HHS Public Access

Author manuscript

Int J Radiat Biol. Author manuscript; available in PMC 2016 January 31.

Published in final edited form as:

Int J Radiat Biol. 2015 July ; 91(7): 568–575. doi:10.3109/09553002.2015.1043753.

Orally Administered DTPA Di-ethyl Ester for Decorporation of ^{241}Am in dogs: Assessment of Safety and Efficacy in an Inhalation-Contamination Model

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Abstract

Purpose—Currently two injectable products of diethylenetriaminepentaacetic acid (DTPA) are U.S. Food and Drug Administration (FDA) approved for decorporation of ^{241}Am, however, an oral product is considered more amenable in a mass casualty situation. The diethyl ester of DTPA, named C2E2, is being developed as an oral drug for treatment of internal radionuclide contamination.

Materials and methods—Single dose decorporation efficacy of C2E2 administered 24-hours post contamination was determined in beagle dogs using a ^{241}Am nitrate inhalation contamination model. Single and multiple dose toxicity studies in beagle dogs were performed as part of an initial safety assessment program. In addition, the genotoxic potential of C2E2 was evaluated by the *in vitro* bacterial reverse mutation Ames test, mammalian cell chromosome aberration cytogenetic assay and an *in vivo* micronucleus test.

Results—Oral administration of C2E2 significantly increased ^{241}Am elimination over untreated controls and significantly reduced the retention of ^{241}Am in tissues, especially liver, kidney, lung and bone. Daily dosing of 200 mg/kg/day for 10 days was well tolerated in dogs. C2E2 was found to be neither mutagenic or clastogenic.

Conclusions—The di-ethyl ester of DTPA (C2E2) was shown to effectively enhance the elimination of ^{241}Am after oral administration in a dog inhalation-contamination model and was well tolerated in toxicity studies.

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Declaration of interest:

Drs. Jay and Mumper are co-founders of Capture Pharmaceuticals, which owns intellectual property related to C2E2. However, no financial support from Capture Pharmaceuticals was provided in these studies.

Keywords

Americium; Alpha emitters; DTPA; Decorporation; Radionuclides

Introduction

In his first speech to the U.N. Security Council in New York, President Obama called nuclear terrorism one of the greatest threats to international security and that the consequences of such an attack would be enormous (Allison, 2010). This increasing threat has spurred the development of radionuclide decorporation agents for treatment of individuals contaminated with various radioactive materials; the transuranic elements Am, Pu and Cm are among these radionuclides of concern (International Commission on Radiological Protection, 1979). In 2004, trisodium calcium diethylenetriaminepentaacetate injection (Ca-DTPA) and trisodium zinc diethylenetriaminepentaacetate injection (Zn-DTPA) received U.S. Food and Drug Administration (FDA) approval for the treatment of internal contamination with these actinides and were subsequently included in the Strategic National Stockpile for use in the event of mass contamination. While Ca- and Zn-DTPA injections have been shown to be efficacious in treating contaminated individuals (Yaes RJ, 2004), they require administration by a skilled professional via either intravenous injection or via nebulization. These are not practical methods of administration in a mass casualty situation. This problem is particularly acute as early access to treatment improves decorporation efficacy by minimizing tissue deposition of the radioactive material. The loss of efficacy caused by treatment delay cannot be overcome by increasing the cumulative dose of decorporation agent (Seidel, 1975). Therefore, there is a need to develop DTPA formulations that can be self-administered and rapidly distributed to a large population. However, DTPA is known to have poor oral bioavailability (~3%) primarily because its charge and hydrophilicity cause permeability-limited absorption (Volf, 1984). Esterification is often used to overcome barriers in drug delivery such as poor solubility (Stella and Nti-Addae, 2007) or absorption (Beaumont et al., 2003). The carboxylic acids present on DTPA provide multiple sites for esterification that we hypothesized could be used to increase lipophilicity and overcome the permeability-limited absorption.

Lipophilic promoieties have previously been added to DTPA or triethylenetetraminehexaacetic acid (TTHA) in order to enhance chelation of intracellular radionuclides, however, no studies previously focused on their use for oral bioavailability enhancement (Markley, 1963), (Guilmette et al., 1979), (Bruenger et al., 1992). The DTPA ligands were not orally administered and although the TTHA ligands were given orally, the research focused on the long-term removal of radionuclides from intracellular deposition sites. None of the polycarboxylic acid ligands investigated were more effective or less toxic than Ca-DTPA and most of the research was abandoned (Durbin, 2006).

Therefore with a focus on bioavalibility enhancement, a series of DTPA esters were synthesized, and two promising candidates emerged following evaluation of their physical-chemical properties and permeability characteristics. These were the penta-ethyl and di-ethyl esters of DTPA, referred to as C2E5 and C2E2, respectively. C2E5 was shown to effectively

decorporate ^{241}Am in a wound-contamination animal model (Sueda et al., 2012), (Sueda, 2014), but its unfavorable pharmaceutical properties and concerns related to its hepatotoxicity caused us to focus our efforts on C2E2.

Here we report the decorporation efficacy of C2E2 following a single dose to beagle dogs contaminated via inhalation of americium. Americium was chosen as a model isotope for efficacy studies due to its wide availability and potential for use in dirty bombs. The toxicity of C2E2 was determined both *in vitro* using classical genotoxicity endpoints and *in vivo* by evaluation of both the acute and ten day maximum tolerated doses in beagle dogs.

Materials and Methods

General

Americium-241 was obtained from the Department of Energy as a nitrate complex stock solution. An aliquot of the stock solution was taken to dryness on a medium temperature hotplate and reconstituted in 0.25 M nitric acid to yield a final activity of 7.44 MBq/mL (201.1 μCi/mL) as determined by gamma pulse height analysis. C2E2 was prepared by first synthesizing DTPA bis-anhydride followed by its subsequent reflux with ethanol as previously described (Guilmette et al., 1979) (Yield - 85%, Purity – 97.7%).

