D.F, $p = 0.1600$, Day 21, $\chi^2 = 6.00$, with 3 D.F, $p = 0.112$, Day 28, $\chi^2 = 6.00$, with 3 D.F, $p = 0.112$. The null hypothesis that samples came from identical populations could not be rejected however due to the small sample size ($n=2$) in each dosage group the statistical validity of this data was not considered meaningful.

Repeated Dose Study - Clinical Observations and Examinations

Stool containing test article-like material was observed in all animals from Day 2 of administration throughout the administration period and one day after the end of administration. Mucous stool, i.e. stool containing a mucoid substance, but not orange in color, was observed in two animals in the 600 and 1800mg/kg/day groups during the administration period and once in one animal in the 1800mg/kg/day group on Day 3 after the end of administration. Vomiting was noted in three animals during the administration period, two in the 600mg/kg/day group and one in the 1200mg/kg/day group. There were no significant changes noted in body weight or food consumption in any group.

Chronic Toxicity Study - Pharmacokinetic Analysis

Pre-treatment plasma total CoQ₁₀, on day one prior to first dosing, was detected in all animals in both treatment groups and the placebo group, with comparable levels in all groups. In the control group, plasma total CoQ₁₀ was detected in all animals at all measurement time points at Weeks 13, 26 and 39.

The mean T_{max} in both sexes on Day 1 ranged from 16.0 to 24.0 hours after administration of CoQ₁₀, whereas the mean T_{max} at Week 39 was markedly shorter in both sexes, ranging from 2.0 to 6.0 hours. On Day 1 both treatment groups had similar mean C_{max} values and similar mean AUC_{0-72h} values. There was no apparent dose dependency between the high and low dosage groups. At Week 39 the mean C_{max} and AUC_{0-72h} were again similar in both treatment groups, but these pharmacokinetic parameters were two to four times higher than those on Day 1 in both groups, indicating accumulation. In summary, after repeated dosing, the mean C_{max} and AUC_{0-72h} increased at both 1200 and 1800mg/kg dosages and the T_{max} shortened. No apparent gender differences were noted between the high and low dosage groups in mean T_{max} , C_{max} , or AUC_{0-72h} throughout the study (Supplemental Tables 6a and 6b and Figures 3, 4 and 5).

Determination of mean plasma levels of total CoQ₁₀ at Week 13 in both treatment groups revealed a gradual elevation of plasma concentration from Day 1, in both sexes, at all measurement time points, and the values (pre-dose, 4 and 24 hours after dosing) at Week 13 reached levels detected at Week 39. C_{max} and AUC_{0-72h} were not determined at either the

Week 13 or Week 26 time point, however trough levels of plasma CoQ₁₀, the lowest concentration that a drug reaches in the plasma before the next dose is administered, were measured and the levels reached at Week 13 were similar to those detected at Week 39 (Table 7). No samples were taken between Day 1 and Week 13 of study. We therefore do not have data to demonstrate the achievement of steady state in plasma; however the findings presented above suggest plasma levels of total CoQ₁₀ had reached steady state by week 13. Simulation of the single dose study results demonstrated steady state in plasma should be reached after three or four weeks and results of the repeated dose study showed steady state was reached after two weeks at all dosages (Table 4). It was therefore considered likely that steady-state would have been achieved by the week 13 time point in the chronic toxicity study.

Chronic Toxicity Study - Clinical Observations and Examinations

No deaths or untoward events were observed in any group. Stool containing test-like article was observed frequently in all male and female dogs in the 1200 and 1800mg/kg/day groups throughout the treatment period. This was thought to be due to the excretion of unabsorbed test article in stools. Mucous stool was observed sporadically in one male and one female in the 1800mg/kg/day group. Vomiting was observed in one male and three females in the 1200mg/kg/day group and in all males and three females in the 1800mg/kg/day group. For the first six weeks vomitus-containing test article-like substance was noted in one female in the 1200mg/kg/day group and in three males in the 1800mg/kg group. These findings were not considered to be significant.

Mean body weight gain (male/female) during the administration period in the control, 1200 and 1800mg/kg/day groups was 2.2/1.2, 2.1/1.1 and 1.8/1.3 kg respectively. There were no statistically significant differences in mean body weight and food consumption between the 1200 and 1800mg/kg/day groups and the control group.

