C2E2: AN ORALLY ADMINISTERED RADIONUCLIDE DECORPORATION ADENT

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ABSTRACT

James E. Huckle: C2E2: An Orally Administered Radionuclide Decorporation Agent
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The increasing threats of nuclear terrorism have made the development of medical countermeasures a priority for international security. Injectable formulations of diethylenetriaminepentaacetic acid (DTPA) have been approved by the FDA, however an oral formulation is more amenable in a situation involving mass casualties. The overall objective of this thesis is to synthesize and characterize the di-ethyl ester of DTPA, named C2E2, and assess the orally bioavailability and radionuclide decorporation efficacy.

The first specific aim investigates the physiochemical properties of C2E2. The solubility, lipophilicity and pKa's were determined. C2E2 was shown to have high aqueous solubility and lipophilicity was moderately increased over DTPA. The americium binding constant for C2E2 was also determined and shown to be higher than that of the plasma proteins, suggesting that C2E2 would retain the ability to chelate americium in the plasma despite having a lower coordination number than DTPA. Secondly, both the stability of C2E2 prior to absorption and within the plasma was investigated. Single dose pharmacokinetics in Sprague-Dawley rats demonstrated that C2E2 was the predominant form in plasma after administration along with lower levels of the mono-ester metabolite and DTPA. The oral bioavailability was determined as 29% in Sprague-Dawley rats compared to that of 5% for DTPA.

The third aim evaluated both the toxicity and oral efficacy of C2E2 in rats and dogs. Oral dose-range finding toxicology studies were performed to identify no-observed-adverse-effect-levels (NOAEL). Single and multiple dose decorporation efficiency of C2E2 was determined when administered 24 hours post-contamination in rats with an intramuscular wound model with ^{241}Am–nitrate. A single dose decorporation efficiency study was performed in dogs with contamination of ^{241}Am–nitrate via inhalation.

Overall C2E2 was synthesized and shown to have improved oral absorption over DTPA and retained the ability to chelate americium from the plasma. Oral administration of C2E2 exhibited low toxicity, a simple metabolic pathway and enhanced ^{241}Am decorporation over control. Based on this research this DTPA analog appears to be an effective orally-administered medical countermeasure for treating individuals contaminated with transuranic elements.

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LIST OF ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ALI	Allowable Level of Intake
ALT	Alanine Aminotransferase
Am	Americium
AMAD	Activity Median Aerodynamic Diameter
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
AUC	Area Under Curve
BCS	Biopharmaceutics Classification System
BID	Twice Daily / bis in die
C2E1	DTPA Mono-ethyl Ester
C2E2	DTPA Di-ethyl Ester
C2E3	DTPA Tri-ethyl Ester
C2E5	DTPA Penta-ethyl Ester
CAD	Charged Aerosol Detector
CEA	The Atomic Energy and Alternative Energies Commission
CHO	Chinese Hamster Ovary
Ci	Curies
Cm	Curium
DSC	Differential Scanning Calorimetry
DTPA	Diethylene Triamine Pentaacetic Acid

ED	Effective Dose
EDTA	Ethylenediaminetetraacetic Acid
F	Bioavailability
FDA	US Food and Drug Administration
FIH	First in Humans
GFR	Glomerular Filtration Rate
SEM	Scanning Electron Microscope
Gy	Gray
HDEHP	Di-(2-ethylhexyl)phosphoric Acid
HED	Human Equivalent Dose
HOPO	Hydroxypyridinone
HPLC	High Performance Liquid Chromatography
IAEA	International Atomic Energy Agency
ICH	International Conference on Harmonisation
ICRP	International Commission on Radiation Protection
IM	Intramuscular
IND	Investigational New Drug
ITDB	Incident and Trafficking Database
IV	Intravenous
LD	Lethal Dose
MCT	Medium-chain Triglyceride
MRSD	Maximum Recommended Starting Dose
MTD	Maximum Tolerated Dose

NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCE	Normochromatic Erythrocyte
NCRP	National Council on Radiation Protection & Measurements
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
ORAU	Oak Ridge Associated Universities
PCE	Polychromatic Erythrocyte
PET	Positron Emission Tomography
PKPD	Pharmacokinetic-Pharmacodynamic
PK	Pharmacokinetic
PO	Orally / per os
Pu	Plutonium
QD	Once Daily / quaque die
RDD	Radiological Dispersal Device
RH	Relative Humidity
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SNS	Strategic National Stockpile
SPECT	Single-Photon Emission Computed Tomography
SV	Sieverts
TK	Toxicokinetic

TTHA	Triethylenetetraaminehexaacetic Acid
UNC	University of North Carolina

CHAPTER I

INTRODUCTION

1.1 The Hazards of Radioactivity

The threat posed by radioactive materials is not new; naturally occurring isotopes, such as radium, continually expose us to low levels of radiation. In the United States the average background absorbed radiation dose is 360 mrem/y, which comes equally from both natural and manufactured sources[1]. Fortunately the consequences of background radiation are minimal as our cellular repair mechanisms are usually capable of responding to the effects of ionizing radiation. Therefore, chronic low-dose radiation is stochastic in nature, whereby some people may experience an effect from the exposure but the risk is low and cannot be attributed to radiation. Exposure to ionizing radiation at high-doses however leads to predictable effects that can be irreversible and often fatal.

The existence of radioactivity was unknown until electrical discharges were first observed from Crooks tubes after application of a high voltage across the tubes. Thomson identified the light emitted from the gas as negatively charged particles which became known as electrons[2]. In 1895 Roentgen also working with cathode rays discovered that the florescence produced was penetrating radiation which he named X-rays[3]. In 1896 Henri Becquerel discovered that alpha particles, beta particles and gamma rays were released from uranium[4]. The same discovery was subsequently made by Marie Curie in 1898 with radon

and coined the term radioactivity[5]. The discovery of these new particles led to further investigations and in 1911 Rutherford identified the nucleus, making it possible to determine the mechanism of these particle emissions[6]. The discovery of the neutron in 1932[7] by Chadwick while studying the beryllium nucleus completed the puzzle and major advances in nuclear research quickly followed: uranium fission and the possibility of releasing vast amounts of energy in 1939[8], self-sustaining fission reactions in a reactor in 1942, explosion of nuclear fission devices in 1945, production of thermonuclear explosives and the first nuclear powered submarine in 1952[9]. Prior to the 1950s only naturally occurring radionuclides were generally available, however the investigations of Glenn Seaborg at the University of California, Berkeley spurred the discovery of ten new transuranic elements including plutonium, americium and curium[10]. In addition to the use of radioactivity for power and weapons, there are now hundreds of medical, industrial and academic applications[11]. The use of Technetium-99m generators within hospitals and the power of computers have revolutionized diagnostic imaging with techniques such as single-photon emission computed tomography (SPECT) and positron emission tomography (PET) in common use. Radiation therapy has treated countless cancer victims, including those with drug resistant tumors. The increase in use of radioactive isotopes however has resulted in a large supply of radioactive sources with millions distributed over the last fifty years, and hundreds of thousands are currently used and produced[12]. Even though there are hundreds of available radioisotopes, only seven are of particular concern with respect to nuclear terrorism due to their widespread use, radiotoxicity and long half-life: ^{241}Am, ^{242}Cf, ^{137}Cs, ^{60}Co, ^{192}Ir, ^{238}Pu and ^{90}Sr[13].

This widespread availability gives rise to potential terrorist threats, which since September 11, 2001 has become a national security priority[14]. The use of unconventional terrorist tactics such as the delivery of anthrax spores in mailboxes in New York, Washington and Florida during 2001[15] illustrates the risk of attacks and there is an increased risk of further attacks that utilize biological, radiological or even nuclear weapons. The use of either radiological or nuclear weapons could result in significant numbers of individuals being exposed to ionizing radiation requiring urgent medical intervention. President Obama highlighted the importance of this threat in his first speech to the U.N. Security Council in New York, where he called nuclear terrorism one of the greatest threats to international security and stated that the consequences of such an attack would be enormous[16].

Terrorist acts could expose a population to radiation by contaminating food/water sources, placing radiation sources in a public location, detonating a radiological dispersal device (RDD, "dirty bomb") or by attacking a nuclear power plant or storage facility. Finally there is also a possibility that a nuclear explosive device (atomic bomb) could be detonated which, in addition to causing major structural damage would also lead to mass contamination from radioactive "fallout". Of these threats, the wide availability of radiological sources and ability to produce homemade bombs makes an RDD the most likely candidate for terrorists. An RDD is a device that causes dispersion of radioactive material without nuclear detonation. In 2013 there were 146 incidents confirmed in the Incident and Trafficking Database (ITDB), 6 of which were related to criminal activities, 47 involved loss and 95 were unauthorized activities[17]. In addition, four of these incidents involved theft of International Atomic Energy Agency (IAEA) category 1-3 sources. (Sources are categorized from 1-5, where a category 1 source is the most dangerous with which an exposure time of a

couple of minutes could be fatal.) The loss of radioactive sources does not only occur overseas, for example nineteen tubes of ^{137}Cs were stolen from a hospital in Greensboro, North Carolina and were never found[18].

The release of radioactivity can result in localized or whole-body exposure and both internal and external contamination with radioactive materials. Exposure to radioactivity occurs when radiation penetrates the body from an external source, whereas contamination occurs when material deposits on the surface of an object. In order to minimize exposure it is important to increase distance from the source, wear shielding and minimize the time of exposure.

In the case of local exposures patients can often survive high absorbed doses[19]. Although burns and blistering may occur, the systemic manifestations are not as severe as at the local area of contamination. Patients should be treated against infection and given pain medication as required. The absorbed dose is measured in Grays (Gy) where one Gy is equivalent to one joule per kg. Whole body exposure is more lethal with exposure to doses above 2 Gy resulting in bone marrow suppression leading to hematopoietic syndrome. The associated reduction in lymphocyte count can be used to estimate the dose of radiation. Acute doses of 10 to 30 Gy cause GI damage due to the death of mucosal stem cells leading to rapid onset of nausea, vomiting and diarrhea. Following a latency period sepsis and electrolyte imbalances lead to death. At doses exceeding 30 Gy cardiovascular and central nervous system damage begins to occur with almost immediate nausea, vomiting, hypotension and convulsions and death may occur within several days[19]. As with local exposure there are limited treatment options. Amifostine has been FDA approved for patients undergoing

radiotherapy, however, it is only effective if given prophylactically and has numerous serious side effects[20]. Treatment of people exposed to radioactivity is thus mainly symptomatic.

When a radioactive nucleus decays it emits ionizing radiation in the form of an α-particle, β-particle, γ-rays, or a combination of these. Alpha particles consist of two protons and two neutrons. They can travel 2-3 cm in air, however they can only travel microns in tissue. They cannot penetrate paper, so are not an external hazard on skin, the outer layers of which are already dead. However, if ingested or inhaled alpha particles can cause serious damage to the adjacent tissues. Beta particles are high-energy electrons and can travel a meter in air and millimeters within tissue. They are an external hazard as they can penetrate the skin and can cause severe burns, if internalized they will also cause significant damage. Finally gamma rays are high-energy rays with short wavelengths that are very penetrating, having the ability to travel many centimeters through tissue[21].

Differences in tissue susceptibility to radiation were first identified by Bergonie and Tribondeau in 1906 who reported that immature, undifferentiated and actively dividing cells are more radiosensitive than those which are mature and are not actively dividing[22]. These differences are a result of the interactions between cells and ionizing radiation, which acts either directly or indirectly with cells. Direct interaction occurs when the cell's macromolecules are hit by the ionizing radiation causing disruption to the whole cell, either leading to cell death or mutation of cellular DNA[23]. Direct hits can cause a wide range of cellular injury depending on the location of the damage. Generally the effects of ionization are not observed until higher doses of radiation are present causing cell replacement to cease[24]. Alternatively cellular damage can occur indirectly as radiation energy causes hydrolysis of cellular water leading to the formation of hydroxyl free radicals. Two hydroxyl

ions can form unstable hydrogen peroxide resulting in the formation of a peroxide hydroxyl radical that can become stable by combining with organic compounds in the cell. The organic hydrogen peroxide can then interfere with essential enzymes leading to cell death.

In addition to being exposed to penetrating radiation following a nuclear accident, some people are likely to become contaminated. External contamination of skin and clothes does not require special medical treatment; the contaminated items should be removed and contained to minimize the spread of activity. The patient can then wash the contaminated areas of skin with soap and water providing that there are no physical injuries. If the patient has not received a very high dose of external exposure and any medical emergencies, such as trauma, have been addressed, then the greatest concern is that of internal radiation contamination.

1.2 Internalized Radionuclide Contamination

There are four main routes by which internal contamination can occur (a) inhalation, (b) uptake via cuts, wounds, burns or contaminated shrapnel, (c) ingestion followed by oral absorption and (d) transdermal absorption. The risk of exposure to radioactive heavy metals via the last two routes is low due to poor oral and transdermal absorption. Accidental contaminations such as syringe pricks or puncture wounds can occur in industrial, hospital or research laboratory environments. After detonation of a nuclear device, inhalation of the radioactive plume and wound contamination from radioactive shrapnel are the most likely sources of internalized contamination.

There have been a number of radiological accidents leading to contamination. The Goiânia incident in Brazil during 1987 highlighted the dangers of unsecure nuclear materials[25]. A radiotherapy source was stolen from an abandoned hospital and sold to a local scrapyard. The source container was opened and the blue cesium-137 was passed among the owner's family and friends. As members of the family began to fall ill, the contents were tested and found to be radioactive; ultimately 249 people had significant contamination on their body of which 129 also had internal contamination. There were four fatalities associated with the incident, all of whom worked at the scrapyard where the radioactive source became exposed.

More recently in 2011 following a tsunami, three nuclear reactors at the Fukashima nuclear power plant in Japan underwent meltdown that led to the release of large quantities of iodine-131 and cesium-137 into the atmosphere resulting in the evacuation of 300,000 people[26]. In addition in 2014 a leak occurred in a nuclear storage facility in New Mexico, USA causing contamination of 13 workers with americium and the release of americium and

plutonium into the atmosphere, though in this case the levels were deemed low enough to not require treatment[27].

The main focus of this doctoral research is the decontamination of americium and plutonium, which are α-particle emitting radionuclides of the actinide series, with americium-241 being the radioisotope of primary interest. Each of these radionuclides has a unique biokinetic profile resulting in different patterns of tissue deposition. As such the disposition is also dependent on the route of contamination, the physio-chemical properties such as size and solubility of the ingested form, and the health status of the contaminated individual. Once internalized the tissues adjacent to the radionuclide are exposed to ionizing radiation until the contamination is removed either by excretion or treatment. The International Commission on Radiological Protection (ICRP) has summarized the distribution of americium and plutonium[28].

Plutonium-239 has a half-life of 24,000 years[29] and is the primary fissile isotope of plutonium used in the production of nuclear weapons and is also one of the main isotopes used as fuel in nuclear reactors. Due to its widespread use, the biodistribution of plutonium has been extensively investigated. Once it leaves the blood 50 % of the material is deposited on bone surfaces, 30 % retained in the liver and only 20 % in other tissues. Removal of plutonium from the bone and liver occurs with half-lives of 8000 and 9 years, respectively[30].

Americium-241 is produced through the degradation of plutonium present in spent nuclear fuel. It is an alpha emitter with a 432-year half-life but also emits a small amount of γ-radiation. Due to its alpha emission it is found in smoke detectors making small quantities widely available. In the body americium exists only in the trivalent Am(III) oxidation state. It behaves in a similar fashion to plutonium, however it's uptake and retention in the liver is

different with 50 % of the radionuclide depositing in the liver and 30 % on the bone. Americium clears from the liver faster than plutonium with a half-life of 2-3 years, however loss from the skeleton is no different[31]. Once the radionuclide is deposited in a tissue it may re-speciate by equilibrating with other ligands and metal ions present, redistributing via the blood stream to a secondary deposition site[32]. It is for this reason that the half-life of americium elimination from the liver begins to increase over time, as americium redistributes from its primary disposition sites to the liver. It is easy to see from the long biological half-lives that internalized americium and plutonium can be expected to continue to irradiate the surrounding tissues for the remaining life of a contaminated individual in the absence of treatment to remove it.

The distribution and elimination kinetics of americium influence many aspects of treatment. Although the rate of tissue distribution is dependent on the site of contamination and the solubility of the internalized form, once solubilized and absorbed into the plasma the distribution and elimination kinetics are predictable and independent on the route of contamination. Therefore, though unlikely to occur, IV ^{241}Am administration has proved useful for investigating the fate of ^{241}Am after uptake from contamination sites. Radionuclide contamination is most likely to occur through either inhalation or a contaminated wound site. Thus during efficacy studies both inhalation and IM contamination models are more suitable as they mimic the continuous uptake of radionuclide into the plasma.

Upon entry into the plasma, americium can associate with a variety of plasma proteins and low molecular weight ligands. There is little difference between the plasma concentrations of electrolytes and plasma proteins across species and although there is a large change in body size, the plasma volume remains fairly consistent[33] (Table 1.1). The main

difference between species is the plasma iron concentration and subsequently the transferrin saturation. In human plasma americium is predominantly associated with transferrin, with the remainder associated with other proteins such as albumin, α- and γ-globulins or low molecular weight ligands[34]. The stability constants for low molecular weight ligand complexes of americium have been determined[35] (Table 1.2). Based on the stability constants and concentrations in plasma, citrate and carbonate are likely to be the predominant low molecular weight species. Any americium present as a citrate complex is ultrafiltratable by the kidneys and will be excreted in the urine.