The general procedures for animal care and housing were conducted in accordance with the National Research Council for the Care and Use of Laboratory Animals and the Animal Welfare Standards. All procedures and protocols used in animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Covance Laboratories Inc. or Lovelace Respiratory Research Institute and were performed in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facilities.

Beagle Inhalation Contamination Model for ^{241}Am

Inhalation contamination was performed in a method similar to that previously described (Doyle-Eisele et al., 2014). The beagles were fasted, sedated (acepromazine i.m.; 0.05 mg/kg.) and anesthetized with Isoflurane (5%) (MWI Veterinary Supply, Boise, ID, USA) prior to exposure to ^{241}Am. A muzzle was fixed to the dog and placed into an exposure plenum where a mixture of oxygen and isoflurane was continually flowing and monitored. Aerosols were generated from the ^{241}Am-nitrate solution (pH - 0.74) using a Hospitak nebulizer (Unomedical Inc. McAllen, Tx, USA). The aerosol was dried by transiting through a tube furnace (Thermo Scientific) operating at 70–80°C to minimize the amount of acid present. Oxygen was maintained at 45–55 % and isoflurane was delivered as needed (2–3 % in oxygen). Aerosol samples were collected on Pallflex Fiberfilm filters (Pall, Port Washington, NY, USA) and analyzed for ^{241}Am content. Dogs were exposed to an ^{241}Am aerosol atmosphere of 42.7 ± 8.3 kBq/L (1.16 ± 0.22 μCi/L) for 8 minutes. The particle size of the aerosol was consistent with previous inhalation studies (Doyle-Eisele et al., 2014) and determined to be 0.63 μm Activity Median Aerodynamic Diameter (AMAD) with a geometric standard deviation (GSD) of 1.72, with each dog receiving an average of 111 kBq (3 μCi) of ^{241}Am.

Single Dose Efficacy of C2E2 in Beagle Dogs

The efficacy for decorporation of ^{241}Am by C2E2 was evaluated in beagle dogs (n = 4/sex/dose) approximately 13 months of age (8.8 ± 1.2 kg). The dogs were acclimatized to metabolic cages 24 hours prior to ^{241}Am administration. Dogs were fasted overnight prior to treatment. C2E2 solutions were administered by oral gavage 24 hours after contamination to mimic a realistic treatment delay (Cassatt et al., 2008). Dogs received either de-ionized (DI) water (vehicle control) or C2E2 solutions at 100, 300 or 500 mg/kg. C2E2 was dissolved in DI water on each day of dosing and dogs were administered between 33–52 mL of solution to achieve the desired dosage. Urine and feces were collected daily for analysis of radioactivity. Fourteen days after contamination the dogs were euthanized and necropsied. The liver, spleen, kidneys, lungs and both femurs were among the tissues collected and analyzed for radioactivity. Samples were thermally and chemically processed as previously described (Sueda et al., 2014), placed into 20-mL scintillation vials and the gamma pulse height from ^{241}Am was analyzed in a gamma counter (2480 Wizard^2 Gamma Counter, Perkin Elmer, Waltham, MA, USA).

Statistical analyses to evaluate the pattern of recovered doses were conducted by one-way analysis of variance (ANOVA). For each sample, differences between untreated controls and treated groups were assessed with individual F-tests based on the ANOVA's pooled estimate of underlying variance between dogs.

Acute Maximum Tolerated Dose of C2E2 in Dogs

Four beagles (Covance Research Products Inc., Madison, WI, USA), (n = 2/sex/dose with 7 day washout between doses), 8 to 10 months old (10.1 – 12.0 kg) were housed individually in stainless steel cages. The dogs were acclimatized to the conditions at least two weeks prior to the study: *ad libitum* access to water and certified canine diet #2027C (Harlan Laboratories Inc., Indianapolis, IN, USA) for 6 hours per day in a controlled environment with lighting on a 12-h light/dark cycle. Prior to dosing, the dogs were fasted overnight and food was returned 2 hours after dosing. The same dogs were used for each consecutive dose level with a minimum 7-day washout period between dose escalations. The initial dose of C2E2 administered was 100 mg/kg with subsequent escalating doses (300 or 750 mg/kg) based on clinical observations and clinical pathology results of the previous dose. C2E2 solutions were prepared by dissolving in DI water to a concentration that allowed each dog to receive 8.3 mL/kg of dosing solution by oral gavage. In addition, the effect of food on toxicity was examined at a single dose level of 300 mg/kg under fed conditions.

Detailed observations were performed at 1, 2 and 4 hours post-dose and then twice daily. Blood was collected from the jugular vein pre-dose and at 0.5, 1, 2, 4, 6, 8, 12 and 24 hours for toxicokinetic analysis and approximately 48 hours post-dose for standard hematology and clinical chemistry analyses as well as trace element analyses. Concentrations of C2E2, the mono ethyl-ester of DTPA (C2E1) and DTPA were determined by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using a Thermo TSQ Quantum Access mass spectrometer (Waltham, MA, USA). Quantification of C2E2 and DTPA was based on the peak areas of analyte to their respective internal standards. As a C2E1 standard was not available, concentrations were estimated by producing a calibration

curve from the 1% C2E1 impurity present in the C2E2 material. The range of quantification was 100 to 100,000 ng/mL for C2E2, 5 to 1,000 ng/mL for C2E1 and 10 to 1,000 ng/mL for DTPA. Toxicokinetic analysis was performed using WinNonlin Professional Edition (Pharsight Corporation, Version 5.2)

Ten-Day Oral Dose Range Finding Study of C2E2 in Dogs

Twenty beagles (Covance Research Products Inc.), (n = 2/sex/dose), 7 to 9 months old (8.4 – 11.4 kg) were housed individually in stainless steel cages. C2E2 doses of 0, 60, 200, 400, and 600 mg/kg/day for 10 days in fasted dogs were selected based on the results of the oral acute maximum tolerated dose study. Animals were fasted overnight prior to dosing and certified canine diet #2027C was returned approximately 3 hours after completion of dosing. Water was provided ad libitum. Cageside observations were performed at 1, 2 and 4 hours post-dose and detailed observations were performed during the predose phase, and prior to dosing on days 1 and 6. As in the single dose study, blood was collected from the jugular vein pre-dose and at 0.5, 1, 2, 4, 6, 8, 12 and 24 hours for toxicokinetic analysis and predose, day 6 and at sacrifice (day 11) for standard hematology and clinical chemistry analyses.