Chronic Toxicity Study - Standard Toxicological Examinations Ophthalmology

No treatment-related changes were detected during ophthalmologic examinations in any group.

Electrocardiography (ECG)

A decrease in heart rate, lower than the value observed in the week prior to administration, by at least 30% was recorded in two males and one female in the 1200mg/kg/day group at Week 39. This finding was not considered to be toxicologically significant since there were no changes in any other parameter, and corresponding histopathological examination revealed no organic changes in the heart.

The following changes were considered to be incidental, as they were similar to their respective pre-treatment values: prolongation of the QRS complex ($p < 0.05$) in females in the 1200mg/kg/day group at Week 13; a low value for the QT interval ($p < 0.05$) in males in the 1200mg/kg/day group at Week 26, and in females in the 1200mg/kg/day group at Week 39. These ECG changes were found to be statistically, but not toxicologically significant from placebo at corresponding time points, because the placebo values at these respective time points had also changed from their pre-treatment values (Supplemental Tables 8a and 8b).

Urinalysis

A positive occult blood and erythrocyte in sediment were detected in two females in the control group, two females in the 1200mg/kg/day group and one female in the 1800mg/kg/

day group at Weeks 13, 26 and 39. These findings were thought to be related to estrus hemorrhage.

Hematology (Supplemental Table 9a)

Increased eosinophils, both in ratio and count were observed in males in the 1800mg/kg/day group at Weeks 26 and 39. The value at Week 26 was determined to be statistically ($p < 0.05$), but not toxicologically significant due to the absence of changes in other leukocytic parameters. A high value for basophils ($p < 0.05$, in ratio) was observed in females in the 1200mg/kg/day group at Week 26 however the value was similar to that pre-treatment (Supplemental Tables 9b and 9c).

Blood Chemistry (Supplemental Table 10a)

An increase in blood urea nitrogen (BUN) was observed in one male in the 1200mg/kg/day group at Week 39. This change was observed in only one animal with no other corresponding BUN-related abnormalities. The following statistically significant abnormalities were not considered to be toxicologically significant as they were similar to their respective pre-treatment values and remained within normal range: a low value for glucose ($p < 0.05$) in males in the 1800mg/kg/day group at Week 39; a high value for BUN ($p < 0.05$) in males in the 1800mg/kg/day group at Week 26; a low value for chloride ($p < 0.05$) in males in the 1200mg/kg/day group at Week 13 (Supplemental Tables 10b and 10c).

Postmortem Data

Gross Pathological Findings—A white focus was observed in the lungs of one female in the control group and one male in the 1200mg/kg/day group. One male in the control group was found to have hypoplasia of the epididymis. These gross pathological findings were not considered toxicologically significant.

Histopathological Findings—Only minimal or mild changes were observed in some organs / tissues of some animals (Supplemental Table 11). These were judged to be incidental based on their pathological nature or feature of occurrence.

In summary there were neither deaths nor CoQ₁₀-related adverse events during the administration and observation periods. No toxicologically significant changes were observed in body weight, food consumption, ophthalmology, ECG, urinalysis, hematology, blood chemistry, gross pathology, organ weight and histopathology.

Discussion

Multiple studies have been conducted to assess the safety and tolerability of high doses of CoQ₁₀ in humans^{9–12, 14, 23–28}. In a 28-subject tolerability trial assessing 1200, 2400 and 3600mg/day of CoQ₁₀, 20 subjects with HD and 8 healthy subjects received four weeks at 1200mg/day, followed by four weeks at 2400mg/day and were then maintained on 3600mg/day for twelve weeks¹². CoQ₁₀ was well tolerated with 82% of subjects achieving the target maximum dosage. In an open-label pilot study, the safety and tolerability of high dose CoQ₁₀ in 17 subjects with mild PD was examined¹⁴. Escalating dosages of CoQ₁₀ at 1200, 1800, 2400 and 3000mg/day were administered with vitamin E 1200 IU daily. Maximum exposure was two months with one month at 2400mg/day or 3000mg/day. CoQ₁₀ levels were measured after each dose and were found to reach a plateau at the 2400mg/day dosage. No further increase was detected at the 3000mg/day dosage. It was concluded that the appropriate dose to test in future efficacy studies should be 2400mg/day. Similar conclusions were reached in studies of HD¹² and ALS¹⁰. Much of the available published

data on clinical trials of CoQ₁₀ at dosages ranging from 30 to 3000mg/day has been previously reviewed^{29, 30}. Adverse effects do not follow a consistent pattern and there is no relationship with adverse events and increasing dosage of CoQ₁₀ in any previous trial. These studies support the strategy of using the highest well-tolerated dosage of CoQ₁₀ that has an incremental biological effect (i.e. increases blood levels of CoQ₁₀).