The rate of radionuclide plasma clearance is largely dependent on its ability to form complexes with transferrin. Actinides that form more stable complexes, such as plutonium, are cleared more slowly than those with lower affinities such as americium. Animal physiology is therefore important with plasma ^{241}Am retention being directly related to body size and inversely related with metabolic rate and renal filtration rate so that:

Human > Dog > Baboon > Monkey > Rat > Mouse

Plasma clearance of ^{241}Am is fast with 90 % complete within 60 minutes and 99% complete in < 600 minutes (Figure 1.1). Only a small fraction is present in the urine at 24 hours and therefore the majority is deposited within the tissues.

The initial distribution of americium has been investigated in six species following IV administration. The tissue distributions are similar across all species (Table 1.3). Entry of metal ions into the liver can occur through facilitated diffusion or receptor-mediated endocytosis. The liver cell internalizes more than 20 % of its volume each hour in a process known as membrane recycling. Through this process any metal with a high affinity for membrane-binding sites will be internalized[36]. Iron is predominantly absorbed through

receptor-mediated endocytosis involving the Tf-Fe complex and a cell surface Tf-receptor. Although both americium and plutonium bind with Tf, this seems to have a negative effect on hepatic uptake[37]. Therefore, hepatic uptake is related to the successful competition of hepatic cell binding sites for ^{241}Am from plasma circulating complexes. Similarly skeletal binding sites, depending on their location, compete with ^{241}Am-complexes present in the plasma or extracellular fluid. Generally there are three types of bone surface, those actively growing, resting, or resorbing and ^{241}Am deposition is higher on resorbing and resting bone surfaces than those actively growing[38]. Ultimately there is continual competition between americium ions, plasma complexes and the tissues resulting in a dynamic equilibrium of species with a continual shift to the most stable complex. However actinides deposited within the bone are only released during limited structural remodeling. Therefore, once deposited within the bone the strong association prevents release back into the circulation even in the continued presence of chelating agents.

The risk associated with this internalized contamination is dependent on the dose of radionuclide. As the harmfulness of each type of radiation is different, the absorbed dose measured in Grays is weighted and defined as equivalent dose, measured in Sieverts (Sv). Susceptibility to radiation is also tissue dependent and therefore the equivalent dose can be further weighted to give an effective dose also quantified in Sieverts[39]. The biological effects resulting from the dose of ionizing radiation can be described as either deterministic or stochastic. Deterministic effects have a threshold below which they are nonexistent, however, once the threshold is reached, the severity of the effect is proportional to the dose. Conversely stochastic effects involve long-term low-level exposures that can increase the

likelihood of cancer or DNA damage leading to genetic mutations. As there is no threshold, an exposure at any level carries a risk of stochastic effects.

Although any degree of contamination can lead to an increase of cancer, the increase in risk may be insignificant compared to other daily health risks. Hence the National Commission for Radiation Protection (NCRP) has suggested radiation levels below which treatment is often unwarranted. These levels are based on a conservative point, whereby treatment should start at the upper limit of radionuclide contamination permitted for radiation workers each year, known as an allowable level of intake (ALI). This ALI corresponds to an effective radiation dose of 5 rem (equivalent to 0.05 Sv), fifty times that of what is allowed for members of the general public, at which there is no measurable risk. For americium and plutonium treatment is indicated when one ALI is internalized which correlates to 0.8 μCi of ingested or 6 nCi of inhaled material[40]. For reference the average smoke detector contains 1 μCi of americium.

Treatment of internal contamination is known as decorporation therapy, and it is intended to minimize absorption and internal disposition and enhance elimination of the radionuclide, thus reducing the future risk of biological effects. Prompt intervention is crucial as some isotopes reach their terminal organ of disposition within hours or minutes of internalization from which removal is difficult.

Table 1.1 Plasma volume, protein and iron concentrations across species (Taken from [33]).

	Mouse	Rat	Beagle	Monkey	Human
Body weight (kg)	0.035	0.25	10	6.2	70
Plasma (mL kg^{-1})	50	36	49	36	43
Serum Albumin (mM L^{-1})	0.56	0.60	0.52	0.64	0.65
Serum Globulin (mM L^{-1})	0.14	0.22	0.19	0.25	0.21
Transferrin (mM L^{-1})	0.036	0.033	0.032	0.040	0.027
Serum Iron (mM L^{-1})	0.053	0.039	0.025	0.032	0.018
Transferrin Saturation (%)	74	59	39	40	33

Table 1.2 Concentrations of relevant low molecular weight ligands in plasma and their stability constants for both americium and plutonium (Adapted from [35a] and [35b]).

Biological Ligand	Blood Serum Concentration (M)	Stability constant (Log K)	
		Americium Am^{3+}	Plutonium Pu^{4+}
Carbonate (CO_3^{2-})	2.5×10^{-2}	8.0	-
Citrate	1.6×10^{-4}	8.6	15.3
Chloride	9.0×10^{-2}	1.1	1.8
Glycine	$2.6 \times 10^{-4*}$	4.1	-

*Concentration from [41]

Table 1.3. Comparison of the initial distribution of injected americium in various tissues across species.

Species	Mass Injected	Days	Fraction of injected americium (%)					
			Tissues				Excreta	
			Skeleton	Liver	Kidney	Other	Urine	Feces
Mouse	0.23	1	27	50	1	6	14	3
Rat	0.12	4	35	42	-	3	10	10
Dog	0.54	1	31	52	0.7	5	10	1
Monkey	0.12	8	25	56	0.6	7	9	2
Baboon	0.06	1	35	31	0.7	22	10	1
Man	0.04	4	31	69	-	-	-	-

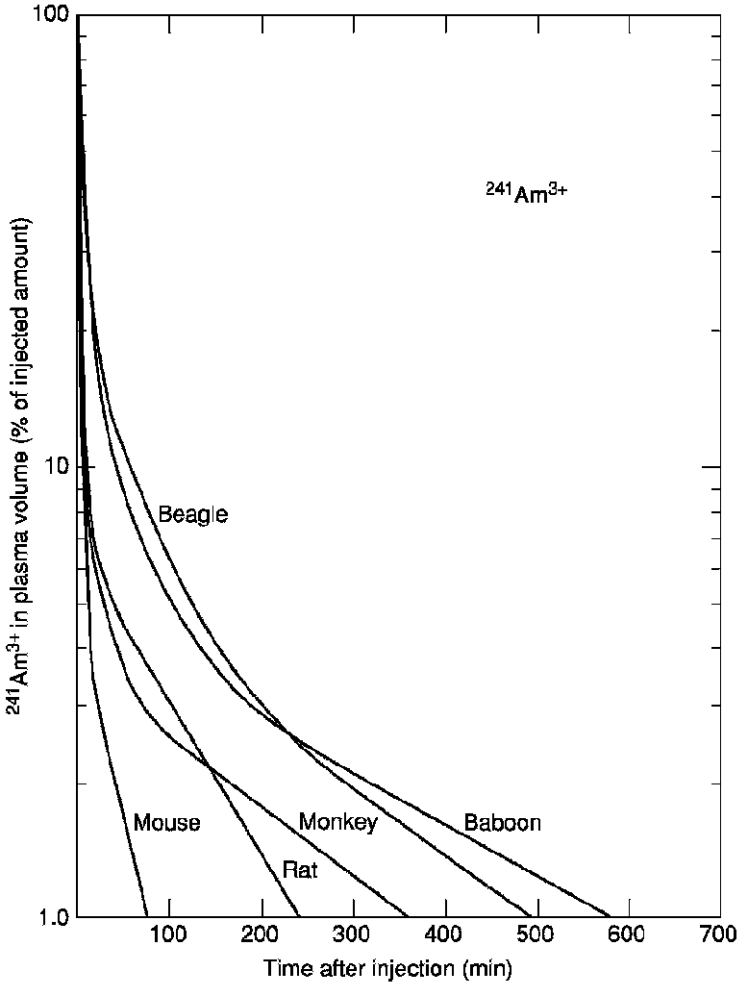

Figure 1.1 Clearance of IV-injected $^{241}Am^{3þ}$ from the plasma volume of animals: rat (Turner and Taylor, 1968a); mouse (P. W. Durbin and B. Kullgren, unpublished data); beagle (Stevens and Bruenger, 1972); monkey (Durbin, 1973); baboon (Guilmette et al., 1980). Figure from [33]

1.3 Decontamination of Internalized Radinuclides

Fortunately the dangers of plutonium and other actinide elements were perceived during early investigations, so decorporation methods were sought out by the health group of the Manhattan Project as early as 1947[42]. For those who become internally contaminated, there are five decontamination treatment options, the use of which depends on the internalized radionuclide. The underlying hypothesis for all five treatments is that accelerating the natural rate of elimination of the radionuclide will reduce the amount of activity remaining in the body and thus reduce the dose received by sensitive tissues and the risk of radiation effects[31]. The currently available oral and IV decorporation treatments are listed in Table 1.4.

The first method is to surgically remove the contaminated area. This method may be employed in situations where wounds are contaminated with a long-lived isotope, such as plutonium, in order to prevent systemic uptake. A second simple measure is to administer large volumes of oral fluids, which help to dilute the radionuclides and increase urinary excretion. For example, treatment with 5-10 L/day of fluid for one week is indicated for tritium contamination[43]. The use of diuretics will also help to further enhance urinary elimination.

For radionuclides that are ingested, gastric absorption can be limited by stomach lavage and/or the use of emetics, however this carries the risk of aspiration. Instead absorption blockers can be used to help minimize uptake without this risk. In addition they may also be effective after inhalation contamination, where a fraction of the dose may become ingested due to mucociliary clearance. Alternatively, the use of fast acting laxatives is the simplest form of absorption prevention by minimizing the time a radionuclide has to be absorbed from the GI tract. An example of an enteral binding agent is ferric hexacyanoferrate (Prussian

Blue), which is insoluble and binds cesium-137 and isotopes of thallium with high affinity, preventing their absorption. In addition to preventing absorption of ingested cesium-137, Prussian blue can play a role in decorporation of patients systemically contaminated with cesium-137 by interfering with its entero-hepatic circulation[44]. In 2003 it received US Food and Drug Administration (FDA) approval for treatment of internal contamination[45]. Other examples of binding agents include barium sulfate, a radiographic contrast agent, and aluminum and magnesium salts, all of which can bind to strontium and radium to decrease absorption[46].

Once a radionuclide is absorbed into the systemic circulation, the first three decontamination methods have limited use. The radionuclide must now be removed by enhancing its elimination either through blocking uptake into the target organ or through the use of a chelating agent. Uptake into target organs can be blocked by supersaturating radionuclide binding sites thereby preventing accumulation and enhancing elimination. Potassium iodide has been approved by the FDA since 2002 for prevention of radioactive iodine sequestration by the thyroid[47]. Calcium gluconate and calcium chloride act in a similar fashion, limiting the incorporation of radioactive strontium or calcium into the bone. Mobilizing agents can be used to help remove deposited radionuclides. Sodium bicarbonate can be used to remove uranium from the kidney and ammonium chloride can help to increase elimination of internalized radiostrontium. Both of these agents work by altering the pH at their target site.

Chelating compounds are organic ligands that are able to exchange less firmly bound inorganic ions for other more favorable ion complexes. The strength of binding for a particular metal varies for each chelator, and, although they are able to form chelates with a

range of cations, some are bound with a higher affinity leading to preferential chelation of the most tightly bound cation. Once bound the cation is part of a stable complex and no longer acts as a free ion, allowing for excretion by the kidney. This basic principle was first reported in 1942 by Kety[48] and has been applied to decorporation therapy since the 1950s. Chelator selectivity for a particular ion is determined by a number of properties including the number of vacant coordination sites, the distance between these sites and the size of the ion to be bound. If the affinity of the chelator for essential metals such as Mg^{2+} is higher than that of the plasma proteins toxicity can occur due to depletion of essential metals.

Chelation therapy is most effective when administered immediately after contamination, while the radionuclide is in the plasma and before deposition within the tissues has occurred. Although the highest decorporation effect is seen with the initial dose, as a large proportion of the radionuclide is in the plasma, chelators are still effective at eliminating radionuclides albeit at lower levels with sustained treatment. The continual benefit of chelation therapy may be due to metabolic recycling of the radionuclide into the extracellular fluid or in some cases be due to intracellular chelation. After exposure via the lung or a wound, the radionuclide will continue to slowly diffuse into the plasma and redistribute from the contamination site to other tissues such as the bone. In these instances chronic chelation therapy may be indicated to continue fostering elimination, reducing the total dose and residual disposition in the bone and liver. Ultimately, the duration of treatment will be guided by the biokinetics of the radionuclide and the degree of contamination.

Chelating agents for radionuclide decorporation have been under investigation since the early 1950s when the dangers of accidental exposure were recognized. Early efforts involved

the use of common carboxylic acids and complexing agents and showed limited efficacy. A good chelating agent should possess the following properties:

- Chelating moieties should be deprotonated at physiological pH
- High number of chelate rings to increase stability of the complex
- Actinide-ligand complex behaves differently from actinide-biological complex
- High affinity for actinides and low selectivity for essential metals
- Low toxicity
- Avoid degradation to metabolites that no longer bind the metal
- High oral bioavailability is desirable[35b]

There are currently 11 FDA approved chelators (Table 1.5) available for the treatment of poisoning with various metals[49]. British-Anti-Lewisite (BAL) or dimercaprol contains two sulfhydryl groups that form stable non-toxic five-membered heterocyclic rings with heavy metals[50]. It was initially used to sequester arsenic during the Second World War and in 1946 received FDA approval for the decorporation of arsenic, mercury and gold[51]. D-Penicillamine and *N*-Acetyl-D-Penicillamine are degradation products of penicillin and have been used for copper[52], lead[53], gold[54], mercury[55] and zinc[56]. Deferoxamine is a trihydroxamic acid secreted by the *Streptomyces polosus* fungus, which has a high affinity for iron and low affinity for other essential metals. Therefore it is used for treatment of acute iron toxicity or chronic iron overload[57]. Two synthetic polyaminopolycarboxylic acids are also used for chelation. Ethylenediaminetetraacetic acid (EDTA) was the first polyaminopolycarboxylic acid used in humans and has received FDA approval for the treatment of lead poisoning. Diethylenetriaminepentaacetic acid (DTPA) can be considered an expanded version of EDTA with an extra amine and carboxylic acid group. The addition of these two extra

chelation sites results in a 100-fold increase in binding affinity for various metals compared to EDTA. In 2004 two forms of DTPA, namely the pentetate calcium trisodium injection (Ca-DTPA) and pentetate zinc trisodium injection (Zn-DTPA), received FDA approval for treatment of internal contamination with plutonium, americium or curium[58].

Table 1.4 Overview of Decorporation Treatments (Modified from[43])

Decorporation Agent	Route	Dosage	Radionuclide
Ammonium Chloride	Oral	1-2 g q.i.d for 6 days	Strontium
Ca-DTPA, Zn-DTPA	i.v.	1 g in 250 ml saline over an hour	Americium, Plutonium, Curium
Calcium	Oral	Generous doses	Radium, Strontium, Barium
Calcium gluconate	i.v.	2.5 g administered in 0.5 L over 4h. Daily for 6 days	Strontium
D-Penicillamine	Oral	250 mg daily between meals and bedtime up to 4-5g daily	Cobalt, Indium, Palladium
Potassium Iodide	Oral	130 mg daily	Iodine
Potassium Phosphate	Oral	250-500 mg q.d.s.	Phosphorous
Propylthiouracil	Oral	100 mg t.d.s. for 8 days	Iodine
Prussian blue	Oral	1 g t.d.s for up to three weeks	Cesium, Thallium, Rubidium
Sodium alginate	Oral	10 g add water and drink	Strontium
Sodium bicarbonate	i.v.	-	Uranium

Table 1.5 Chelators currently approved by the FDA

Chelator	Target Radionuclide(s)
Deferasirox	Iron
Deferiprone	Iron
Deferoxamine mesylate	Iron
Dimercaprol (BAL)	Arsenic, Gold, Mercury
Edetate calcium disodium (Calcium EDTA)	Lead
Penicillamine	Copper
Pentetate calcium trisodium (Ca-DTPA)	Americium, Plutonium, Curium
Pentetate zinc trisodium (Zn-DTPA)	Americium, Plutonium, Curium
Prussian blue	Caesium, Thallium
Succimer (DMSA)	Lead
Trientine hydrochloride	Copper

1.4 DTPA

Diethylenetriaminepentaacetic acid (DTPA) is a polyaminopolycarboxylic acid chelating agent that was originally synthesized in 1954 as an alternative EDTA ligand with the aim of increasing the affinity towards multivalent cations[59]. The additional nitrogen and carboxylic acid on DTPA provide eight potential metal coordination sites that allow binding to a wide range of metals with high affinity[60] (Table 1.6). The additional binding sites improve the stability of metal chelates compared to EDTA, while the coordination of Ca ions remains similar therefore the $K_{ML}^{M} / K_{CaL}^{Ca}$ ratio is enhanced resulting in increased efficacy. Due to having a lower affinity for calcium compared to zinc, the Ca-DTPA salt is used preferentially within the first 24 hours after contamination where it is ten times more effective at actinide elimination than the Zn-DTPA salt[61]. After the initial 24-hour period both salts are equally effective, however Zn-DTPA is preferred due to zinc's stronger affinity for DTPA, which minimizes toxicity by limiting the loss of essential traces metals.

Due to its hydrophilic nature DTPA is poorly absorbed from the GI tract (3-5%)[62], however when administered intraperitoneally or intramuscularly it is completely and rapidly absorbed[63]. Following IV injection DTPA rapidly distributes in the plasma and extracellular fluid, though its hydrophilicity prevents it acting intracellularly. The plasma concentration in humans can be described by three exponential components with half-lives of 1.4, 14.5 and 94.4 minutes. DTPA is rapidly cleared by glomerular filtration into the urine ($t_{½}$ 19 min); 99 % of the administered dose is excreted in the urine within 24 hours, with the remaining DTPA being slowly excreted over the following days[64]. This remaining fraction of the DTPA dose (< 1%) may remain in excess of radionuclide due to the minute quantities present and be responsible for enhanced urinary elimination of the radionuclide after 24 hours.