Genotoxicity of C2E2

A reverse mutation assay in *Salmonella typhimurium and Escherichia coli* (Ames test), a chromosomal aberration assay in Chinese hamster ovary (CHO) cells and an *in vivo* rat bone marrow micronucleus assay were performed in accordance with standards established by International Conference on Harmonisation (ICH) guidance S2(R1) (Food and Drug Administration, 2012). The Ames test was conducted with *Salmonella* histidine auxotrophs TA98, TA100, TA1535, and TA1537 and the *E. Coli* tryptophan auxotroph WP2*uvr*A, using a plate incorporation procedure as previously described (Ames et al., 1975),(Green and Muriel, 1976). C2E2 was evaluated at doses of 5 to 5000 μg/plate with or without rat liver homogenate metabolic activation (Aroclor-induced Sprague-Dawley rat liver S9, NADP and Glucose-6-phosphate). Positive and vehicle controls were concurrently evaluated, and triplicate plates were used for all samples. Positive controls (Sigma, St. Louis, MO, USA) included, 2-nitrofluorene, sodium azide, ICR-191, 4-nitroquinoline-N-oxide, benzo[a]pyrene and 2-aminoanthracene. Dose concentration was confirmed by high-performance liquid chromatography (HPLC). C2E2 was considered to have produced a positive response if a dose-dependent increase in revertant frequency was ≥2.0-fold vehicle control values for strains TA98, TA100, and WP2*uvr*A, or ≥3.0-fold vehicle control values for strains TA1535 and TA1537.

The chromosomal aberration assay was performed in a CHO cell line derived from an ovarian biopsy of a female Chinese hamster (CHO-WBL). C2E2 was prepared in cell culture grade water, and stocks were prepared by serial dilution in vehicle so that all treatments were administered into 10 mL cultures in a volume of 10%. Treatment periods continued for 3 hours with or without metabolic activation, or for approximately 20 hours without metabolic activation. Solutions containing C2E2 ranging from 7 to 500 μg/mL were prepared and concentrations were confirmed by HPLC. Visual observations of cultures for general cell health and confluence were made prior to termination. Mitomycin C (1.5 μM for the 3-hour treatment, and 0.6 μM for the 20-hour treatment) were used as positive controls

for the assays without metabolic activation (rat liver S9, NADPH and isocitric acid) and cyclophosphamide (19 μM) in the assay with metabolic activation. At culture termination, viable cells were counted and population doubling was calculated for measurement of cytotoxicity to support selection of dose levels for aberration analysis. For each concentration, 1000 cells were assessed for toxicity and at least 200 cells in metaphase were scored and tabulated for aberrations. To control bias, slides were identified by an abbreviated code unknown to the scorer. Statistical analysis employed a Cochran-Armitage test for linear trend and Fisher's Exact Test to compare the percentage of cells with aberrations in treated cells to the results obtained for the vehicle controls.

The *in vitro* micronucleus test was performed in male Sprague Dawley rats. C2E2 was formulated in cell culture grade water vehicle and the dose volume for all treatment groups was 20 mL/kg. Male rats were administered vehicle control or C2E2 at 500, 1000, or 2000 mg/kg/day for two days separated by approximately 24 hours. The 2000 mg/kg high dose is the limit dose for this assay recommended by ICH S2(R1) guidance. A positive control group received a single 60 mg/kg cyclophosphamide treatment on the second day of dosing. Animals were observed at least twice daily for toxic signs and/or mortality. Bone marrow was extracted approximately 24 hours after the last treatment in all groups and at least 2000 polychromatic erythrocytes (PCE) per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of polychromatic erythrocytes and normochromatic erythrocytes (NCE) observed while scoring at least 500 erythrocytes per animal.

Results

Single Dose Efficacy of C2E2 in Beagle Dogs

When radionuclide exposure is conducted via the inhaled route, fractional recoveries cannot be calculated; therefore, the actual administered activity of ^{241}Am must be calculated. This is determined based on aerosol concentration samples as well as physiological and radiochemical measurement data. The estimated mean delivered ^{241}Am activity was calculated to be 102.5 and 95.5 kBq (2.77 and 2.58 μCi) for female and male dogs, respectively.

Statistically significant increases in ^{241}Am elimination over control were observed in all treatment groups with the highest dose of C2E2 (500 mg/kg) producing significant increases in decorporation over the 300 mg/kg dose ($p<0.01$). As shown in Figure 1, urinary excretion of ^{241}Am increased in a dose-dependent manner. Urinary excretion was 8-fold higher than control for 100 and 300 mg/kg doses and 13-fold higher at 500 mg/kg. At all dose levels, fecal elimination showed modest enhancement for three days after treatment before returning to control levels (Figure 2). Treatment with doses ≥ 300 mg/kg resulted in a 50 % increase in fecal elimination of ^{241}Am, significantly higher than control ($p<0.05$).