In a previous contract research study performed by the Kaneka Corporation, the reduced form of CoQ₁₀, ubiquinol, was administered by gavage to six beagle dogs per dose for 13 weeks at dosages of 0, 150, 300 and 600mg/kg/day, with one dosage of the oxidized form of CoQ₁₀, ubiquinone, at 600mg/kg/day³¹. The concentration of CoQ₁₀ in plasma increased in a dose-related manner from Day 1 to Week 7 and then remained at steady state until Week 13. The no-observed adverse effect level (NOEAL) was estimated to be more than 600mg/kg/day for male and female dogs. Two further rat studies, one 13-week and one 52-week toxicity study showed that dosages up to 1200mg/kg/day administered by gavage were well tolerated with no deaths or toxicological side effects over both 13- and 52-week study periods. Histopathology of the treated rats was normal except for some exudate in the nasal turbinates and lung granulomas attributed to incidental exposure to crystallized test material. Although these pre-clinical studies had been submitted to the regulatory authorities as evidence to support the phase 3 human trial of CoQ₁₀ in people with HD, it was not deemed adequate to support such a long (60 months), and large (n = 608), clinical trial^{32, 33}. Three studies of CoQ₁₀ in beagle dogs were therefore conducted with two shorter preliminary dog studies designed to confirm the appropriate dosage for the longer, 39-week, dog study. Dogs were chosen as they are widely used in safety studies in pharmaceuticals and are suitable for time-course examinations. In addition, the predominant form of endogenous CoQ in dogs is, as in humans, CoQ₁₀.

Based on the simulated total plasma CoQ₁₀ concentration curves the single dose study determined the appropriate length of the treatment period for the repeated dose study to be four weeks. Due to the nature of CoQ₁₀, i.e. its low solubility in water and high molecular weight, large individual differences and poor dose response in plasma CoQ₁₀ concentrations were expected in the repeated dose study. Therefore, four dosage levels from 600 to 2400mg/kg/day were selected to determine the maximum tolerated dose. In terms of administration volume per dose the 2400mg/kg/day dosage is double the expected maximum feasible dose of 1200mg/kg/day (12gm/10kg of body weight). A dose of 1200mg/kg/day corresponds to nearly 5% of a dog's daily diet (200 to 300gm/dog) and would therefore be expected to have significant nutritional effects³⁴. Results of the four-week repeated dose study showed that plasma CoQ₁₀ levels reached steady state after two weeks at any dosage (600, 1200, 1800 or 2400mg/kg/day). Similar results have been shown in a four-week clinical safety study of high dose CoQ₁₀ in healthy subjects and in another four-week study with ubiquinol in healthy volunteers. The latter study also showed after daily administration is stopped plasma CoQ₁₀ concentrations decrease to near baseline levels, suggesting that CoQ₁₀ does not accumulate in the body after cessation^{35–37}. A decline in plasma CoQ₁₀ concentrations was observed after the cessation of CoQ₁₀ on Day 28 of the repeated dose study.

The plateau in plasma exposure was the basis for choosing the high dosage for the definitive chronic toxicity study, i.e. there was no need to test doses beyond a dosage that resulted in a maximum achievable plasma exposure. The highest appropriate dose for future chronic toxicity studies was therefore determined to be 1800mg/kg/day (900 × 2). Application of safety factors to adjust for differences between humans and test animals, using a 50-fold margin of safety would suggest an acceptable daily intake of 36mg/kg/day in humans³⁸. This would equate to 2520mg/person/day in a person weighing 70kg. It is understood however that metabolic and physiological differences between humans and dogs exist that

may influence tissue utilization or responses to CoQ₁₀. A previous review of the available published clinical trial data on CoQ₁₀ concludes it is safe to use as a dietary supplement in humans³⁰. Application of customary safety evaluation methods suggests daily dosages up to 1200mg/day are safe and that higher levels, which have been tested in clinical trials without adverse effect, may also be safe³⁰.