Historically DTPA been used for the treatment of lead poisoning and in the paper industry for chelation of metals in pulp. In addition, Gd-DTPA is used medically as an MRI contrast agent. Its efficacy for radionuclide decorporation was established through animal studies and accidental human contaminations[65].

The efficacy of DTPA has been extensively demonstrated in animal studies using mouse, rat and dog models for evaluation of decorporation. The experimental conditions used for efficacy studies have varied widely, though overall they demonstrate that chelation therapy can reduce radiation damage, increase tumor latency and survival[66]. In rats, the efficacy of ^{241}Am decorporation has been investigated using both Ca- and Zn-DTPA salts over a series of concentrations and after a range of treatment delays. Administration of a single 30 μmol/kg IV Ca-DTPA dose, 1.5 minutes after IV contamination with ^{241}Am-citrate, the liver burden was reduced from 43 to 7 % and skeletal burden from 22 to 5 % compared to untreated controls[61]. Increasing the dose of chelating agent up to 300 μmol/kg continues to improve efficacy, however there is a diminishing return. This is due to the chelator levels being in excess of mobilized ^{241}Am even at low doses. The influence of treatment delay on ^{241}Am retention in the liver and skeleton is shown in Figure 1.2. As the time interval between contamination and treatment increases, the effect of DTPA diminishes[61]. The effect of treatment delay is more distinct when the rate of radionuclide translocation is high (americium and curium) and when binding to the target site is strong, such as on the bone surface[67]. The effect is not as pronounced for liver disposition as the actinide remains available for a longer period of time as demonstrated by the removal of americium from the liver with DTPA in both beagles[68] and man[69].

In beagles contaminated by IV administration of ^{241}Am-citrate, a single 30 μmol/kg IV

Ca-DTPA dose administered 30 minutes after contamination resulted in significant increases in elimination. After seven days, urinary elimination was increased from 10 to 70 % of the total injected dose and fecal elimination from 1.3 to 9.8 %. In addition the liver disposition was significantly reduced from 47 to 9.7 %[70]. The relationship between efficacy and initial dose was established in beagles to justify a 30 μmol/kg human initial dose. Body retention of ^{241}Am was inversely proportional to the dose, with a linear relationship seen over doses up to 300 μmol/kg. The 30 μmol/kg dose human dose was selected as doubling the dose resulted in < 25 % change in total body burden after seven days[71].

In addition to animal studies there have been many cases of accidental exposure to actinides in the workplace, however there are very limited numbers of incidents in which detailed *in vivo* measurements have been recorded[72]. The 1976 Hanford incident resulted in an exposure that was around 1000 times more severe than any previous incident, the patient was contaminated solely with ^{241}Am and the case provides the best human evidence for the extended use of DTPA therapy[73].

The incident occurred at the plutonium finishing plant on the Hanford site in Washington. An ion exchange column containing around 100 g of ^{241}Am exploded in a glove box and small shards of glass, nitric acid and the contents of the column were projected onto a radiation worker, especially his face which was uncovered. The explosion caused the worker to be contaminated with 1-5 Ci of ^{241}Am, of which 6 mCi still remained after de-clothing and initial external decontamination before transport to the hospital. The thermoluminescent dosimetry badge indicated that he had been exposed to 500 mrem (5 mSv) during the explosion[74]. On arrival at the hospital the first dose of Ca-DTPA was administered by i.v. injection (1g over 3-5 min), however it is estimated that due to the high solubility of

$Am(NO_3)_3$ more than 20 and 40 μCi had already deposited in the bone and liver respectively[75].

As the ^{241}Am was slowly absorbed into the blood stream this case was suited for chelation therapy. Over the course of the next 4.5 years the patient received a total of 584 g of DTPA. For the first 25 days up to three 1 g doses were given per day, this was followed by daily injections until day 332 where injections were administered 2-3 times per week. When treatment was suspended for a year, ^{241}Am excretion in the urine dropped and biweekly DTPA therapy was resumed as liver retention was seen to be increasing[69]. In total approximately 900 μCi was eliminated via the urine, 59 % of which was removed in the first 6 days. If left untreated only 10 % of the activity would be expected to be eliminated naturally with the remainder being deposited in the liver and bone[76]. The administration of DTPA ensured that after 5.3 years only 1% of the ^{241}Am that reached the plasma was retained in the bone and liver, demonstrating that in this case DTPA blocked 99 % of the ^{241}Am predicted to deposit in the tissues[69].

Due to the slow absorption of ^{241}Am from the skin to the bloodstream the majority was prevented from depositing in the tissues. The only effects of radiation seen were depressed leukocyte, neutrophil and lymphocyte counts in the blood, which had no observable health effects. Due to the contamination at age 64, the threat of tumor development was low due to the latency period exceeding the patient's life expectancy. In addition after intensive and extended therapy, ^{241}Am deposited within the bone was not effectively removed. Therefore this corroborates the fact that therapy should be initiated as early as possible after exposure.

DTPA toxicity has been investigated in mice, rats, dogs, baboons and humans. The calcium salt of DTPA is more toxic than the zinc salt due to the weaker association of

calcium resulting in increased depletion of natural metals such as zinc and magnesium. When examined *in vivo,* the differences in toxicities were less pronounced in non-dividing cells. In proliferating Chinese hamster cells the Zn-DTPA was non-toxic whereas Ca-DTPA resulted in significant toxicity, suggesting that it interferes with protein or DNA synthesis by altering the composition of the extracellular metal ions of the culture media. In mice, the zinc salt was 2.5 times less toxic than the calcium salt[77]. The LD_{50} values for mice administered a single IP Zn-DTPA dose a is 12.5 mmol/kg (6484 mg/kg)[63], which is 1.4 times higher than that of EDTA. In general DTPA can lead to histopathological lesions in the kidneys and similarly to EDTA can be considered nephrotoxic[78].

The doses at which toxicity was noted during animal studies are significantly higher than the 30 μmol/kg used in humans, and therefore it is unsurprising that in all human cases of prolonged DTPA administration, no severe side effects have been observed. The Atomic Energy and Alternative Energies Commission (CEA) in France has administered 1158 injections to 469 people from 1970 to 2003, with over 70 of these cases involving multiple injections. From all these exposures the one-single adverse event was an allergic skin reaction that resolved with no long-term effect[79]. In addition the Oak Ridge Associated Universities (ORAU) maintain a DTPA registry and between the period of 1958 – 1987 reported that 3077 doses of DTPA were administered to 485 patients, with the temporary loss of some essential elements being the only reported effects[80].

The FDA approval of DTPA was granted as part of a government initiative to increase the number of therapeutic agents available in the Strategic National Stockpile. Although no formal efficacy studies were conducted, extensive animal studies had been conducted and

evidence of enhanced elimination and lack of toxicity obtained during unlicensed use in accidental contamination cases was used to support regulatory approval.

As DTPA is only available for treatment via IV infusion or nebulization, both of which require a health care professional. This is a severe limitation in a mass casualty situation during which a large population of patients may require decorporation therapy, in addition to those who have more acute trauma requiring immediate attention. Treatment of internal contamination is likely to be initially deprioritized as it results in a more chronic risk than the acute risk from trauma injuries. These delays in chelation therapy will limit treatment effectiveness, therefore there is a need to develop formulations that can be self-administered and rapidly distributed. In addition there is likely to be a significant population of "worried well" who, although do not need chelation treatment, may receive a benefit. A safe non-toxic oral formulation that has limited strain on medical resources may therefore help to calm the panic of this population of patients. Overall an oral product is more amenable to a mass casualty situation and will aid in minimizing treatment delays, which as shown previously will enhance decorporation.

Table 1.6 DTPA Binding Constants

Element	Log K_M
Americium	22.9
Berkelium	22.8
Californium	22.6
Curium	23.0
Neptunium	23.6
Plutonium	23.4

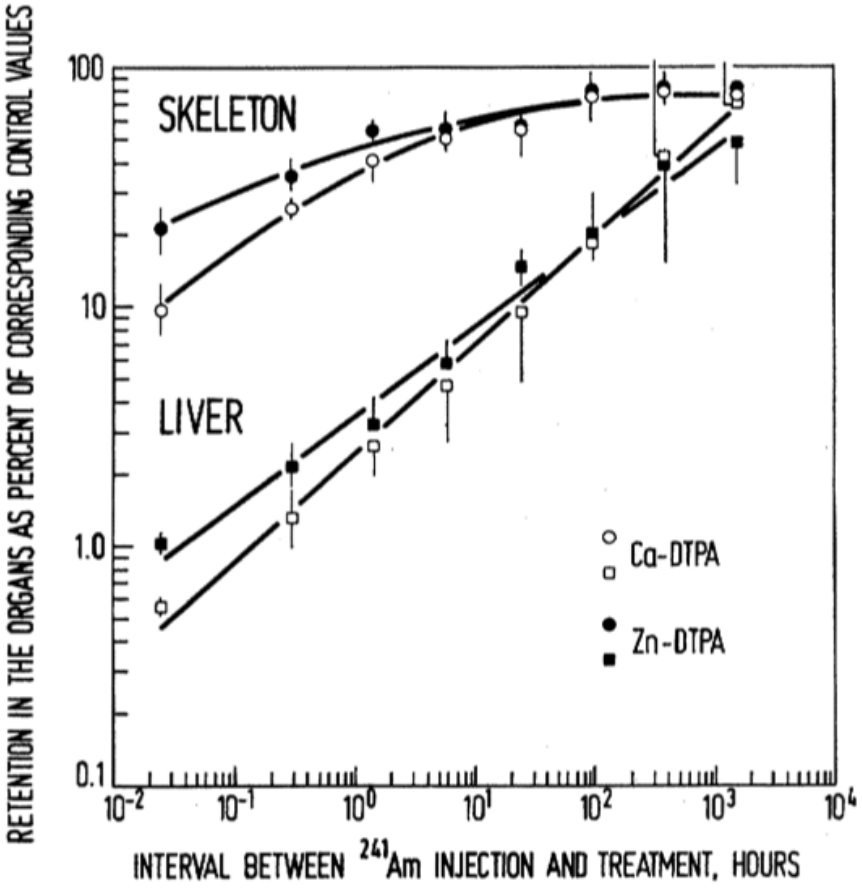

Figure 1.2 Retention of ^{241}Am in rats after various treatment delays between contamination and administration of either Ca- or Zn-DTPA. Tissue concentration was assessed 7 days after DTPA treatment (n = 6)[61].

1.5 Pro-drugs

A drug's bioavailability (F) is the fraction of unchanged drug that reaches the systemic circulation. In the case of an IV this is 100% of the administered dose and therefore its bioavailability is 1. In all other cases of drug delivery, a drug must first overcome a cellular barrier during absorption and only a fraction of the unchanged drug reaches the systemic circulation. The composition of this barrier and the difficulty in achieving permeation varies depending on the delivery route. In order to be absorbed, the drug molecule must first be in solution. Consequently the physiochemical properties of the molecule that affect both solubility and lipophilicity play a critical role in determining its bioavailability.

Although drug absorption is predominately considered to be affected by both the solubility and permeability of the drug molecule, there are additional factors that play an important role in defining the bioavailability. The presence of efflux pumps such as P-gp on the basolateral membrane[81] and first-pass metabolism also contribute to a reduction in the fraction of unchanged drug reaching the circulation.

There are a variety of methods used to improve oral bioavailability, including formation of salts[82], use of novel formulations[83,84,85] and prodrugs[86,87] all of which have been thoroughly reviewed. Prodrugs are biologically inactive versions of a drug that must undergo a chemical transformation in order to release the pharmacologically active drug. They have been used for many years to overcome drug delivery challenges such as poor solubility, poor permeability or rapid metabolism. In the majority of cases a prodrug only requires one or two metabolic steps to yield the active form. The central hypothesis of this dissertation is that synthesis of a DTPA prodrug will enhance the oral bioavailability of DTPA due to both increased lipophilicity and consequential reduction in charge.

Although effective at binding radionuclides in the plasma, the majority of chelators are highly hydrophilic, restricting entry into cells and limiting efficacy. Lipophilic prodrug chelators have previously been investigated in hope of removing radionuclides deposited within the tissues such as the liver and bone. Markley[88] was the first to produce a DTPA pro-drug with the aim of promoting intracellular penetration. Esterification was chosen to allow intracellular hydrolysis, restoration of the carboxylic acids and therefore chelation sites[89]. Markley chose the penta-ethyl ester of DTPA so that toxicity from the liberated alcohol would be minimized. The results indicated that although the penta-ethyl ester was no better than DTPA at removing plutonium from the liver alone, when given in combination with DTPA there was an additive effect suggesting that there were two mechanisms of removal and that the penta-ethyl ester was acting intracellularly.

Although able to act intracellularly the penta-ethyl ester was far more toxic than DTPA, possibly due to chelation of natural metals within the cells[88]. Therefore partially esterified DTPA analogues were investigated for their potential to remove intracellular plutonium[90]. The effect on plutonium deposition in mice was investigated using di-methyl, -ethyl, -butyl and –octyl esters given five days after contamination. None of the esters showed benefit in terms of liver burden, however the methyl and ethyl esters did show a reduction in skeletal burden compared with untreated controls. The study also confirmed that in general the toxicity of esters increased as chain length increased, the exception being that the ethyl ester was less toxic than the methyl ester. All were significantly less toxic than the penta-ethyl ester, which suggests that toxicity is affected primarily by lipophilicity.

More recently the prodrug concept has been applied to the enhancement of oral bioavailability. Triethylenetetraaminehexaacetic acid (TTHA) is a further extension of the

DTPA molecule containing an additional nitrogen and carboxylic acid increasing its coordination number to 10. TTHA molecules have been esterified with chain lengths varying from C_8-C_{22} in order to both improve their oral absorption and traverse cellular barriers. These chelators were designed to increase removal americium or plutonium retained within the tissues and efficacy was assessed in rats contaminated with ^{239}Pu and ^{241}Am by IV injection. The chelators were administered fourteen days after administration of activity to allow deposition in tissues. For 30 days, rats received 80-85 mg of the C_{22}-TTHA ligand orally, DTPA control rats were administered an equimolar concentration of DTPA by S.C. injection twice a week. At completion of the study the activity of ^{239}Pu and ^{241}Am in all tissues and bones was no better than DTPA therapy[91]. Interestingly the prodrug is predominantly excreted via the fecal route, suggesting a biliary excretion pathway, less lipophilic prodrugs however result in a larger fraction of urinary excretion[92]. Preliminary toxicity studies indicated that there was no apparent toxicity following 10 daily 200 mg/kg (200 μmol/kg) doses of the C_{22}-TTHA ligand[93].

Similarly to the TTHA prodrugs, a range of penta-ester substituted DTPA analogues has previously been investigated with the penta-ethyl ester selected due to its lower potential toxicity due to the liberation of ethanol and to keep the molecular weight close to 500 as per lipinski's rules. Although previously studied for removal of intracellular plutonium, the improvement in oral bioavailability remained uninvestigated. The penta-ethyl ester was highly absorbed[94,95] and in rats contaminated with ^{241}Am-Citrate (IV) the elimination of ^{241}Am was doubled compared to untreated controls when a 75 μmol/kg oral dose was administered one hour after contamination. Unfortunately the penta-ethyl ester was not fully metabolized to DTPA in the plasma and the tetra- and di-ethyl esters were the predominant

circulating forms[95]. The increased lipophilicity resulted in efficient membrane transport, which may be a contributing factor to the high toxicity. Although successful the oral penta-ethyl ester research ceased due to a complex metabolic profile involving ten potential metabolites and due to the high toxicity. The penta-ethyl ester prodrug has also been examined topically with the aim of establishing sustained delivery of chelator to eliminate the continual release of radionuclide from the contamination site[96]. Topical administration and subsequent metabolism by skin esterases was also hypothesized to limit the fraction of lipophilic metabolites reaching the plasma resulting in reduced toxicity and improved efficacy. In rats contaminated with Am-nitrate (IM) and treated with a 1120 μmol/kg topical gel applied over a 6 cm^2 area 24 hours after contamination, statistically significant increases in elimination of ^{241}Am were observed[97]. Liver and skeletal burdens were also reduced compared to untreated control animals, demonstrating that the transdermal route may be a feasible option for chelation therapies.

Though there has been much research into alternatives for DTPA over the past fifty years there are still no new products in medical use. This may have been due to the lack of funding for a product with a limited patient population and the lack of animal rule pathway for regulatory approval.

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1.6 Approval of Medical Countermeasures

In late 2001 the National Institutes of Health (NIH) and National Institute of Allergy and Infectious Disease (NIAID) began to formulate a comprehensive biodefense research program, which culminated with the publication of a strategic plan for medical countermeasures against radiological and nuclear threats in 2005. One of the aims outlined in this plan was to promote research of new decorporation products that can be easily administered, safe for repeated dosing, have a long shelf life and be easy to manufacture[98]. In addition to the creation of a biodefense research program, the FDA introduced a new pathway for drug approval to accelerate the development of medical countermeasures (MCM) known as the "Animal Rule"[99]. Prior to the introduction of this rule there was no pathway for approval for these MCMs where the patient population is too small to conduct well-controlled clinical trials and it is unethical to contaminate healthy individuals in order to determine the efficacy.

In order to gain regulatory approval via the animal rule the product must meet the following criteria[100]:

1. A detailed understanding exists regarding the pathophysiological mechanism for toxicity of the agent in humans and also regarding the mechanism by which the proposed product reduces or prevents adverse effects in humans.
2. The prophylactic or therapeutic effect of the product is demonstrated in more than one animal species, unless the effect is demonstrated in a single animal species that represents a sufficiently well characterized animal model for predicting human response.
3. The animal study end points correlate to increased survivability or prevention of significant morbidity in humans in the face of lethal challenge.