The ability of a decorporation agent to prevent uptake of ^{241}Am into body tissues, particularly the liver, kidney and bone, is also important. Significant reductions in liver, kidney and lung ^{241}Am burden were observed at all dose levels ($p<0.05$). Figure 3 demonstrates that a dose-dependent decrease in tissue burden was only observed in liver and

lung tissues. At 500 mg/kg there was >65% decrease in liver and spleen ^{241}Am burden, >50% decrease in kidney and lung burden and >30% decrease in total ^{241}Am bone content compared to untreated controls.

Acute Maximum Tolerated Dose of C2E2 in Dogs

Beagle dogs received single C2E2 doses of 100, 300 or 750 mg/kg under fasted conditions and 300 mg/kg under fed conditions. Across all groups, transient emesis, excessive salivation and fecal abnormalities occurred that did not affect the overall health of the dog and, therefore, were not considered significantly adverse. C2E2 administered at ≤ 300 mg/kg had no significant effects on clinical pathology test results. Two days after administration of 750 mg/kg, two of four dogs exhibited increased alanine and aspartate aminotransferase (ALT and AST) activities indicating hepatocellular injury. Systemic exposure to C2E2 (Area under the concentration time curve from 0–24 hours ($AUC_{0–24}$)) generally increased in a dose proportional manner (Table 1), and there was no greater than a 2-fold difference in mean C2E2 maximum plasma concentration (C_{max}) and $AUC_{0–24}$ values between sexes. C2E2 exposure was lower under fed conditions compared to the fasted state; C_{max} and $AUC_{0–24}$ values were at least 22-fold and 7-fold lower, respectively, in fed dogs. Under fed conditions the fraction of C2E2 metabolized to C2E1 doubled but was only responsible for 2 % of the total exposure. No adverse clinical observations were made in male or female dogs and the maximum tolerated single dose of C2E2 by oral gavage was identified as 750 mg/kg.

Ten Day Oral Dose Range Finding Study of C2E2 in Dogs

Daily administration of C2E2 to beagle dogs by oral gavage for 10 days at doses of 60, 200, 400 and 600 mg/kg/day resulted in the early sacrifice of one male in both the 400 and 600 mg/kg/day groups due to weight loss and declining health. Clinical pathology identified hepatocellular necrosis as the cause of toxicity in these dogs. Due to these early sacrifices the doses ≥ 400 mg/kg/day exceed the maximum tolerated dose. Exposure to C2E2 increased with increasing dose and no accumulation of C2E2, C2E1 or DTPA was observed after administration of multiple doses. As in the acute maximum tolerated dose study, transient emesis and/or fecal abnormalities (bulky stools, mucus in stools, loose stool or mild diarrhea) occurred in dogs receiving doses ≥ 200 mg/kg/day. In animals that survived until necroscopy, mild to moderate (1.0 – 3.6 x control) increased AST activity was observed on day 6 at doses ≥ 600 mg/kg/day. Mildly to markedly (6.8 – 90 x control) increased ALT activity was observed on days 6 and 11 in animals receiving doses ≥ 400 mg/kg/day. The increases in AST and ALT levels were associated with microscopic evidence of liver injury and considered adverse. Tubule cell necrosis was present in one female in both the 400 and 600 mg/kg/day groups. This was not noted in the males at terminal sacrifice but was noted in both males sacrificed early. The liver and kidney were identified as the target organs of toxicity at ≥ 400 mg/kg/day. Based on these findings, an No-observed-adverse-effect level (NOAEL) of 200 mg/kg/day for 10 days of oral dosing in beagles was established.

Genotoxicity of C2E2

C2E2 was evaluated for its potential to induce reverse mutations at the histidine locus of *Salmonella typhimurium* and at the tryptophan locus of *E. coli* in the Ames test and for its ability to cause chromosome aberrations in CHO cells. These *in vitro* tests are required by the FDA for all small molecule drugs prior to their use in human clinical safety trials. Additionally, an *in vivo* rat bone marrow micronucleus test was performed to evaluate C2E2 for clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes.

The mutagenicity of C2E2 was evaluated in five bacterial tester strains at doses from 5 to 5000 μg/plate with or without S9 activation. Compared to vehicle controls, reductions in the mean numbers of revertant colonies, indicative of C2E2 treatment-related toxicity, was noted at the 5000 μg/plate level in TA100, TA1535, TA1537 and WP2uvrA under conditions without S9 and at ≥ 500 μg/plate in TA1535, TA1537 and WP2uvrA under conditions with S9. There were no relevant increases in the number of revertant colonies observed at any dose level with any strain with or without S9 metabolic activation (Table II). All vehicle and positive control values were within acceptable ranges. Thus, C2E2 was not mutagenic in the Ames assay for bacterial gene mutation up to 5000 μg/plate with or without metabolic activation.

In the chromosome aberration assays after 3-hour exposure, either with or without metabolic activation, no cytotoxicity was observed as evident by no dose-related reductions in cell counts or calculated population doublings compared to the concurrent vehicle control. All vehicle and positive controls were in acceptable ranges. Chromosomal aberrations were analyzed from the cultures treated with 245, 350 and 500 μg/mL of C2E2 and there were no statistically significant increases in the number of cells with chromosomal aberrations, polyploidy, or endoreduplication.

For the 20-hour test chromosomal analysis, cytotoxicity was observed. Based on mitotic indices, the 6.92, 9.89 and 14.1 μg/mL dose levels were selected for aberration analysis. The 14.1 μg/mL dose level produced a 53% reduction in mitotic index compared to the concurrent vehicle control. Chromosomal analyses again showed that there were no biologically relevant or statistically significant increases in the number of cells with aberrations observed at any dose level examined. Therefore, C2E2 was determined to be negative for the induction of chromosomal aberrations with and without S9 when tested up to cytotoxicity-limiting dose levels and the 500 μg/mL limit dose for this assay.