In all three studies described, dosages of more than 1200mg/kg/day were given in two divided doses by gavage approximately six hours apart for ease of administration. Where possible a single daily dosing regime was employed in an attempt to maintain animal welfare and reduce any undue stress to animals that may be related to gavage administration. It is not known if and how a divided daily dosing schedule in some groups affected the comparisons with groups administered single daily dosing as this issue was not specifically examined in these studies. However it has been previously reported that 200mg of CoQ₁₀ given in equally divided doses (100 mg twice a day) causes a greater increase in the plasma CoQ₁₀ level than a single dose of 200mg in humans³⁹. This suggests that dogs administered divided doses would be exposed to higher levels of CoQ₁₀ than following a single dose, an idea supported by results from the repeated dose study where the 1800mg/kg/day dosage induced a higher plasma CoQ₁₀ concentration than the 1200mg/kg/day dosage. Early morning trough plasma levels are commonly used to monitor a test drug in repeated dose studies. Measurement of trough levels in the chronic toxicity study described above allowed determination of adequate exposure to CoQ₁₀ over 39 consecutive weeks and was a good indication of the achievement of steady state.

A review of the few published reports on the pharmacokinetics of CoQ₁₀ in humans and in rodent models shows mean T_{max} values ranging from two to six hours^{40, 41}. On Day 1 of the chronic toxicity study the mean T_{max} ranged from 16 to 24 hours, falling to between two and six hours after 39 weeks of daily CoQ₁₀ administration by gavage indicating that CoQ₁₀ may be absorbed slowly from the gastrointestinal tract, in part due to its hydrophobic nature and relatively high molecular weight, and that the rate of CoQ₁₀ absorption increases with chronic exposure. Studies in rats administered CoQ₁₀ by gavage have shown it appears in liver tissue in a dose dependent manner and in the spleen⁴². Chronic toxicity studies of large doses of CoQ₁₀ in rats have shown increased concentrations in brain mitochondrial fractions⁸. In our chronic toxicity study orange (test article-like) material was observed in stools of all dogs in the 1200 and 1800mg/kg/day dosage groups, indicating that CoQ₁₀ is incompletely absorbed. The pharmacokinetic results presented above may therefore be a reflection of incomplete absorption. Although different formulations of CoQ₁₀ have been used in previous studies most have used ubiquinone, the form of CoQ₁₀ used in the studies described above^{9–12, 14, 23–28, 31}. Other studies have used ubiquinol, the active antioxidant form found endogenously in the body^{31, 35}. A safety and bioavailability study of ubiquinol in healthy human volunteers indicated that ubiquinol is better absorbed from the gastrointestinal tract compared to ubiquinone, however once ingested and absorbed almost 90% of ubiquinone is converted into ubiquinol³⁵. Some studies have used Vitamin E in combination with CoQ₁₀ as Vitamin E may increase CoQ₁₀ absorption^{11–12, 14, 43}. A single dose comparison study of four different CoQ₁₀ formulations conducted to assay the amount of active CoQ₁₀ in each formulation however reported no such increase⁹. Formulations included two Vitaline CoQ₁₀ chewable wafers (one with 300IU of Vitamin E and one without), one hard gelatine capsule and one gel cap containing an oil-based vehicle. Assay of the four preparations revealed that all contained the claimed amount of CoQ₁₀, review of the blood draws did not reveal any major differences in plasma CoQ₁₀ curves generated and pharmacokinetic results were all within the same range⁹. All three studies employed small sample sizes which precludes meaningful statistical comparisons. Nevertheless from the results of the chronic toxicity study, we are able to conclude that no untoward changes related to CoQ₁₀ administration were observed with regard to body weight, food