4. Pharmacokinetic and pharmacodynamics data for the proposed product are available and can be used to select an effective dose in humans.

For radionuclides the mechanism of toxicity is established and extensively detailed in published data. The kinetics of americium and plutonium distribution have predominantly been investigated in rats and dogs. Not only is the choice of animal important but the route of contamination must also be considered, as the animal model must mimic the effects of contamination in humans. In addition to selecting the most appropriate contamination models, the timing of treatment administration should mimic that which will be used in humans. A single model is unlikely to simulate the human response and two species can help to increase the confidence in the animal models response[101]. The development of a pharmacokinetic-pharmacodynamic model will help to identify the dose range likely to be effective in humans.

Currently five products have received FDA approval through the animal rule. The first two products to be approved, pyridostigmine bromide (PB) and Cyanokit, both contained drugs that had previously received approval for alternative uses. PB was approved in 2003, only seven months after the introduction of the animal rule, for use by the army for prophylaxis against soman toxicity[99]. Cyanokit contains hydroxocobalamin and is used as an antidote for cyanide poisoning. It was approved in 2006, based on a single placebo-controlled study in dogs[102]. There was a six-year lull before the approval of additional products, though three products achieved approval during 2012-13, namely Levaquin, raxibacumab and botulism antitoxin. Interestingly raxibacumab was the first completely novel product to gain approval solely via the animal rule, demonstrating the changing regulatory environment over the last decade[103] and is promising for the future approval of novel medical countermeasures.

1.7 Perspective

In response to the NIH/NIAID call for improvements of DTPA formulation, three research groups received funding, including SRI International (Menlo Park, CA), Nanotherapeutics Incorporated (Alachua, FL) and the lab of Dr. Jay at the University of Kentucky[51]. SRI has developed orally bioavailable formulations of DTPA by incorporating permeation enhancers into an oral capsule. Various permeation enhancers have been evaluated *in vitro* and the oral absorption of DTPA has been shown to increase from 5 to 12 % in rats[*104*]. The toxicity and efficacy of this novel oral formulation has also been investigated. The Nanotherapeutics approach involves the development of an orally administered nanoparticle formulation. Preclinical studies of this oral 'NanoDTPA™ capsule formulation have demonstrated good pharmacokinetic and safety profiles in rodent and dog models[105] and the product received additional funding from Biomedical Advanced Research and Development Authority (BARDA)[106]. For a more detailed discussion on the results of these studies in relation to the current work the reader is referred to chapter VII.

The research presented herein is an extension of the DTPA prodrug research initiated at the University of Kentucky. As described previously the research in Dr. Jay's lab has focused on two novel DTPA products. Both the oral and topical formulations were based on the penta-ethyl ester of DTPA, named C2E5. C2E5 was shown to effectively decorporate ^{241}Am in a wound-contamination animal model[94-95], but its unfavorable pharmaceutical properties and concerns related to its hepatotoxicity caused us to focus our efforts on the di-ethyl ester of DTPA, named C2E2.

1.8 Specific Aims

Specific Aim 1: To synthesize and characterize the di-ethyl ester pro-drug of DTPA, C2E2, and its major metabolite the mono-ethyl ester C2E1. In order to study C2E2 as a potential DTPA pro-drug and to determine its pharmacokinetics, the compounds of interest must be synthesized. Both C2E2 and its primary metabolite C2E1 have previously been described in the literature, however their use as an oral pro-drug of DTPA is novel. Once synthesized the compounds will be characterized by NMR, MS, HPLC, DSC, XRPD, elemental analysis and water content to determine their purity. The majority of active pharmaceutical ingredients (APIs) are administered as a solid dosage form. Regulatory agencies place a heavy emphasis on safety and efficacy; therefore the chemical purity of solids is often the primary focus. However the physical aspects of the dosage form such as stability are equally important. The measurement of pKa, solubility and lipophilicity are an integral part of lead compound profiling. Properties to be investigated include solubility, pKa, stability constants, Log P and metal binding kinetics.

Specific Aim 2: To investigate the metabolism of C2E2 *in vitro.* The stability of C2E2 will be determined in simulated intestinal and gastric fluids to ensure the esters are not hydrolyzed prior to absorption. Plasma binding, and stability will be determined using human, beagle dog and rat plasma. The metabolism of C2E2 by human, beagle dog and Sprague-Dawley rat S9 fractions will be evaluated to determine the conversion of C2E2 to it's metabolites C2E1 and DTPA.

Specific Aim 3: Determine the pharmacokinetics and decorporation efficiency of C2E2, C2E1 and DTPA *in vivo* using a Sprague-Dawley rat and Beagle Dog models. The pharmacokinetics of C2E2 and its metabolites C2E1 and DTPA will be determined in both

Sprague-Dawley rat and Beagle dog models. The parameters obtained such as the C_{Max} and AUC can be incorporated into a pharmacokinetic model. The bioavailability and decorporation efficiency of C2E2 will also be determined. Tissue accumulation will also be performed.

The successful completion of these specific aims is expected to lead to a greater understanding of the efficacy and pharmacokinetics of C2E2 which, combined can assist in the development of a physiologically based pharmacokinetic model for the oral decorporation efficacy of C2E2**.** Due to the complexity of the dual pro-drug and the fact that C2E2 and C2E1 in addition to DTPA are expected to have some metal chelating activity, it would be helpful to predict the therapeutic outcome before large scale lengthy and expensive pre-clinical and clinical trials are performed. A pharmacokinetic-pharmacodynamic model combines a model that describes drug concentration vs time (PK) with a model describing the relationship of effect vs concentration and a statistical model describing intra and inter individual variability of the PK/PD models. The parameters investigated above can be incorporated into a pharmacokinetic-pharmacodynamic model. The *in vitro* species dependent metabolism can then potentially be used to predict the human metabolism based on interspecies scaling calculations. Binding constants for C2E2 and its metabolites can be incorporated into the model in order to correlate the exposure of each compound with the observed effect. The ultimate aim is to produce a model that is capable of predicting the human dose required to produce the desired decorporation as human efficacy studies are not possible for decorporation agents.

CHAPTER II

C2E2: A DI-ETHYL ESTER PRODRUG OF DTPA AS AN ORALLY BIOAVAILABLE RADIONUCLIDE DECORPORATION AGENT

2.1. Introduction

Diethylenetriaminepentaacetic acid (DTPA) is a chelating agent with eight coordination sites making it effective at binding a wide range of metals with high affinity. It is available as both calcium and zinc tri-sodium salts (Ca-/Zn-DTPA) for intravenous injection both of which have been approved by the FDA for treatment of individuals internally contaminated with isotopes of the transuranic elements of americium (Am), curium (Cm) and plutonium (Pu)[58]. Once administered DTPA is rapidly distributed to the extracellular fluids where the calcium or zinc is exchanged for the higher affinity radioactive metals enhancing their elimination via urinary excretion.

Americium, curium and plutonium are α-emitters which rapidly deposit on the bone surface where they irradiate the radiosensitive bone marrow causing increase the risk of cancer and leukemia in a dose dependent manner[107]. If left untreated the biological half-lives of radionuclides can be up to 50 years[28b]. DTPA is relatively ineffective at removing skeletally deposited radionuclides and therefore is most effective when given soon after

exposure while the metal is in the circulation and soft tissue[43]. Any delay in treatment has a profound effect on the retention of americium (^{241}Am). In beagles contaminated with IV ^{241}Am citrate and treated with a single dose of IV Ca-DTPA, the percentage of ^{241}Am retained increased from 3% after a 1 minute delay to 29, 58 and 73% after 30 minutes, 8 hours and 1 day delays, respectively[108]. Similar effects have been shown with both plutonium and curium. The radionuclide deposition kinetics are dependent on the method of contamination, species, and chemical form of the heavy metal[33]. Contamination is most likely to occur via inhalation or wound sites and therefore the percentage decorporation achieved after a single dose is likely to be lower than that described above due to retention of the metal in the lung or wound site. For this reason it is not uncommon for DTPA to be given over a period of days or months and in extreme cases for years[73]. The efficacy of DTPA in humans has been investigated after unintentional contaminations such as during the Hanford Americium Accident[109]. Due to the increased threat of nuclear terrorism both forms of DTPA have been added to the United States Strategic National Stockpile as radiological countermeasures.

DTPA is highly ionized and therefore poorly absorbed (~5%) [110], thus it is usually given by intravenous injection or infusion. Following inhalation exposure the solution may be given via a nebulizer, however this also is more expensive and requires specialized equipment. An orally administered DTPA product would offer an advantage over the current treatment. Health care professionals are required for administration of the IV product which is time consuming and costly. This is a limitation during emergency situations such as the release of radioactivity from a nuclear power (Fukushima) and waste facilities, or by terrorists using a radiological dispersal device or improvised nuclear device (“dirty bomb”)

where there may be mass contamination and casualties. During an emergency scenario delivery of oral dosage forms of DTPA would enable rapid distribution and cost-effective treatment saving resources for patients who need more urgent care. The ease of administration should also help to decrease the delay in receiving the first dose thereby improving decorporation and reducing the length of treatment required. In addition those who are severely contaminated may need months of treatment. Daily IV injections will reduce the patient's quality of life, which would not be the case with an oral product.

Prodrugs have frequently been used for bioavailability enhancement[111], with ester groups frequently being employed to add lipophilic moieties or remove charge. The ideal prodrug would retain high solubility, enhance permeability, and be stable in the GI tract prior to absorption and subsequently metabolize to DTPA. The five carboxylic acids present on DTPA provide multiple sites for esterification. We therefore hypothesized that by reducing the net charge of DTPA through formation of esters using these free acid groups and concurrently increasing lipophilicity, the permeability limited absorption could be overcome. The use of ester prodrugs should allow subsequent metabolism in the plasma due to pH or esterases yielding DTPA. Ethyl groups were chosen to keep the molecular weight of the prodrug below 500 as per Lipinski's rules[112]. In addition cleavage of larger aliphatic alcohols are generally more toxic[113], whereas shorter chains such as ethanol although less hydrophobic also have less toxicity. The di-ester prodrug was chosen as previous studies in our lab with the penta-ethyl ester although exhibiting good efficacy had shown incomplete metabolism to DTPA with the majority of the metabolites remaining as the tetra-ethyl ester[95].

The following experiments were conducted to examine the physiochemical properties of the di-ethyl ester prodrug of DTPA in relation to oral delivery. In addition a preliminary

efficacy study has been performed to evaluate the prospect of DTPA di-ethyl ester as an oral decorporation agent. These studies will be used to identify suitable formulations based on both the physiochemical properties determined and the doses required to achieve decorporation.

2.2. Experimental Methods

2.2.1 Synthesis & Identification

The synthesis of C2E2 is a simple two-step procedure as depicted in Figure 2.1. Initially, diethylenetriaminepentaacetic bisanhydride (DTPA-BA) was synthesized in a method similar to that used by Raut et al.[114]. DTPA (3.93 g, 10 mmol) and acetic anhydride (5.72 g, 56.9 mmol) were added to 6.2 mL of pyridine and heated to reflux 65-70^{o}C for 14 hours. The resulting material was filtered through a Buchner funnel and rinsed with diethyl ether. An off-white powder (DTPA-BA, 1) was collected and dried overnight. The di-ethyl ester of DTPA (2, C2E2) was synthesized by reacting DTPA-BA (2.0 g, 5.6 mmol) with ethanol (0.97 ml 0.77 g, 16.7 mmol) in pyridine (1.33 ml 1.32 g, 16.7 mmol). The reaction was stirred under nitrogen at RT for 24 hours, the product precipitated in DCM (200 mL) at -40^{o}C, filtered and dried to give an off-white powder. The identity of the product was confirmed to be the di-ethyl ester by ^{1}H NMR, mass spectrometry (MS) and elemental analysis. ^{1}H NMR was performed using a Varian 400 MR NMR spectrometer (Agilent Technologies, Santa Clara, CA) operating at 400 MHz. MS was performed on a TSQ Quantum (Thermo Scientific, Waltham, MA) by direct injection of the C2E2 dissolved in acetonitrile. Electrospray ionization with nitrogen gas was used and the ion trap mass analyzer was operated in positive-ion scanning mode. Elemental analysis was performed for C, H, N and O elements by combustion and pyrolysis at ALS Environmental (Tuscon, AZ). A 25-minute HPLC method was developed to quantify C2E2 during pre-formulation experiments. Analysis was performed on a prominence HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with a Corona Ultra charged anion detector (CAD) (Thermo

scientific, Sunnyvale, CA). A reverse phase gradient separation was performed using an Alltima C18 column 250 x 2.1 mm^2 internal diameter with 5 μm particle size (Grace) at 40 ^oC and a flow rate of 0.25 mL/min. The mobile phases were composed of water with 0.1% trifluoroacetic acid (A), acetonitrile/isopropanol 2:1 (B). The mobile phase followed a linear gradient from 94:6 to 75:25 over 14 min, the gradient then increased for 0.5 min to achieve a flow of 0:100 for 3.5 minutes followed by re-equilibration of the system at 94:6 for 6 min. The CAD analysis was performed at 25 ^oC with nitrogen flow at 35.1 psi.

2.2.2 Characterization of Solid C2E2

Two thermal methods were used to characterize the DTPA di-ethyl ester. Differential scanning calorimetry (DSC) was performed using a Perkin Elmer heat flux DSC (Waltham, MA). This enabled determination of the melting point and can was also used to detect the presence of solvate species. Briefly a few mg of solid powder was weighed into aluminum pans placed in the DSC and heated to 145^oC at 5^oC/min followed by a 1 min hold at 145^oC before cooling. Thermogravimetric analysis (TGA) was also performed using a Q5000 Thermogravimetric Analyzer (TA Instruments, New Castle, DE). Briefly a few mg of solid was weighed into open aluminum pans placed on the TGA and was heated from 25 to 100^oC at a rate of 10^oC/min. An isothermal step was introduced at 100^oC for 5 min to ensure all water loss had occurred, and then the temperature was further increased at 10^oC/min to 170 ^oC. The experiment was stopped at 170 ^oC to avoid decomposition. In addition X-Ray powder diffraction was used to further characterize the solid and determine crystallinity. A Cu Kα radiation with a wavelength of 1.5405 Å at 45 kV and 40 mA from an X'Pert PRO diffractometer (PANalytical, Almelo, The Netherlands) was used. Samples were placed on

zero-background silicon plates and scanned from 2-50° 2θ with a step size of 0.016 and a time of 10 s per step.

2.2.3 Solubility

The apparent solubility of C2E2 was determined in deionized water. C2E2 was added to the water in excess and left to stir, at various time-points a sample of supernatant was taken and filtered to remove undissolved solid. The concentration of the supernatant was then determined by HPLC-CAD. The solubility was measured until two concurrent readings were achieved and the experiment repeated in triplicate.

2.2.4 Lipophilicity

The pH-dependent partition coefficients were calculated by measuring the partition of C2E2 between 1-octonol and aqueous buffers. Phosphate (pH 2, 3 and 7), Acetate (pH 4 and 5), Succinate (pH 6), TRIS (pH 8 and 9) were prepared at 200mM concentrations (I=0.6, NaCl). Initial experiments were performed to determine the optimum octanol-aqueous buffer ratio of 10:1. Prior to combining the phases octanol solutions were pre-saturated with the corresponding aqueous buffer overnight. After the phases were combined, they were gently agitated at room temperature for 24 hours using a rotisserie-style shaker and upon completion were separated by centrifugation and the C2E2 content of aliquots from each phase was measured by HPLC-CAD with all samples were analyzed in triplicate.

2.2.5 Stability

The stability of C2E2 was investigated in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The simulated fluids were prepared as outlined in the United States Pharmacopeia using pepsin and pancreatin (Acros organics). C2E2 was dissolved in pre-heated simulated fluids, and the reaction mixture retained at 37.5^{o}C for the duration of the experiment. At various time-points and aliquot was taken and the concentration of C2E2 was determined by HPLC-CAD. In addition C2E2 capsules were evaluated under accelerated stability conditions at both 25^{o}C/65%RH and 40^{o}C/75%RH. Size 0 HPMC capsules were filled with 200 mg C2E2 and placed in HDPE bottles. At 0, 1, 2, 3 and 6 months the dissolution performance of the capsules was evaluated. The C2E2 content of the capsules was measured at each of the aforementioned time-points and at 12 months. Dissolution was performed in a using dissolution bath (Hansen) equipped with USP II paddle apparatus. Capsules were placed in 900 mL of 0.1N HCl solution with constant stirring at 50 rpm with all samples were analyzed in triplicate.

2.2.6 Single Dose ^{241}Am Decorporation

In order to evaluate the potential of C2E2 as an oral decorporation agent, an efficacy study was performed in rats contaminated with ^{241}Am. Male and female Sprague Dawley rats (32/gender) were contaminated with $^{241}Am(NO_3)_3$ (0.25 μCi) by intramuscular injection (0.1 mL, 0.1 M HNO_3) into the right hind leg while under isoflurane anesthesia. Immediately after injection, body weights were recorded and animals were placed in individual metabolic cages. Rats (n = 4/gender/group) received a single oral gavage dose of 0, 200, 600, or 1000 mg/kg C2E2 (10% w/w solution in water) 24 hours after contamination. The study was

performed under both fed and fasted conditions to determine if there were any food effects. Approximately 16 hours before administration of C2E2, rats assigned to the fasted groups had access to food restricted with replacement occurring 1 hour after oral gavage treatment. At all other times all animals had *ad libitum* access to food. A treatment control group received a single intravenous dose of Ca-DTPA solution (13.3 mg/kg, 0.67 mL/kg) via a jugular vein catheter 24 hours after contamination under fed conditions. All animals were observed at least once daily for morbidity, mortality, and general appearance.