During the *in vivo* micronucleus test, one animal from the 2000 mg/kg/day group was noted to have audible respiration and hypoactive behavior on day 2. No other adverse signs of clinical toxicity were observed in any other C2E2 treated animal. There were no statistically significant decreases in the C2E2 treatment group PCE:NCE ratios compared to the vehicle control value indicating an absence of treatment-related bone marrow cytotoxicity.

Discussion

Although there has been much research investigating the decorporation of radioactive actinides, DTPA remains the agent of choice for elimination enhancement of internalized americium, plutonium and curium. Recently there has been a focus on the development of novel orally active chelators and improved formulations to overcome the shortcomings of DTPA. Tablets containing DTPA and permeation enhancers increased oral DTPA absorption from 5 % to 12% in rats (Shankar et al., 2013). Another approach involves the development of an orally administered DTPA nanoparticle formulation. Preclinical studies of this oral 'NanoDTPA'™ capsule formulation demonstrated favorable pharmacokinetic and safety profiles in rodent and dog models (Reddy et al., 2012). Radionuclide decorporation efficacy was also demonstrated with a transdermally administered formulation of the DTPA penta-ethyl ester prodrug (C2E5), however, toxicological assessment was not conducted (Zhang et al., 2013a,b).

In this work we detail the development of an orally bioavailable ester of DTPA for use in radiological emergencies. For medical countermeasure development programs where efficacy can only be demonstrated in animal models, particular emphasis is placed on the appropriateness of the models used. Here we have demonstrated that orally administered C2E2 enhances urinary and fecal elimination of ^{241}Am from beagle dogs in a model that was designed to mimic a realistic response to a mass casualty contamination. Specifically, the inhalation route of contamination was selected based on the most likely scenario for contamination of a large number of people, the species used was selected for the similarity of its ^{241}Am biokinetics to humans, and a 24-hour delay before treatment was used to approximate the time at which the initial response treatment would commence. The clearance of radioactive particles from the lungs following inhalation exposure is a complex and species-dependent process (Snipes, 1989). Particle clearance can occur by a mechanical process (e.g. muccocilliary action) or by the dissolution and subsequent absorption of the radionuclide into the systemic circulation. For moderately soluble materials, such as most americium salts, mechanical removal is strongly species-dependent whereas dissolution-absorption is species-independent (Bailey et al., 1989). The rate of particle clearance in dogs and monkeys are a better approximation of human clearance than rodents (Snipes, 1989). For these non-rodent species, human ^{241}Am biokinetics in other compartments are better approximated in dogs (Durbin, 2006), hence their use in the present study.

The elimination of ^{241}Am was significantly increased over controls in all treatment groups. Americium was predominantly eliminated by C2E2 in the urine, although statistically significant increases in fecal elimination were observed in the mid and high dose groups. This is in contrast to other studies performed with more lipophilic (C_{22}) polyaminocarboxylic acids, where the predominant elimination was fecal via the biliary pathway (Miller et al., 2010). This difference may be attributed to a smaller increase in lipophilicity generated by addition of ethyl esters rather than longer alkyl chain promoieties. The inhalation contamination model may also mask some of the fecal elimination effects due to the high percentage of activity (27 %) cleared from the lung in control animals via mucocillary clearance.

As efficacy cannot ethically be tested in human volunteers, proposed radionuclide decorporation agents need to comply with the Animal Rule (21 CFR 314.600) (Food and Drug Administration, 2009) for efficacy determination before receiving approval for use in humans. The studies described herein were performed as the initial steps to meet regulatory requirements to demonstrate that C2E2 is safe for first–in-human studies and efficacious for studies performed under the Animal Rule. Dose escalating toxicity studies were conducted to evaluate the acute toxicity of C2E2 and the data was used to support dose level selection for subsequent repeat-dose toxicity testing. The NOAEL established in dogs indicates that large doses of C2E2 are well tolerated. Administration of C2E2 with food resulted in a reduction in systemic exposure; this is an important factor that must be considered in the design of human clinical trials. In dogs, the liver was identified as a target organ of toxicity due to elevation of ALT and AST levels after a single dose of 750 mg/kg and at ≥ 400 mg/kg after 10 daily doses. At high doses or after multiple injections, DTPA is also associated with liver and kidney toxicity (Fukuda, 1984 & 1987). As with DTPA, the rapid excretion of C2E2 in the urine, may explain some of the nephrotoxicity. On occasion, oral administration of C2E2 at doses ≥ 200 mg/kg/day resulted in transient emesis. Emesis in future studies may be minimized by formulating C2E2 in enteric coated tablets or capsules. Although this may delay release, it may also help to minimize loss of drug and further increase bioavailability.

The maximum tolerated dose of 750 mg/kg is seven fold higher than the lowest effective single dose tested, though upon repeat dosing the NOAEL was determined to be 200 mg/kg/day. The therapeutic index is determined by the ratio of a drug's effective dose and lethal dose. Unlike traditional diseases in which the effective dose is measured by a quantal effect, the goal in decorporation therapy is to maximize radionuclide removal and the effect is measured on a continuous scale. As with DTPA, the retention of ^{241}Am is inversely related to dose; though each doubling of dose results in a diminishing return in efficacy (Lloyd RD. et al, 1979). Consequently a balance must be struck between decorporation efficacy and toxicity. To maximize the therapeutic window and maintain efficacy, a modified dose regimen could be used, in which a high initial dose is given followed by lower daily doses.

A comprehensive assessment of the genotoxic potential of the drug is required to obtain regulatory approval for human clinical trials. As no single test is capable of detecting all of the genotoxic mechanisms relevant to carcinogenicity, a standardized battery approach is recommended by ICH (Food and Drug Administration, 2012). These tests were completed as part of this study and the results indicate that C2E2 is not mutagenic or clastogenic.