consumption, clinical observations, electrocardiograms, ophthalmologic examinations, urinalysis, hematologic and blood chemistry examinations, organ weights or histopathology, including sampling of multiple brain regions, after 39 consecutive weeks of treatment at the maximum practical dosage by gavage. The results of this chronic toxicity study in beagle dogs support the safe use of a 2400mg/person/day dosage of CoQ₁₀ in two phase 3 National Institute of Neurological Disorders and Stroke (NINDS)-sponsored clinical trials that are currently ongoing. When calculated for a 70kg person the maximal safe dosage, of 2400mg/kg/day, administered to beagle dogs in the repeated dose study is 70 fold greater than a 2400mg/person/day dose in man. The chronic dose study also supports the current NIH-sponsored phase 2 multi-center, randomized, double-blind, parallel-group tolerability study of CoQ₁₀ in people with pre-symptomatic gene positive HD⁴⁴. The information gained from these toxicity studies in beagle dogs will prove valuable for future human clinical trials of CoQ₁₀ in multiple disorders in which mitochondrial dysfunction and oxidative damages are implicated, including HD and PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. AUC values in dogs administered a single dose of CoQ₁₀ at 600, 1200, 1800 and 2400mg/kg by gavage

Bars denote mean value and dots denote individual values of two animals per dosage group, except in the 600mg/kg dosage group where one animal showed abnormal plasma concentration, likely due to a dosing error (value not shown).

Figure 2. AUC values in dogs administered 600, 1200, 1800 and 2400mg/kg of CoQ₁₀ daily by gavage for 28 consecutive days
Bars denote mean values and dots denote individual values of two animals per dosage group.

Figure 3. C_{max} Values at Day 1 and at Week 39 in Male and Female Dogs Administered Placebo, 1200mg/kg or 1800mg/kg of CoQ10 daily by gavage for 39 weeks

C_{max} was greater at Week 39 than at Day 1.

Bars denote mean values and dots denote individual values of four animals per dosage group.

Figure 4. T_{max} Values at Day 1 and at Week 39 in Male and Female Dogs Administered Placebo, 1200mg/kg or 1800mg/kg of CoQ10 daily by gavage for 39 weeks

T_{max} was shorter at Week 39 than at Day 1

Bars denote mean values and dots denote individual values of four animals per dosage group.

Figure 5. AUC Values at Day 1 and at Week 39 in Male and Female Dogs Administered Placebo, 1200mg/kg or 1800mg/kg of CoQ10 daily by gavage for 39 weeks

AUC was greater at Week 39 than at Day 1.

Bars denote mean values and dots denote individual values of four animals per dosage group.

Table 4
Dogs received daily gavage dose of CoQ₁₀ for 28 days: AUC_{0-24hr} determinations at various intervals

Steady state AUC was reached after two weeks in all dosage groups.

Dose (mg/kg/day)	Pre-dose plasma CoQ ₁₀ (µg/ml)*	AUC _{0-24hr} (µg·hr/ml)					
		Day 1	Day 7	Day 14	Day 21	Day 28	
600	0.28	30.3	77.0	74.7	69.1	72.0	
1200	0.45	54.1	146.0	150.6	151.2	146.3	
1800	0.48	71.4	170.8	204.0	245.1	208.2	
2400	0.36	57.1	129.9	132.0	133.9	139.4	

Each value shows mean of two animals

* Pre-dose plasma CoQ₁₀ concentrations are shown for comparison

Table 7
Morning trough concentration levels of CoQ10 ($\mu\text{g/mL}$) in plasma during 39 Week Chronic Toxicity Study in Dogs

Dosage (mg/kg/day)	Male				Female			
	Day 1	Week 13	Week 26	Week 39	Day 1	Week 13	Week 26	Week 39
0 (Placebo)	0.39±0.09	0.34±0.09	0.36±0.08	0.35±0.03	0.30±0.04	0.39±0.14	0.35±0.04	0.44±0.07
1200	0.41±0.11	5.43±2.15*	2.33±0.56**	4.28±1.52*	0.37±0.09	5.27±2.04*	2.14±0.65*	5.97±2.01*
1800	0.42±0.09	5.06±1.94*	2.80±0.39**	6.27±1.38**	0.34±0.02	4.28±0.20**	2.68±0.27**	5.34±1.48**

Each value shows mean $\pm$ SD of four animals

* $p \leq 0.05$ vs Day 1 (Paired t-test)

** $p \leq 0.01$ vs Day 1 (Paired t-test)