Excreted urine and feces were collected daily. Feces were allowed to dry overnight before transfer to 20 mL scintillation vials and weighing. Following necropsy selected tissues were removed and weighed. The ^{241}Am gamma activity was determined by quantifying the 59.7 keV photons emitted by ^{241}Am using a gamma counter (Wizard2 2480, Perkin Elmer). The total amount of ^{241}Am administered to the animals was determined by counting duplicate aliquots (100 µL) of the injection solution. A counting window from 40 – 80 keV and a 60-second counting time were used for acquisition and each reading was corrected for background at acquisition. All experimental tissues and samples were counted using the same gamma counter and protocol. For all samples, ^{241}Am content was expressed as a percentage of the initial dose. The femur from the leg opposite to the injection site was scaled by a factor of 20 to estimate total skeletal ^{241}Am burden.

Statistical analysis was performed for final decorporation parameters; total decorporation, liver burden, wound retention and estimated skeletal burden. All animals treated with C2E2 were analyzed by ANOVA to evaluate the effects of gender, dose and fed state. Non-contributing effects were removed from the model and control data (untreated and Ca-DTPA treatment groups) were included for subsequent analysis. ANOVA was performed

and Least squares means calculated for all the groups in this model. Comparison of means was made using the Tukey-Kramer adjustment for multiple comparisons, with $p < 0.05$ considered significant.

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 The University of North Carolina at Chapel Hill and were performed in AAALAC accredited facilities.

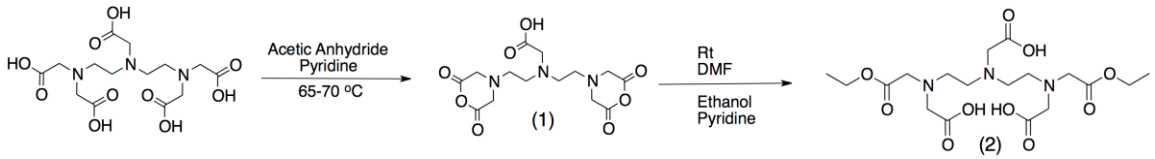


Figure 2.1 Synthesis of DTPA di-ethyl ester (C2E2).

2.3. Results

2.3.1 Synthesis & Identification

The synthesis of DTPA-BA (**1**) resulted in a cream colored powder. Yield = 50 %; ^{1}HNMR (400MHz, d6 DMSO) δ 3.65 (8H, s), 3.44 (2H, d), 2.74 (4H, t), 2.59 (4H, t). Addition of ethanol in the presence of pyridine yielded the DTPA diethyl ester (**2**), named C2E2. Yield =95.6 %; ^{1}HNMR (400MHz, d6 DMSO) δ 4.07 (4H, q), 3.53 (4H, s), 3.45 (2H, s), 3.43 (4H, s), 2.89 (4H, d), 2.84 (4H, d), 1.19 (6H, t). MS, *m/z* calculated 450.2; found 450.13. Elemental analysis: Predicted for $C_{18}H_{31}N_3O_{10}$ – C, 48.10; H, 6.95; N, 9.35; O, 35.60; Actual – C, 47.59; H, 7.22; N, 8.67; O, 36.29.

2.3.2 Solid Characterization

DSC identified a single-phase transition for C2E2 at 115.8^{o}C (Figure 2.2). The use of TGA confirmed that the transition observed during DSC studies was the melting point. There was a small loss in weight observed during TGA analysis, prior to decomposition (Figure 2.2). The loss was gradual, not attributed to a specific temperature, and non-stoichiometric suggesting that surface/incorporated water was the cause. The XRPD chromatogram was obtained and confirmed that the synthesized C2E2 was crystalline (Figure 2.3).

2.3.3 Solubility

The apparent solubility of C2E2 in water was measured after 2, 4, 6 and 24 hours at room temperature. There was no change in concentration observed after 4 hours. The apparent solubility was 149.3 ± 1.9 mg/mL at pH 2.4. In order to determine a pH-dependent

solubility profile a range of buffers were prepared however, due to the high solubility of C2E2 the buffer capacities were exceeded even at high buffer concentrations and so the pH dependent solubility profile could not be determined.

2.3.4 Lipophilicity

The lipophilicity of C2E2 was determined by its partitioning between aqueous buffers of pH 2-9 and 1-octanol. The log D profile in Figure 2.4 shows that lipophilicity increases with decreasing pH. The Log D values ranged from -2.1 to -3.8. This is expected for C2E2, the three acids and bases have an isoelectric point at pH 2.74, therefore the highest lipophilicity would be expected at this point.

2.3.5 Stability

In order to ensure that the ethyl-ester promoeities were not hydrolyzed prior to absorption their stability was determined in both simulated gastric (SGF) and intestinal fluids (SIF). Over a 24-hour period there was a 34 % reduction in C2E2 concentration in SGF and only a 5 % loss in SIF (Figure 2.5). Therefore, with gastric emptying occurring within 2 hours in the average adult[115], only a small fraction of the dose (~5 %) is expected to be lost to hydrolysis prior to absorption.

The stability of HPMC capsules containing C2E2 was assessed over 12 months under accelerated stability conditions. In addition to measuring the content of C2E2, dissolution performance was also monitored for the first 6 months. Under both 25 ^{o}C /65% RH and 40 ^{o}C /75 %RH conditions there was no loss of C2E2 over the 12 months. In addition there were no significant changes observed in the dissolution profiles of capsules stored under either

condition, with > 90 % release achieved in 15 minutes for all capsules tested (Figure 2.6), meeting FDA immediate release acceptance criteria under all the conditions tested[115].

2.3.6 Single Dose ^{241}Am Decorporation

Sixty-four rats completed the study, of these 61 rats (32 male and 29 female) were included in data analysis. Three fasted female rats (1x 600 mg/kg; 2x 1000 mg/kg) were excluded from the dataset as they did not receive a complete dose. C2E2 enhanced the elimination of americium in a dose dependent manner in male and female rats (Figure 2.7). Analysis of the total decorporation achieved in the seven days after contamination for all the rats treated with C2E2 showed significant effects for gender ($F_{(1,44)}$= 17.54, $p < 0.001$) and C2E2 dose ($F_{(2,44)}$= 40.71, $p < 0.001$) but not for the fed state at treatment ($F_{(1,44)}$=1.51, $p = 0.23$). Interactions between food and other main effects were not considered significant and therefore, for subsequent analysis *ad libitum* and fasted groups were combined.

In male rats the total decorporation was significantly increased compared to untreated controls at doses of 600 mg/kg and above ($p < 0.001$) and increasing the dose from 600 mg/kg to 1000 mg/kg also significantly increased decorporation ($p < 0.05$). A similar trend was observed in female rats with doses ≥ 600 mg/kg inducing significantly enhanced decorporation ($p < 0.001$), the 1000 mg/kg dose appears to be more effective than the 600 mg/kg dose although this did not reach statistical significance.

In both male and female rats, C2E2 reduced the americium burden in liver, skeleton, and at the wound site. In male rats, the liver burden was significantly reduced compared to untreated controls at doses of 200 mg/kg ($p < 0.05$) and 1000 mg/kg ($p < 0.001$). Although the liver burden following a 600 mg/kg C2E2 dose did not reach statistical significance (p =

0.056) it is consistent with the trend of C2E2 treatment reducing liver burden. A dose-dependent reduction in ^{241}Am liver burden was observed in female rats although, only the reduction following the 1000 mg/kg dose was statistically significant compared to untreated controls ($p < 0.01$).

Estimated skeletal americium burden in male rats decreased in a C2E2 dose dependent manner with significant reduction compared to untreated control animals at doses of 600 mg/kg and above. Skeletal burden in female rats was lower than in male rats for all groups though was not significantly changed by C2E2 treatment (Figure 2.8).

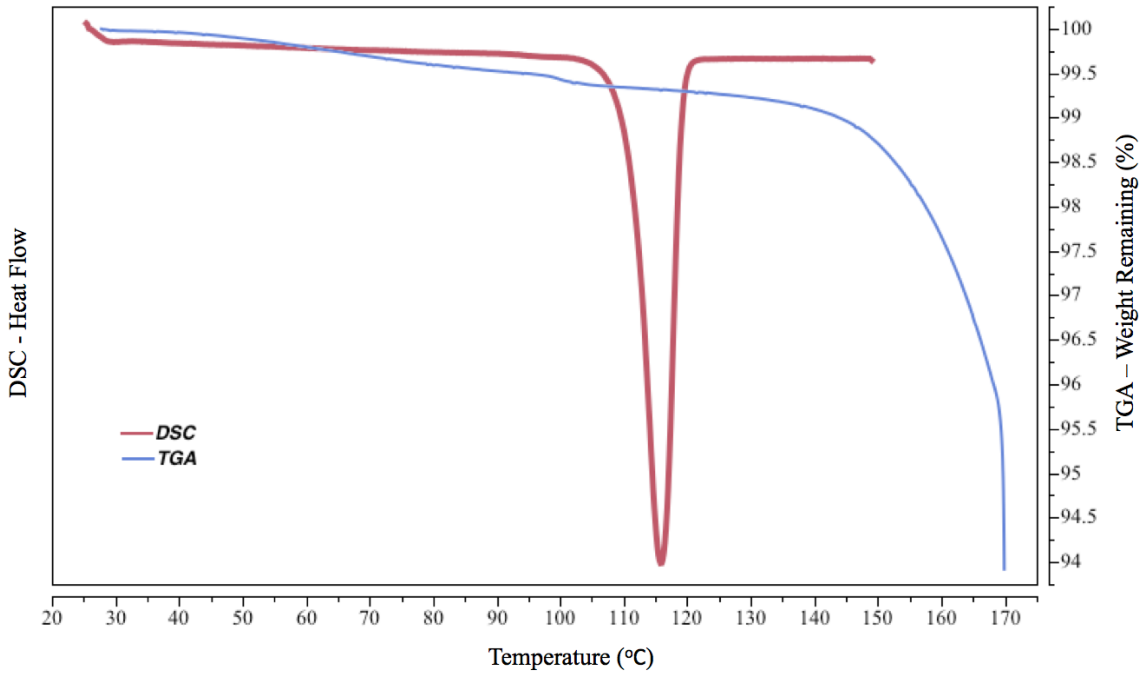


Figure 2.2 Differential scanning calorimetry (DSC, Red) and thermogravimetic analysis (TGA, Blue) chromatograms of C2E2.

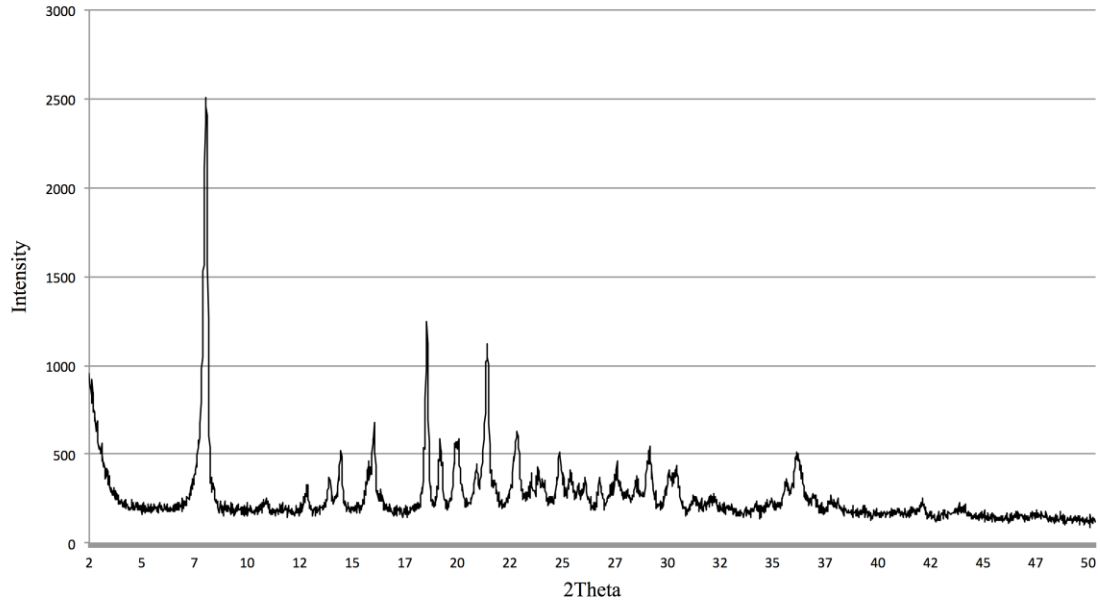


Figure 2.3 X-Ray powder diffraction (XRPD) chromatograph depicting crystalline nature of C2E2.

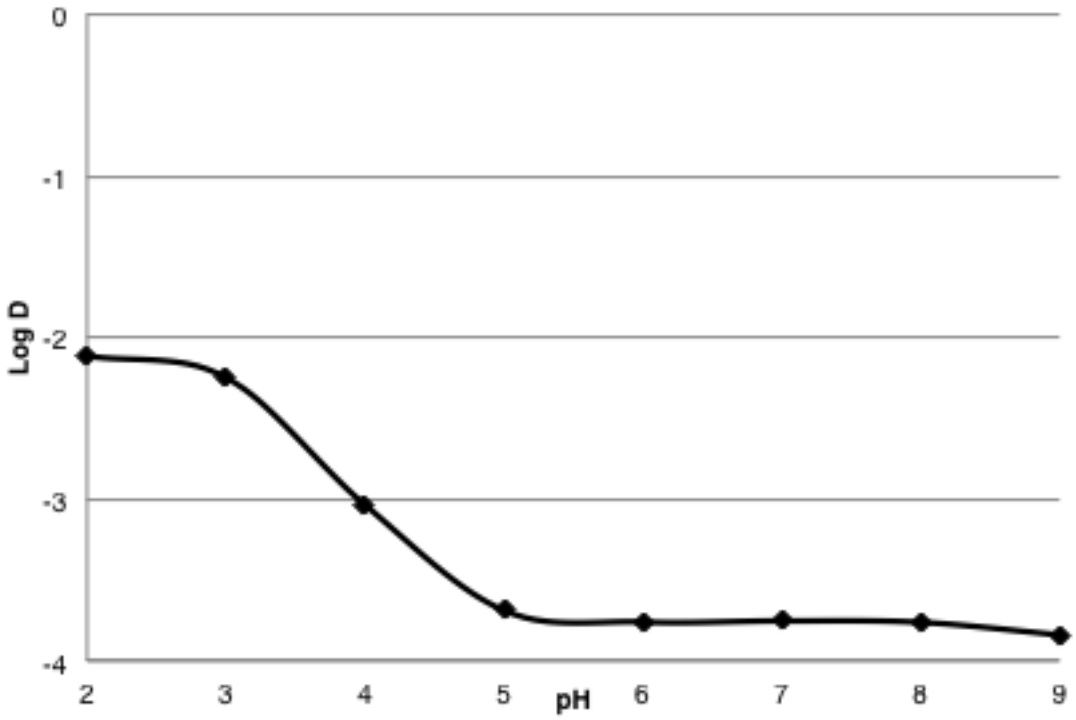


Figure 2.4 Log D profile for C2E2.

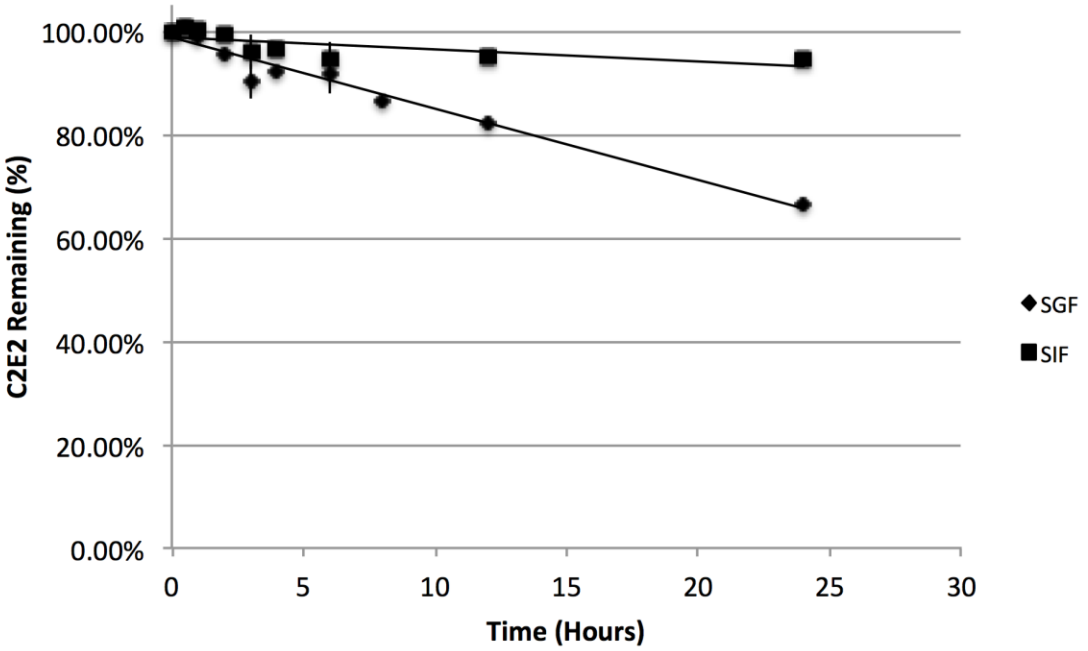


Figure 2.5 Stability of C2E2 in simulated gastric and intestinal fluids.