As with DTPA, repeated dosing with C2E2 is expected to further enhance the elimination of ^{241}Am. Extended daily treatment will enable chelation of radionuclides that continue to translocate from the lung into the systemic circulation. Besides providing ease of access to treatment, the oral formulation is advantageous for long-term treatment because repeated intravenous injections are avoided. The efficacy and acute toxicity of C2E2 in rats and multiple dose toxicity over 28 days in both species are currently being investigated.

Conclusions

Single doses of C2E2 administered orally to dogs 24 hours after contamination by inhalation of ^{241}Am nitrate significantly increased the urinary elimination and reduced the tissue burden of ^{241}Am. The toxicity of acute oral doses of C2E2 was evaluated in dogs, and the NOAEL were identified for single and multiple dosing. C2E2 is neither mutagenic or clastogenic, based on the Ames test, chromosome aberration assay and micronucleus test data. Thus, this DTPA analog appears to be a promising orally-administered medical countermeasure for treatment of individuals contaminated with transuranic elements. Based on these studies as well as preliminary acute and genetic toxicity testing, C2E2 is a promising candidate for inclusion in the Strategic National Stockpile.

Acknowledgments

This work was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and U.S. Department of Health and Human Services under contracts HHSN266200500045C and HHSN272201000030C.

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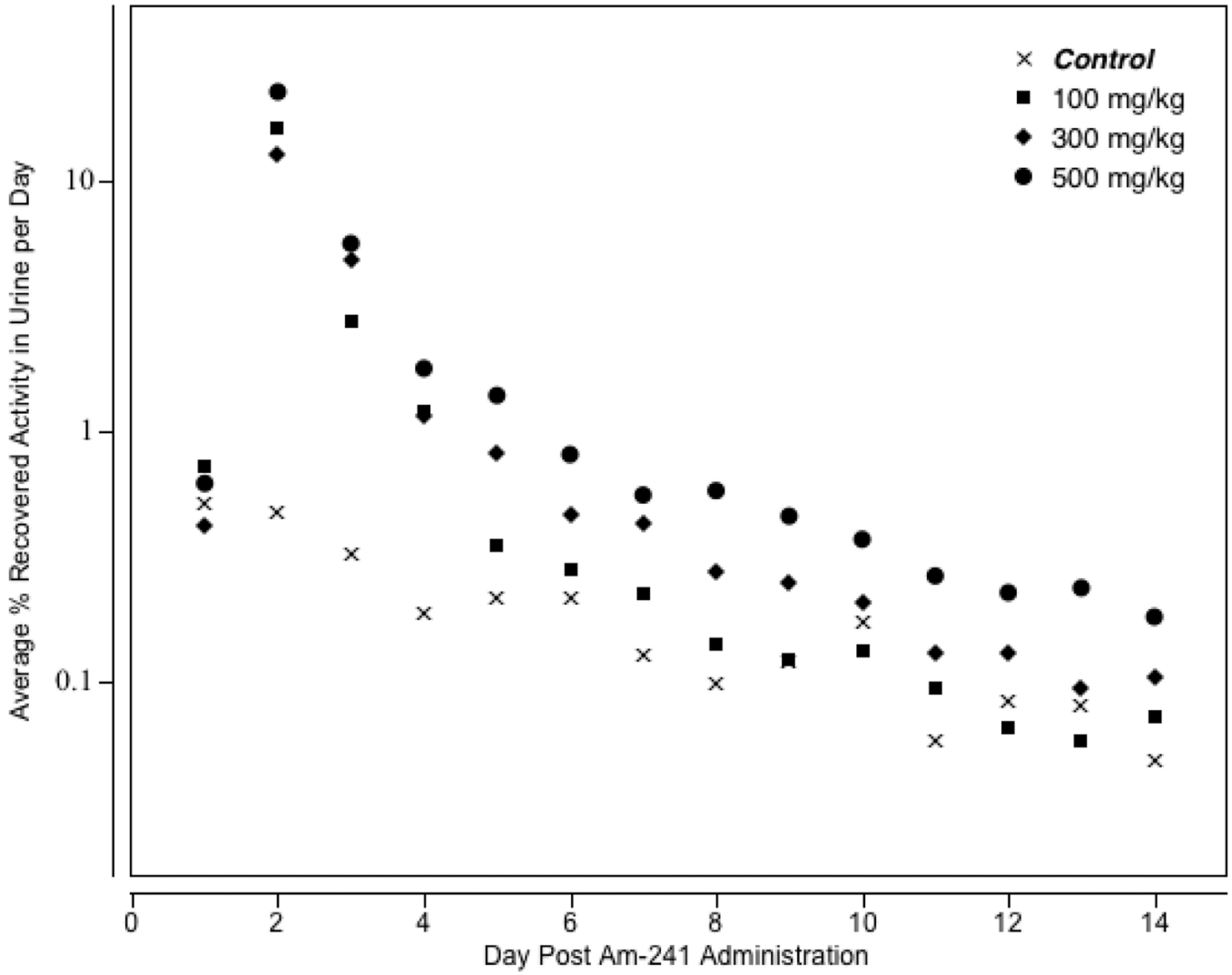

Figure 1.

Daily urinary excretion after a single oral administration of C2E2 to dogs 24 hours after inhalation contamination with ^{241}Am nitrate. Each point represents the average result obtained from 4 dogs.