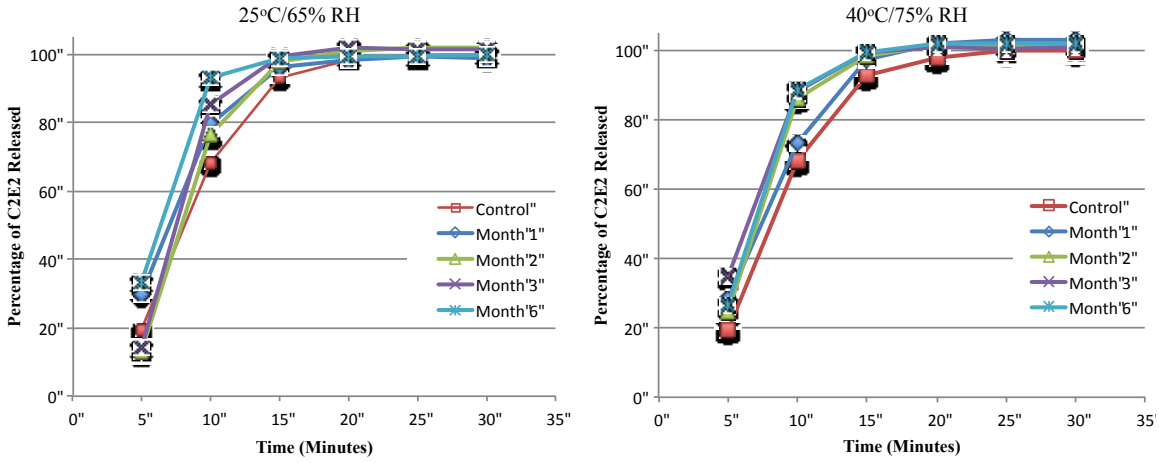


Figure 2.6 Dissolution profiles of HPMC C2E2 capsules after storage under accelerated stability conditions for six months.

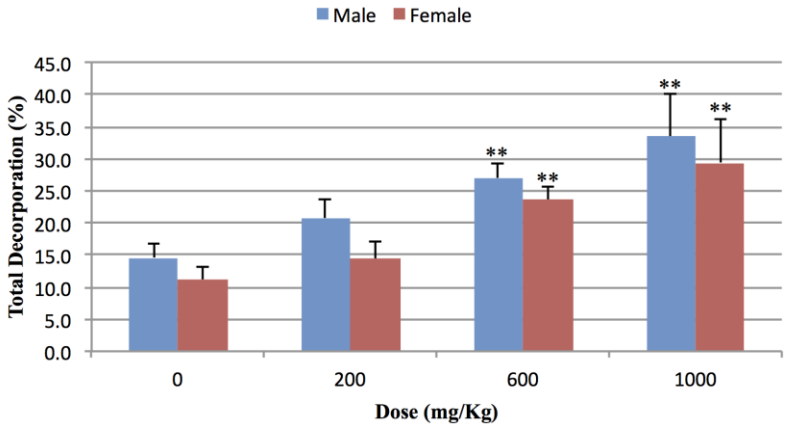


Figure 2.7 C2E2 dose response curve showing total ^{241}Am decorporation in male and female Sprague-Dawley rats seven days after contamination.

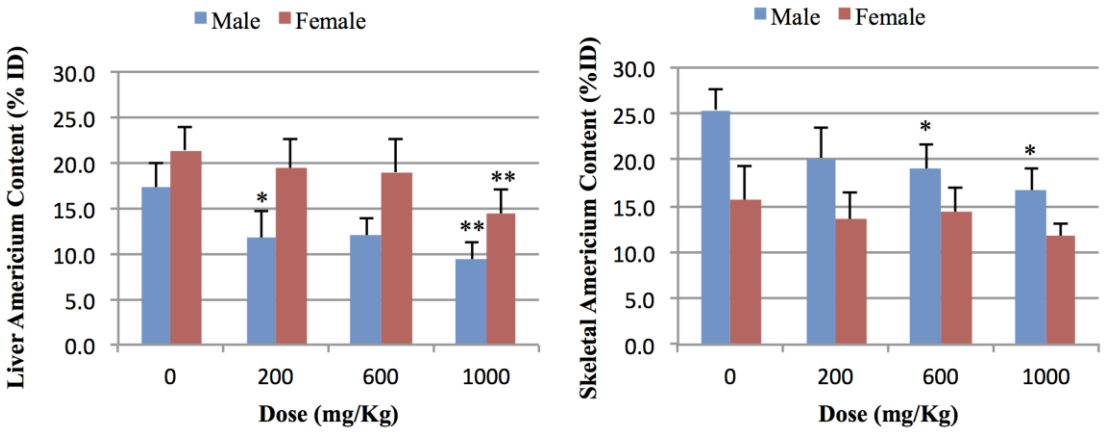


Figure 2.8 C2E2 dose response curve showing ^{241}Am liver (left) and skeletal (right) burdens in male and female Sprague-Dawley rats seven days after contamination.

2.4. Discussion

An oral product provides significant advantages in the case of decorporation therapy. The ability to rapidly distribute and administer the product soon after contamination will minimize deposition of radionuclides in the skeleton and liver from which they are hard to remove[61]. Additionally long term decorporation therapy may be prescribed for highly contaminated patients for whom the oral route is more acceptable and far less disrupting. Finally, after IV administration DTPA is rapidly eliminated with 90 % of the dose being cleared within the first 2 hours[116]. Oral dosing provides further therapeutic benefit by extending exposure due to the extended duration of absorption.

Lipophilic prodrug chelators have previously been investigated in hope of removing radionuclides deposited within the tissues such as the liver and bone. Markley[88] was the first to produce a DTPA pentaethyl ester pro-drug with the aim of promoting intracellular penetration. Although able to act intracellularly the pentaethyl ester was far more toxic that DTPA, possibly due to chelation of natural metals within the cells[88]. More recently the prodrug concept has been investigated for enhancing oral bioavailability. The pentaethyl ester was highly absorbed[94,95] and in rats contaminated with ^{241}Am-Citrate (IV) the elimination of ^{241}Am was doubled compared to untreated controls when a 75 μmol/kg oral dose was administered one hour after contamination. Unfortunately the pentaethyl ester was not fully metabolized to DTPA in plasma and the tetra- and di-ethyl esters were the predominant circulating forms[95]. The increased lipophilicity resulted in efficient membrane transport, which may have been a contributing factor to the high toxicity. Although successful the oral pentaethyl ester research ceased due to a complex metabolic profile involving ten potential metabolites and due to the high toxicity.

C2E2 was chosen as an alternative to the penta-ethyl ester due to its simpler metabolic profile, ease of synthesis and solid form. It is an amphoteric molecule containing three carboxylic acids and three basic tertiary amines. As with most amphoteric molecules C2E2 will be charged across the physiological pH range. The presence of charge on the zwitterions means that the solubility of C2E2 is high however they correspondingly contribute to reduced oral absorption potential. The biopharmaceutics classification system (BCS) is used by the FDA to classify drugs based on their solubility and permeability. The high aqueous solubility and low permeability of C2E2, brands the molecule as BCS class III. This is similar to DTPA, however the permeability (Log P) has been increased from -2.90 (Clog P) to -2.1 through the addition of two ethyl esters[117]. Formulations containing permeation enhancers or efflux blockers have previously been used to further enhance the permeation of class III compounds[118]. A novel formulation of DTPA containing permeation enhancers is under investigation by SRI International (Menlo Park, CA) with oral bioavailability increased from 5 to 12 % in rats[104]. Permeation enhancers may not be appropriate for decorporation therapy as absorption of inhaled radionuclides deposited in the GI tract through muccocillary clearance may increase. Furthermore, high doses of radiation can damage the intestinal mucosa that would be further exacerbated by permeation enhancers.

To assess the oral potential of the prodrug a preliminary decorporation study was performed. The study demonstrated that compared to untreated controls ^{241}Am retention could be significantly reduced orally using C2E2. There was a dose dependent increase in the percentage of ^{241}Am eliminated primarily through the urinary route. This is consistent with other highly charged hydrophilic compounds such as DTPA and contrary to the more

hydrophilic polyaminocarboxylic acids (PACA), which are primarily eliminated through the fecal route[92].

For all of the tissues no statistical difference in americium burden was observed between rats with *ad libitum* access to food and rats fasted overnight prior to C2E2 treatment. This may be due to the fact that over the pH range 5-9 the net charge on C2E2 is constant. The ingestion of food is therefore unlikely to sufficiently change the pH of the intestinal absorption site, alter the charge on C2E2 and effect its passive diffusion. As C2E2 is highly soluble the fed state will not affect the dissolution rate. The log D pH-profile suggests that the predominant site of C2E2 absorption may be the upper small intestine, where the molecules net charge is closest to zero. Under these conditions a fraction of C2E2 will be present as a zwitterion and a small fraction present as the uncharged drug. The absorption of C2E2 is higher than expected for a hydrophilic molecule and may be due to the presence of transcellular drug transporters. Although no food effects were seen lower liver burdens were observed in male rats than females. In contrast skeletal burden was lower in female rats across all groups. As these differences were also observed in untreated controls, they were likely due to variation in americium biokinetics between each gender and not attributed to C2E2.

Oral administration was less effective than the IV DTPA control group, this is to be expected as C2E2 must first cross the intestinal barrier and be metabolically activated to DTPA. Although doses of 600 mg/kg in rats were required to significantly increase americium elimination over control, this is expected to be higher than the doses necessary for treatment in humans based on renal clearance rates of DTPA between species. The effectiveness of DTPA has been shown to differ across species due to differences in

transferrin binding between species with beagles and humans requiring lower concentrations than rats[119]. Additionally administration of multiple doses will also enable to dose to be lowered and result in enhance efficacy.

Although initially designed as a prodrug, C2E2 may retain the ability to bind ^{241}Am albeit with an affinity lower than DTPA making it an active compound. After addition of two esters, six of DTPA's metal coordination sites remain available for chelation. This may result in metal binding affinities on an order similar to ethylenediaminetetraacetic acid (EDTA) thereby eliminating the need for metabolic activation upon absorption. However any ester hydrolysis after absorption will increase the metal coordination number and enhance chelation of americium.

Given that the DTPA is given intravenously at doses of 1 g per day it is unsurprising that large doses of C2E2 are required to achieve chelation. Due to large doses a formulation encompassing minimal excipients is desirable. Based on the determined physiological properties, C2E2 is suited towards an immediate release oral formulation. The high solubility, rapid dissolution and long-term stability of the powder assist in this goal. C2E2 may be formulated as a capsule with minor excipient additions to aid processing. Alternatively, C2E2 could be formulated as a powder in a bottle and reconstituted upon administration. Other marketed products (sodium alginate for strontium decorporation[120]) have used a similar approach to overcome large oral doses.

The absorption of both C2E2 and DTPA are limited by their charge, however masking of all five acids leads to increased toxicity due the increased intracellular absorption and chelation of essential metals. DTPA analogues with more than two ethyl esters exist in a liquid form at RT rather than in a solid form reducing aqueous solubility. Increasing the

length of the esters promoieties can also be used to increase lipophilicity, however once hydrolyzed long chain alcohols are more toxic than ethanol. Increased lipophilicity can also enhance cellular uptake resulting in chelation of essential metals and toxicity. Ultimately in the search for an oral DTPA prodrug a balance must be struck between increased absorption, stability and toxicity.

2.5. Conclusions

The physiochemical properties of C2E2 are more favorable for oral delivery than DTPA. Oral C2E2 solutions were shown to be effective when administered to rats 24-hours after contamination with ^{241}Am-nitrate via i.m. injection. A single oral dose of C2E2 can induce significant improvement in total ^{241}Am decorporation and reduction in ^{241}Am liver and skeletal burden. Thus, this DTPA analog appears to be an effective orally administered medical countermeasure for treating individuals contaminated with transuranic elements. Based on the dose and stability profile, C2E2 may be formulated in either oral capsules or as a powder for reconstitution. The oral bioavailability, metabolism and efficacy in an additional species must be established to continue development of this drug. Additionally, the ability of C2E2 and its metabolite the mono-ethyl ester of DTPA to chelate ^{241}Am must be investigated. Overall C2E2 is a promising candidate for oral decorporation of ^{241}Am.

CHAPTER III

SCALE-UP AND CHARACTERIZATION OF POLYMOPRHIC C2E2

3.1. Introduction

The experiments above were conducted using C2E2 synthesized at UNC as described in section 2.2. However once efficacy was established larger batch sizes were required in order to conduct additional studies. The synthesis was scaled-up by Synexsis Inc. (RTP, NC) producing batches on a kilogram scale. In order to aid the processing of larger batches, the synthesis method was altered.

Diethylenetriamine pentaacetic acid (DTPA) (2029 g, 5.16 mole) was dissolved in acetonitrile (1100 mL) with agitation. Acetic anhydride (1450 mL, 15.3 mole) and pyridine (1660 ml, 20.5 mole) were added and the reaction was heated to 60°C for 4 hours. The reaction mixture was cooled to 22°C and t-butyl methyl ether (MTBE) (800 mL) was added. The reaction mixture was filtered and the solid obtained was washed with MTBE (3000 mL). The solid was dried in a vacuum oven at 40°C. Yield: 1792 g, 97%. The bis-anhydride (1792 g, 5.02 mole) was slurried in absolute ethanol (9000 mL) and heated to reflux with agitation for 1.5 hour. The heated reaction mixture was filtered through Celite and cooled below 20°C. The resulting slurry was filtered and washed with cold ethanol (2800 mL) followed by MTBE (3500 mL). The Celite cake was slurried in ethanol (800 mL) and heated to 70°C and

filtered. On cooling, the slurry obtained was filtered and washed with ethanol and MTBE. The combined isolated solids were dried in a vacuum oven at room temperature. The resulting slurry was filtered and washed with cold ethanol (2800 mL) followed by MTBE (3500 mL). The Celite cake was slurried in ethanol (800 mL) and heated to 70°C and filtered. On cooling, the slurry obtained was filtered and washed with ethanol and MTBE. The combined isolated solids were dried in a vacuum oven at room temperature. Yield: 1488 g, 66%. From this point forward, unless otherwise stated, all studies were conducted with C2E2 synthesized at Synexsis.

Upon receiving the Synexsis material differences in solubility were seen. Precipitation of solutions occurred at concentrations below the previously identified solubility. Therefore in order to identify the cause of this problem the material was characterized in the same manner as the UNC material. In addition SEM images were taken of both batches. After identifying the cause of the solubility differences additional pharmaceutical characterization methods were employed to further improve our understanding of C2E2s properties.

The stability of C2E2 was previously determined in simulated gastric and intestinal fluids with no significant ester hydrolysis is expected to occur during gastric transit. To further understand the ester hydrolysis kinetics, a pH stability profile was obtained for C2E2 over the pH range 2-9. The metabolic stability of C2E2 was also investigated using both intestinal and hepatic S9 fractions and plasma to understand species differences in metabolism.

3.2. Methods

3.2.1 Solid Characterization

The structure of C2E2 was confirmed by elemental analysis, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy. Differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) and X-Ray powder diffraction were performed using the same methods as described in section 2.2. Additionally, scanning electron microscopy was performed on both solids. The solids were coated with a thin layer (5 nm) of metal using a sputter coater (Cressington 108) and images collected on a Hitachi S-4700 SEM.

3.2.2 Buffer Stability

Buffers were prepared across a range of pH values at constant ionic strength (600 mM). To determine the hydrolysis kinetics of C2E2 at each pH, approximately 9 mg of C2E2 was weighed into a 7 mL vial and the desired buffer was added to achieve a concentration of 3 mg/mL. The samples were stored at 40^{o}C for the duration of the experiment. The pH of the samples was checked and an initial concentration determined by HPLC. At each time-point 15 µl of sample was withdrawn and diluted in 285 µl of water and stored at 4^{o}C until analysis by HPLC. The pH of the samples was checked periodically to ensure the samples were effectively buffered. All conditions were run in triplicate.

3.2.3 *In vitro* Plasma Stability

Pooled rat, beagle and human plasma (Xenotech) were used for stability testing of C2E2. Prior to the study a fraction of the plasma was inactivated for negative control samples. This was achieved by heating plasma at 56ºC for 1 hour. For each sample 975 µl of

plasma (Rat, Beagle and Human) was preheated to 37ºC in eppendorff tubes. The test compound (C2E2 or diltiazem) was prepared in DMSO (10 mg/ml). To initiate the study 25 µl of the test solution was added to the pre-heated plasma and briefly vortexed to mix. At 0, 15, 30, 60 and 120 minutes a 150 µl sample of plasma was taken and an equal volume of cold acetonitrile added and mixed to precipitate plasma proteins. The sample was then centrifuged at 4 ºC and 14,000 x g for 10 minutes to remove precipitated proteins, the supernatant collected and the concentration determined by HPLC-CAD (C2E2) or HPLC-UV (diltiazem). All conditions were performed in triplicate. Diltiazem was used as a positive control compound, and heat-inactivated plasma as the negative control.

3.2.4 *In vitro* S9 Stability

C2E2 (5 mM final concentration) and buffer (phosphate pH 7.9) were pre-incubated at 37^{o}C for 5 minutes. S9 fractions (1 mg/mL final concentration) were added to the buffer and incubated at 37^{o}C for 0, 5, 10, 15, 30, 60 or 120 minutes. Metabolism was terminated at each time point by the addition of 100 µl of ice-cold ACN. The samples were vortexed and kept on ice before being centrifuged at 14,000 x g for 10 minutes. The supernatant was collected and the concentration of C2E2 analyzed by HPLC.