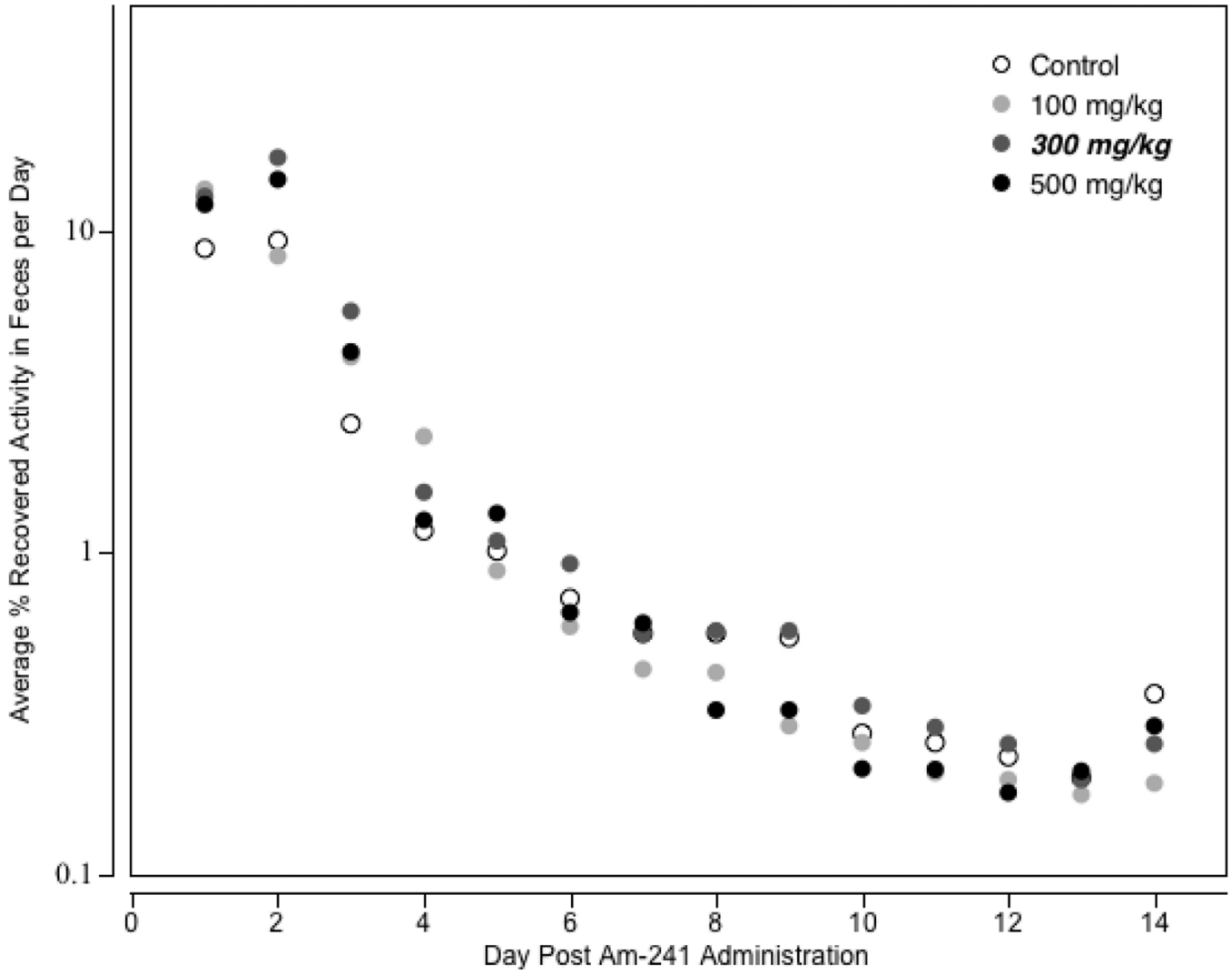

Figure 2.

Daily fecal excretion after a single oral administration of C2E2 to dogs 24 hours after inhalation contamination with ^{241}Am nitrate. Each point represents the average result obtained from 4 dogs.

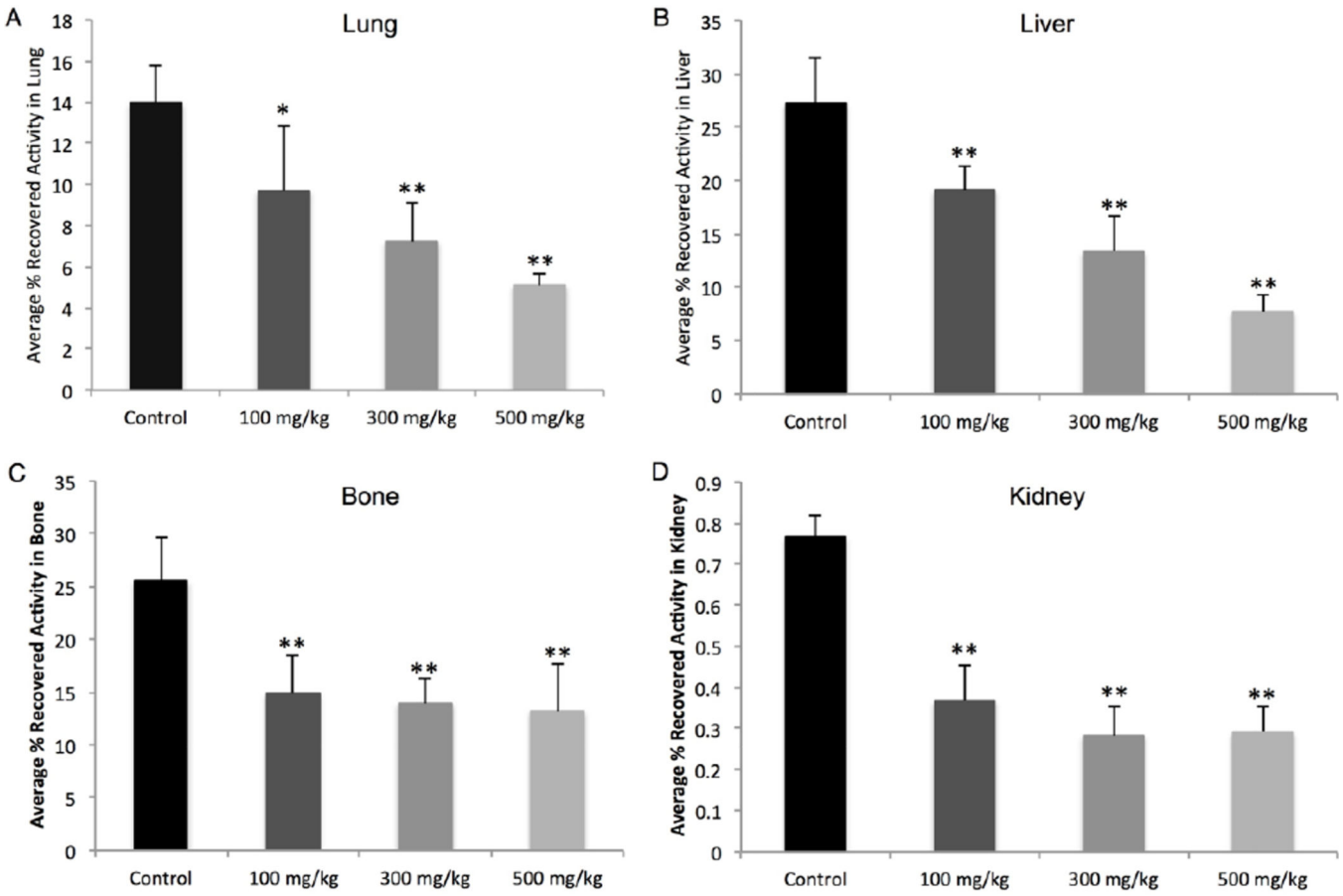

Figure 3.

Percent of respirable ^{241}Am dose remaining in lung (A), liver (B), total bone (C) and kidney (D) in dogs administered different doses of C2E2 at 24 h after inhalation contamination with ^{241}Am nitrate (Error bars indicate standard deviation (SD) n=4, *p<0.05 against control group, **p<0.01 against control group).

Table I

Systemic C2E2 exposure following oral administration of single escalating doses in dogs (Two per sex, with 7 days washout between dose escalation).

Dose Level (mg/kg/day)	Sex	C2E2		C2E1		DTPA	
		C_{max} (ng/mL)	AUC_{0-24} (ng.hr/mL)	C_{max} (ng/mL)	AUC_{0-24} (ng.hr/mL)	C_{max} (ng/mL)	AUC_{0-24} (ng.hr/mL)
100	M	39900	91950	503	949	207	480
	F	30700	79780	483	996	196	453
300	M	74650	230600	381	1692	351	894
	F	108800	263100	644	1559	516	1064
750	M	267000	530400	1895	3570	716	1721
	F	245500	498900	2325	3701	786	1747
300*	M	3335	32860	48	660	BQL	BQL
	F	3555	25930	45	517	BQL	BQL

* Indicates dosed under fed conditions

Table II

C2E2 Mutagenicity in Bacterial Strains of the Ames Test

Dose (μg/plate)	Mean ± SD revertant colonies/plate									
	TA98	**TA100**	**TA1535**	**TA1537**	**WP2uvrA**	**TA98**	**TA100**	**TA1535**	**TA1537**	**WP2uvrA**
	Without S9					**With S9**				
0	12.3 ± 3.2	90.7 ± 8.6	10.0 ± 1.0	7.3 ± 3.2	11.7 ± 1.2	22.0 ± 1.7	101.0 ± 5.0	7.0 ± 1.0	8.3 ± 2.5	14.7 ± 4.7
5.00	11.0 ± 2.6	97.7 ± 11.0	9.7 ± 1.5	4.7 ± 2.3	11.3 ± 0.6	19.0 ± 2.6	112.0 ± 19.5	7.7 ± 2.9	11.3 ± 1.5	15.0 ± 2.6
16.0	10.3 ± 4.0	94.3 ± 12.7	9.7 ± 2.5	5.3 ± 3.5	15.0 ± 2.6	16.7 ± 3.5	126.7 ± 21.5	7.3 ± 3.2	5.3 ± 1.5	13.7 ± 4.0
50.0	14.0 ± 2.0	103.7 ± 7.8	9.3 ± 2.3	4.0 ± 2.0	10.3 ± 2.1	20.7 ± 8.0	93.0 ± 9.8	10.0 ± 0.0	5.7 ± 1.2	14.7 ± 4.0
160	12.0 ± 1.0	65.0 ± 9.5	9.3 ± 1.2	4.0 ± 3.6	8.7 ± 2.1	18.0 ± 4.6	94.0 ± 10.8	9.3 ± 2.1	6.7 ± 3.1	12.7 ± 3.8
500	12.7 ± 1.5	94.7 ± 21.6	7.3 ± 5.0	2.7 ± 1.2	13.7 ± 1.2	21.0 ± 4.4	83.3 ± 12.0	7.0 ± 2.6	4.3 ± 0.6*	8.7 ± 4.7*
1,600	9.3 ± 3.1	72.0 ± 6.2	7.3 ± 3.5	8.3 ± 5.7	11.3 ± 1.0	21.0 ± 2.6	88.7 ± 11.7	8.3 ± 2.1	5.0 ± 1.7*	6.7 ± 1.5*
5,000	10.0 ± 4.6	23.7 ± 17.6*	1.7 ± 0.6*	2.7 ± 2.1*	3.3 ± 1.2*	15.3 ± 3.1	77.0 ± 15.1	4.3 ± 2.5*	4.0 ± 3.6*	5.0 ± 1.0*

(Errors indicate the standard deviation (SD) for n=3, *Reduction in the number of reverant colonies compared to control)