Reaction Conditions

Hepatic S9		Intestinal S9	
Reagent	Volume (µl)	Reagent	Volume (µl)
S9 fraction	5	S9 fraction	25
C2E2 (50 mM)	10	C2E2 (50 mM)	10
Buffer	85	Buffer	65

3.3. Results

3.3.1 Solid Characterization

The identity of C2E2 was confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy with results consistent with the structure. Elemental analysis was performed with the following results found C: 48.23%; H: 6.81%; N: 9.25%. Theoretical C: 48.10%; H: 6.95%; N: 9.35%. As with the C2E2 synthesized at UNC, DSC identified a single-phase transition for C2E2. Although, the UNC synthesis method had a melting point of 115.8^{o}C and the endothermic heat loss was 95.3 J/g compared to the Scynexsis batch with a melting point of 133.5 ^{o}C and an endotherm of 86.3 J/g (Figure 3.1). The use of TGA, again confirmed the endotherms observed during DSC studies was the melting point as there is no significant weight changed observed on the TGA thermogram for either compound at the associated temperature, which is indicative of a phase change rather than decomposition or desolvation. Similarly there was a small loss in weight observed during TGA analysis, prior to decomposition (Figure 3.2). The loss was gradual, not attributed to a specific temperature, and non-stoichiometric suggesting that surface/incorporated water was the cause and that the difference in properties was not due to the present of a solvate. Both solids begin to decompose around 170^{o}C as seen by the rapid weight loss. The differences in melting points however, suggest the presence of polymorphs. From this point forward the C2E2 produced at UNC will be designated polymorph I, and that synthesized by Scynexsis, polymorph II. In addition to being used for identification of solid phases, XRPD can be used to detect polymorphs. Figure 3.3 shows compares the XRPD patterns obtained for both solids. The results demonstrate that both solids are crystalline albeit with distinctive structures. Confirmation of the structural difference and the presence of two polymorphs was obtained

by SEM (Figure 3.4). Polymorph I is scaly in nature, as opposed to the divergent habit of polymorph II, which possesses a triclinic crystal form. The solid characterization data suggests that C2E2 has two polymorphic forms. The higher melting point and lower solubility (observed as precipitating out during storage in the refrigerator) suggests that polymorph II is the most stable. This was also confirmed by a competitive ripening experiment, where upon stirring of a mixed solution of the two forms overnight polymorph II is the resultant form.

3.3.2 Buffer Stability

The pH-dependent stability of C2E2 was evaluated across the pH range 2-9. The loss of C2E2 followed pseudo-first order kinetics and the rate observed rate constants (K_{obs}) were calculated by least squares regression. Figure 3.5 shows the U-shaped pH-rate profile determined in 200 mM buffer with the greatest C2E2 stability observed between pH 5-6. Based on this profile the hydrolysis of C2E2 is anticipated to occur as depicted in Figure 3.6. Below pH 4 where the triprotic form and above exists acid-catalyzed hydrolysis (K_H) is the predominant mechanism. The diprotic form is both acid (K'_H) and base (K'_{OH}) catalyzed and the monoprotic form undergoes base-catalyzed hydrolysis (K''_{OH}). The pK_a values obtained in section 4.3 and nonlinear regression analysis was used to fit that data to the equation[121]:

$$K_{obs} = \frac{[H^+]^4 K_H + [H^+]^3 K'_H K_{a1} + [H^+] K'_{OH} K_{a1} K_w + K''_{OH} K_{a1} K_{a2} K_w}{[H^+]^3 + [H^+]^2 K_{a1} + [H^+] K_{a1} K_{a2}}$$

The rate constants were calculated to be $K_H = 0.05\ h^{-1}\ M^{-1}$, $K'_H = 2.39\ h^{-1}\ M^{-1}$, $K'_{OH} = 2.62\ h^{-1}\ M^{-1}$ and $K''_{OH} = 749.5\ h^{-1}\ M^{-1}$.

3.3.3 Plasma Stability

The stability of C2E2 was investigated using rat, beagle and human plasma *in vitro*. C2E2 is stable in plasma with no significant loss observed in both rat and beagle samples over two hours. Similarly in human plasma 90 % remained intact at the conclusion of the study. Degradation of the positive control (diltiazem) was seen across all species, with the rat producing the highest metabolism. The heat inactivation procedure for production of negative control data was unsuccessful as diltiazem was metabolized as efficiently in these samples. However degradation of both positive and negative control samples demonstrates that enzymes were functional.

3.3.4 S9 Fraction Stability

The metabolism of C2E2 by rat and beagle hepatic and intestinal S9 fractions was determined *in vitro*. As with plasma samples no significant metabolism of C2E2 was observed. In general greater metabolism was seen with the intestinal S9 fractions. Although significant loss is seen in rats at 120 minutes this result does not follow the trend and is assumed to be due to poor sample processing. The lack of C2E2 metabolism in plasma and S9 fractions supports the fact that C2E2 is the most predominant form in the plasma after administration.

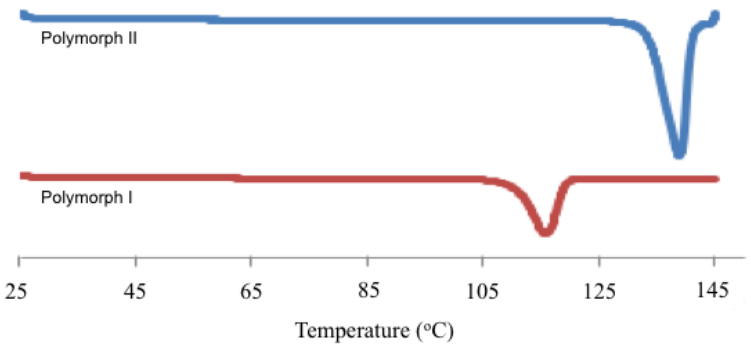


Figure 3.1 Differential scanning calorimetry chromatograph of polymorph I (red) and II (blue).

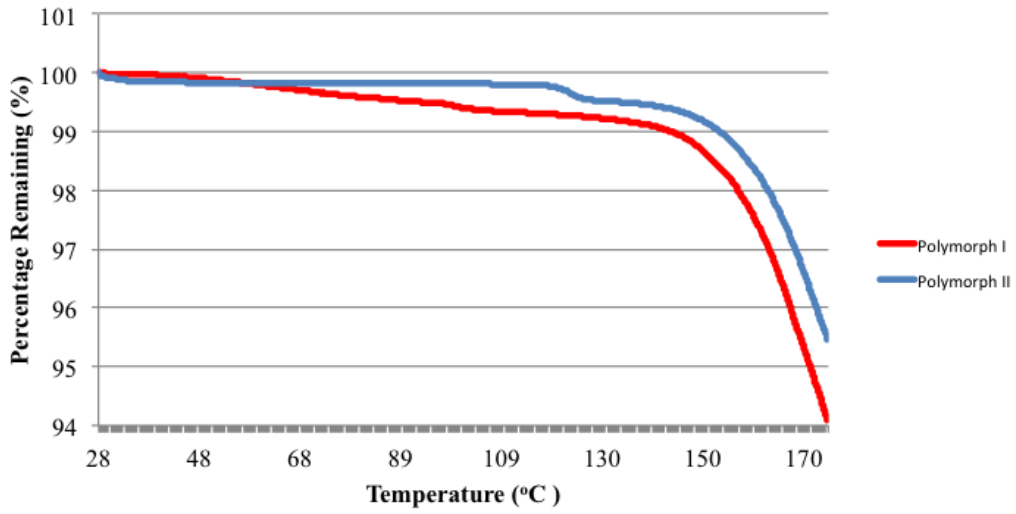


Figure 3.2 Comparison of polymorph I (red) and II (blue) by thermogravimetric analysis.

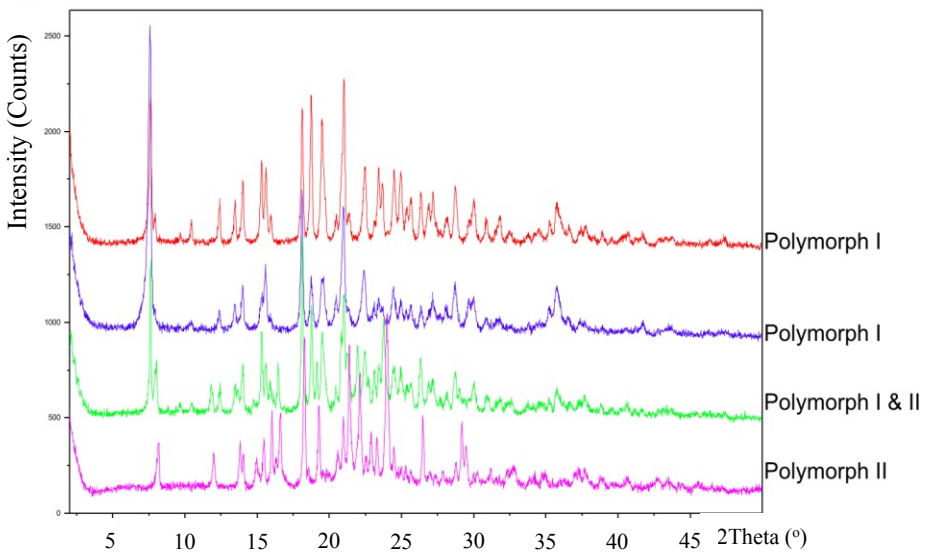


Figure 3.3 X-Ray powder diffraction (XRPD) chromatographs for polymorphs I and II depicting the differences in crystal structure.

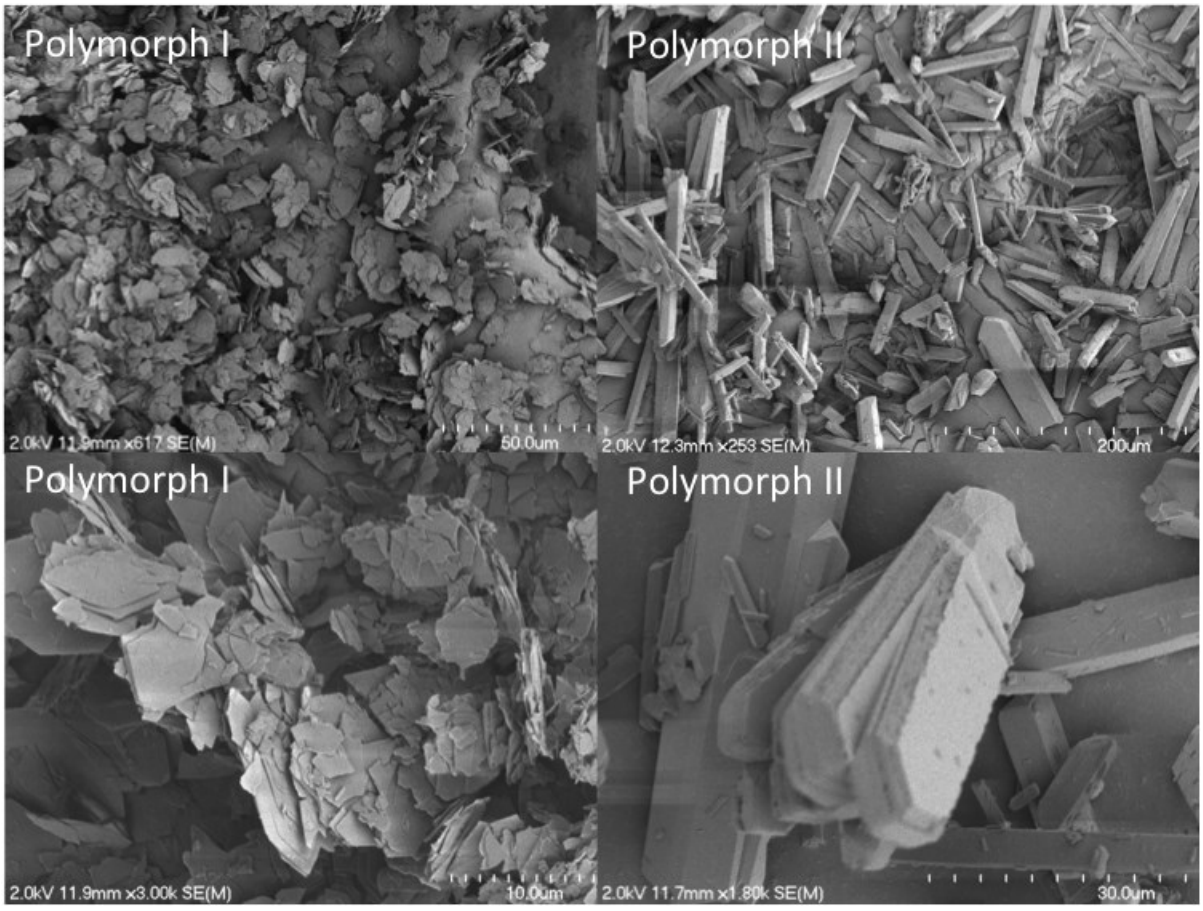


Figure 3.4 Scanning electron microscopy images of polymorph I and II, displaying the different crystal habits of each form.

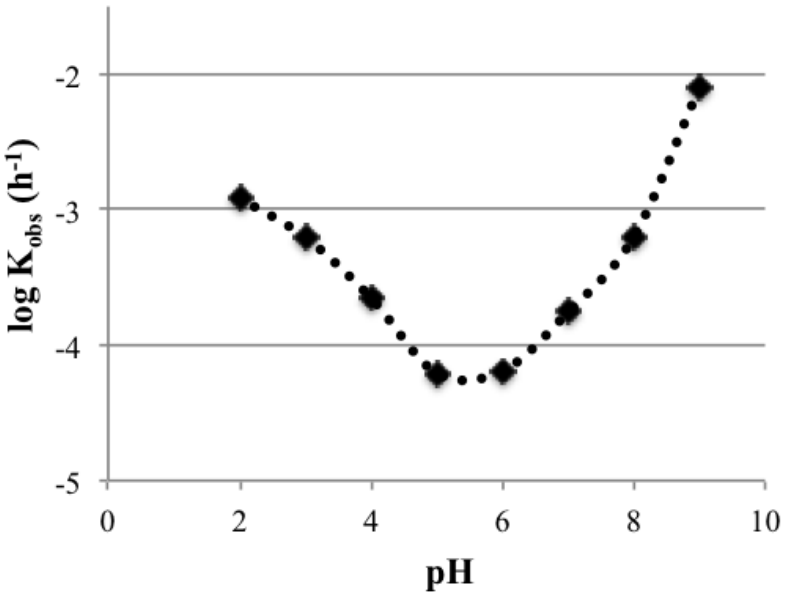


Figure 3.5 pH-rate stability profile of C2E2 at buffer concentrations of 200 mM with constant ionic strength (I = 0.6 M).

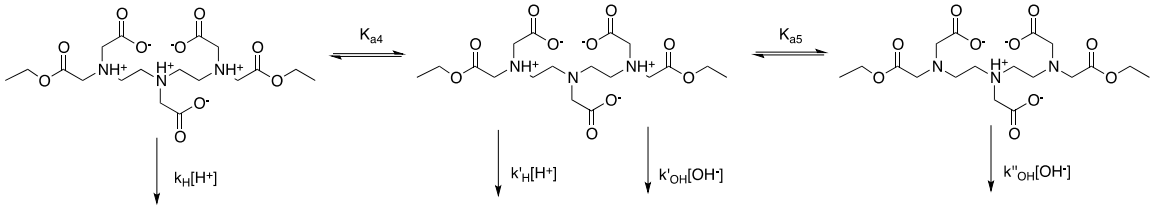


Figure 3.6 Schematic of the proposed acid and base catalyzed hydrolysis pathways for C2E2.

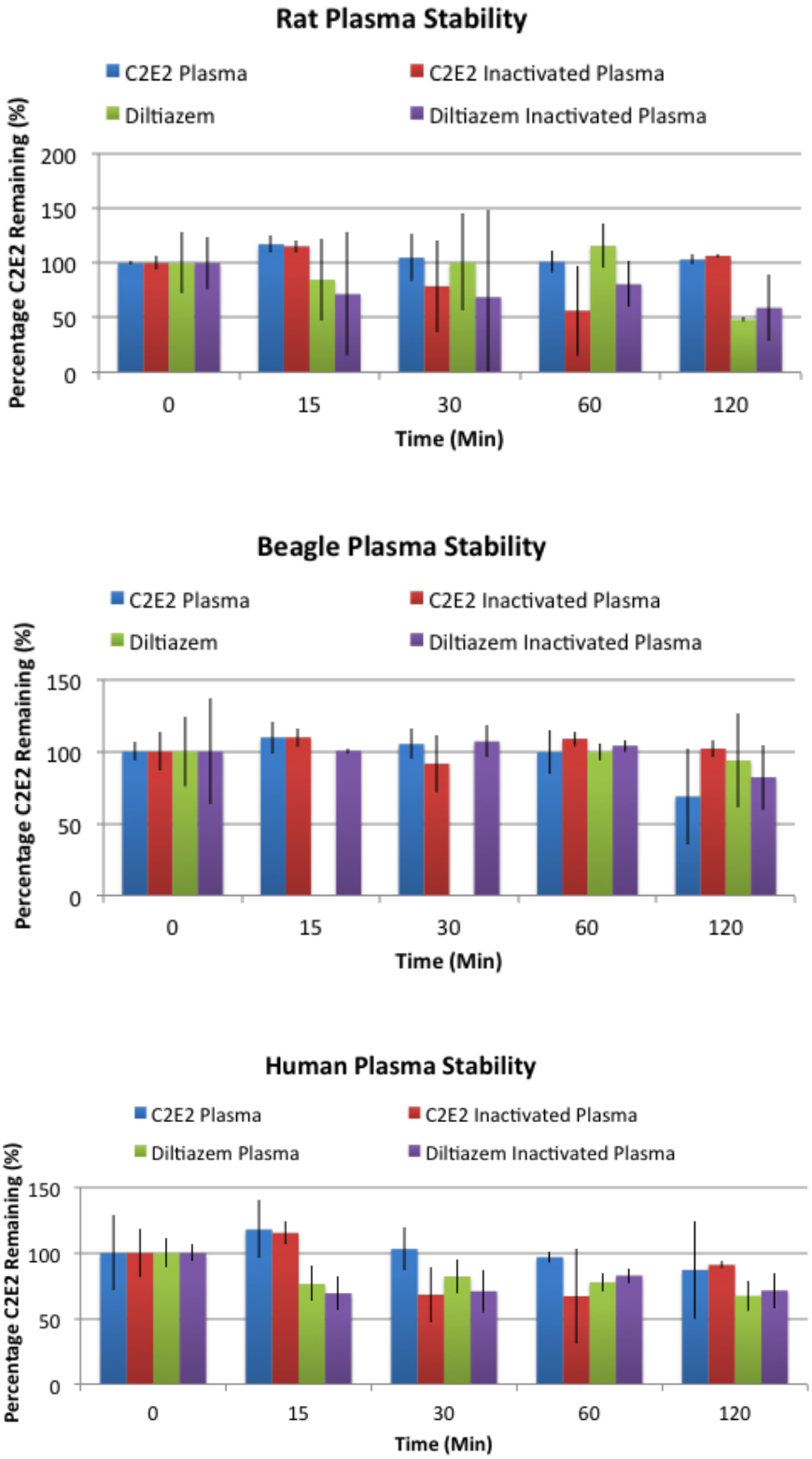


Figure 3.7 Stability of C2E2 in rat, beagle and human plasma.

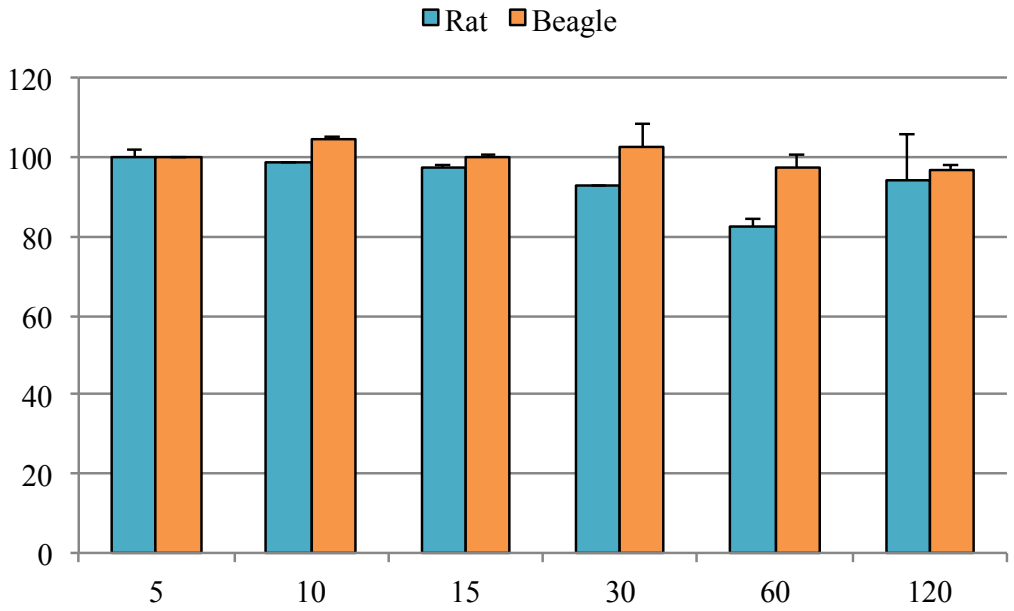


Figure 3.8 Metabolism of C2E2 by rat and beagle hepatic S9 fractions. Graph depicts mean of triplicate samples (+ SD).

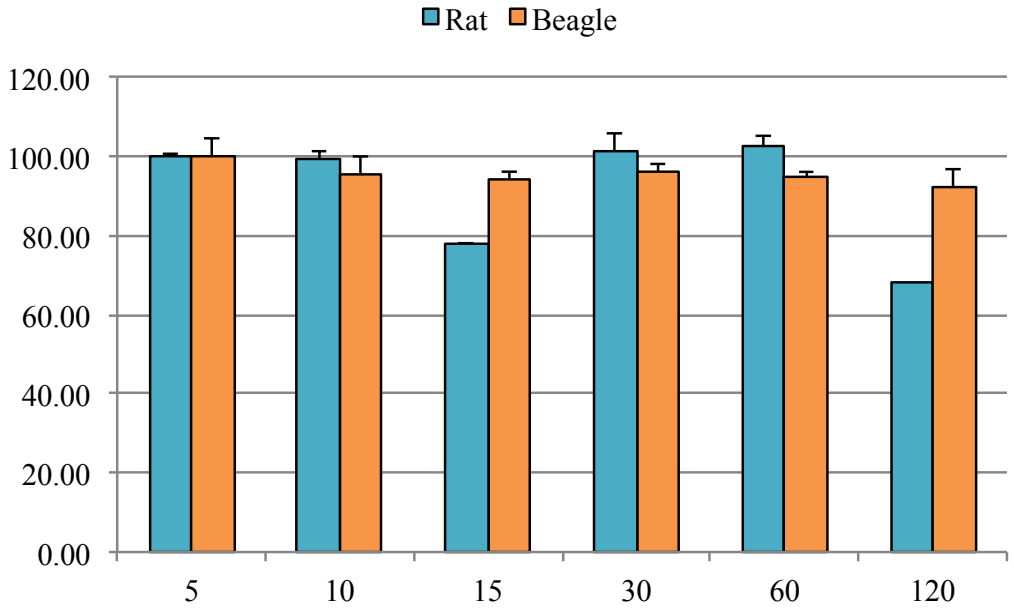


Figure 3.9 Metabolism of C2E2 by rat and beagle intestinal S9 fractions. Graph depicts mean of triplicate samples (+ SD).

3.4. Conclusion

The scaled-up synthesis of C2E2 led to the formation of a more stable polymorph. All the subsequent studies in this thesis were conducted with the most stable polymorph. The stability of C2E2 was also further characterized across the pH range with greatest stability observed between pH 5-6. In addition to being stable in simulated gastric and intestinal fluids metabolism of C2E2 by hepatic and intestinal S9 fractions is insignificant. Furthermore there is no significant hydrolysis of the esters in plasma. These results suggest that C2E2 is poorly metabolized and is likely to be the predominant species present in plasma after administration.

CHAPTER IV

SPECIES-DEPENDENT CHELATION OF ^{241}AM BY DTPA DI-ETHYL ESTER

4.1. Introduction

Recent nuclear accidents such as the meltdown at the Fukushima nuclear power plant in 2011 and the increasing threat of terrorism has spurred research interest in medical evaluation and treatment of contaminated individuals[51]. In the event of a public health emergency the US maintains a Strategic National Stockpile containing medical supplies used protect the public. Of particular interest is diethylenetriamine pentaacetic acid (DTPA), one of only three drugs with FDA approval for treatment of internal contamination. DTPA is an aminopolycarboxylic acid chelating agent with eight potential metal coordination sites that allow binding to a wide range of metals with high affinity[122]. Two forms of DTPA, namely the pentetate calcium trisodium injection (Ca-DTPA) and pentetate zinc trisodium injection (Zn-DTPA), received FDA approval for treatment of internal contamination with Plutonium, Americium or Curium. However, due to its poor bioavailability (~3%)[110], DTPA requires intravenous administration by medical professionals, which would prevent timely distribution in a mass casualty scenario. Americium rapidly clears from the plasma to the tissues with > 90 % cleared 60 min after an IV injection and only a small fraction is excreted in the urine[123]. Early access to treatment therefore improves decorporation by minimizing tissue deposition

of the radioactive material. The loss of efficacy caused by treatment delay cannot be compensated for by increasing the cumulative dose[124], therefore there is a need to develop patient-friendly formulations that can be easily distributed and self-administered.

In order to improve the oral bioavailability of DTPA a number of novel formulation approaches have been investigated such as nanoDTPA capsules[105] and permeation enhancers[104, 119]. Alternatively in our lab we have focused on the development of DTPA pro-drugs[94, 125]. These penta-ethyl ester prodrugs of DTPA although highly absorbed are limited by their toxicity and the potential to form ten metabolites complicating their regulatory approval. Therefore, in order to avoid these difficulties the di-ethyl ester of DTPA, named C2E2, was selected for further development due to its cleaner metabolic profile and lower toxicity.

In the case of all of these approaches there is an insufficient patient population to conduct well-controlled clinical trials. Furthermore, it is unethical to contaminate healthy individuals in order to determine efficacy. Therefore, to encourage the development of such products the FDA introduced the 'Animal Efficacy Rule' 21CFR(314.600), which provides a pathway for approval of a drug if its efficacy is demonstrated in more than one animal species in conjunction with safety data from animals and healthy human volunteers[126]. For this pathway, the mechanism of action needs to be understood and the animal models selected to establish efficacy must be demonstrated as appropriate. In order to fully understand the mechanism of action of these agents the binding affinities should be determined. Additionally, previous research with DTPA revealed that there are species differences in the plasma concentrations of DTPA required for chelation of ^{241}Am[119, 120]. Consequently, to

justify the animal models used for efficacy studies the binding of novel prodrugs should also be evaluated in animal plasma to confirm the appropriateness of the species.

Although C2E2 is designed to be a pro-drug of DTPA, due to its three free acids and three tertiary amines acting as metal coordination sites, it is also likely to act as a chelators itself. Similarly, its primary metabolite, the mono-ethyl ester of DTPA, named C2E1, is also expected to bind metals with affinities between those of DTPA and C2E2. The binding affinities of C2E2 and C2E1 for Am^{3+} are of significance, although they are expected to be lower than DTPA, as the presence of these ligands in plasma could contribute to the overall decorporation efficacy of C2E2 therapy. Furthermore, even if the binding affinities are lower than those of DTPA they may still be several orders of magnitude greater than those of the plasma proteins to which ^{241}Am is bound.

Below the ability of C2E2 and its metabolite C2E1 to chelate ^{241}Am is investigated. The differences of plasma concentrations required for efficacy in rat, beagle and human plasma is also determined. The *in vitro* plasma concentrations are then combined with pharmacokinetic data from dogs to demonstrate the dose required to achieve efficacy and the likely duration of action. This work will be used to support justification of animal model selection in future efficacy studies and aid development of a pharmacokinetic-pharmacodynamic model to help establish the required human efficacious dose.

4.2. Methods

4.2.1 Materials

The di- and mono-ethyl esters of DTPA were obtained from Synexsis Inc (Durham, NC). The synthesis of the diethylester was achieved by first synthesizing DTPA bis-anhydride followed by its subsequent reflux with ethanol as previously described[90]. The monoethyl ester of DTPA was obtained as an impurity during the synthesis and was separated using chromatography. The $^{241}AmNO_3$ stock solution was obtained from Eckert & Ziegler Isotope Products (Valencia, CA).

4.2.2 Ionization Constants

The protonation constants for C2E2/1 were determined by preparing a 5 mM solution of free ligand in 0.15 M KCl. The constants were calculated from three replicates, with each experiment consisting of a titration with acid, followed by a titration with base. After each addition of titrant, a 30 second equilibration time passed before pH measurement with a Seven Easy pH meter and (Ag/AgCl reference) glass electrode (Metler Tolledo). At the end of the titrations the presence of C2E2/1 was confirmed by HPLC-CAD.

4.2.3 Solvent Extraction Studies

The formation constant for the ^{241}Am/C2E2 complex was determined by a solvent extraction method similar to that described previously[127]. The experiments conducted with tracer amounts of ^{241}Am (16.7 $MBq.L^{-1}$) using an organic phase of 0.1 M HDEHP in dodecane and aqueous phase consisting of 0.1 M NaCl at pH 1.3. The concentration of ligand in the aqueous phase ranged from 0.05 to 100 μM. On addition of aqueous phase the samples

were vortexed and then maintained at 25°C for 60 minutes by means of thermomix automatic shaker. Following incubation the two phases were separated by centrifugation (10,000g, 10 min) and a 100 µL sample of each phase was collected and the ^{241}Am content in each sample fraction was measured by gamma scintillation counting (Perkin Elmer Model 2470 Gamma Counter). Background-corrected radioactivity was quantified over 30 min using a 40-80 keV energy window to measure the 59.7 keV photon emission associated with ^{241}Am decay. Experiments were performed in triplicate. In addition to determining the distribution in the presence of C2E2, C2E1 or DTPA, the HDEHP solvent loaded with ^{241}Am was contacted with a 0.1 M NaCl solution at pH 1.3, free of chelating ligand to determine D_O.

4.2.4 Competitive Binding Studies

The ability of C2E2 and its primary metabolite C2E1 to chelate plasma bound ^{241}Am in blood was investigated by *in vitro* experimentation using a method developed in our lab for DTPA[119, 120]. As previously described various concentrations of chelating ligand were combined with ^{241}Am in rat, beagle or human plasma before separation and evaluation. All samples utilized 385 µl of plasma, 8 µl of ligand and 7 µl of ^{241}Am solution. The ligand stock solutions were prepared in 10 mM sodium phosphate buffer at pH 7.4. The final concentrations of ligand used ranged from 0.5 to 750 µM in rat, beagle and human plasma for both C2E2 and C2E1. The ^{241}Am stock solution was prepared by diluting $^{241}AmNO_3$ in 1 M hydrochloric acid. The resulting concentration of ^{241}Am nitrate once combined with plasma and ligand was 3 nM.

Once combined the samples were incubated for 60 minutes in a heating block at 37 ^{o}C with mild agitation before being transferred to an Amicon Ultra centrifugal filter with a

Ultracel 3K membrane (Milipore, Billerica, MA) and centrifuged at 14,000 x g for 30 min to extract the protein bound fraction. The filtrates were subsequently transferred to Pierce Strong Cation Exchange Spin Columns (Thermo Fisher Scientific, Rockford, IL) that had been conditioned with 0.8 M ammonium hydroxide and water to separate positively charged ^{241}Am species from ligand-bound ^{241}Am. Elution on the ligand-bound ^{241}Am species was achieved by centrifuging at 2000 x g for 5 min. The positively charged ^{241}Am species were collected by further centrifugation at 2000 x g with 1 M nitric acid. The ^{241}Am content of each fraction was determined by gamma scintillation counting (Pelkin Elmer Model 2470 Gamma Counter, Waltham, MA). Background corrected radioactivity was quantified over 30 min using a 40-80 keV energy window to measure the 59.7 keV photon emission associated with ^{241}Am decay.

The dose-response curves were produced by calculating the fraction of total ^{241}Am that was bound to C2E1, C2E2 or plasma proteins. The sigmoidal regression profiles were generated by Gauss-Newton non-linear least squares regression analysis using SAS 9.3 (SAS, Cary, NC). The regression applied the logistic equation[128];

$$y = \frac{c}{1 + e^{a(b-x)}}$$

where; *a* describes the slope as the curve approaches the asymptote, *b* is the inflection point, *c* is the asymptote, and *x* is the log transformed concentration of C2E1 or C2E2 that corresponds to *y*, the fraction of bound ^{241}Am. The EC_{50} values were determined by setting *y* equal to 50% of the *c* term and calculating for *x*. The same regression analysis was applied to the data for ^{241}Am-protein binding; however, a transformation was applied to $y_{protein}$ such that,

$$y' = 100 - y_{protein}$$

where *y'* was used for the regression analysis. Subsequent reversal of the transformation produced the final binding curves.

4.2.5 Pharmacokinetics of C2E2 in Beagle Dogs

Two male and two female beagle dogs were administered a single 100 mg/kg dose of C2E2 via oral gavage (12.05 mg/mL). The dogs were 8 to 10 months old, and their body weights were 11.4 and 12.0 kg for the males and 10.1 and 10.9 kg for the females. During the study they were housed individually and fasted overnight prior to dosing with food returned approximately 2 hours after dose administration. Blood (0.7 mL) was collected via a jugular vein pre-dose and at 0.5, 1, 2 4, 6, 8, 12 and 24 hours post dose in vacutainer tubes containing 5mg sodium fluoride and 4 mg potassium oxalate. The tubes were pre-chilled, once added the sample was inverted several times prior to centrifugation at 4 ^{o}C for 10 minutes (1300 x g) within 15 minutes of collection. 125 µL of plasma was transferred to a micro centrifuge tube containing 125 µl of 20 % formic acid, vortexed and stored on dry ice prior to being stored in a freezer (-80 ^{o}C) until analysis was conducted.

The plasma concentrations of C2E2 and its metabolites, C2E1 and DTPA were quantified using two LC/MS/MS methods. One method was used for C2E2 and C2E1 and another for DTPA. For C2E2, 100 µl of the acidified samples was treated with 400 µL acetonitrile containing internal standard ($^{13}C_5$-C2E5) followed by mixing and centrifugation. 400 µL of the supernatant was subsequently removed, evaporated and reconstituted with 500 µL water/acetonitrile/formic acid (85/15/0.1) for injection. The separation of the components was performed on a YMC ODS-AM (100 x 2.1 mm, 3.5 µm) column with a mobile phase gradient starting with 0.1% formic acid in water and ending with 0.1 % formic acid in

acetonitrile. The compounds were detected on a triple quadrapole mass spectrometer using heated electrospray ionization in the positive-ion mode. For DTPA, an excess of Fe(III) was utilized to ensure formation of an Fe(III)-DTPA complex for quantification. A $^{13}C_5$-DTPA internal standard was used. 100 µl of the acidified samples was treated with 50 µL of 2 mM $Fe(III)Cl_2$ and allowed to incubate for 90 minute. Acetonitrile (400 µL) containing internal standard was added. After mixing and centrifugation, 350 µL of the supernatant was removed, evaporated and reconstituted with 100 µL of mobile phase. Separation was performed using a Phenomenex Luna phenyl-hexyl (150 x 2mm, 3 µm) column with a mobile phase gradient starting with water/methanol (90/10) with 1 mM acetic acid and 1 mM tributylamine and ending with acetonitrile/methanol (50/50) with 1 mM acetic acid and 1 mM tributylamine. The DTPA was detected on a triple quadrapole mass spectrometer using heated electrospray ionization in the negative-ion mode.

Quantification of C2E2 and DTPA was based on the peak areas of analyte to their respective internal standards. 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. The approximate precursor-product ion transitions and approximate retention times are described in Table 4.1.

4.3. Results

4.3.1 Ionization Constants

Evaluation of the titration curve for C2E2 (Figure 4.1) identified 6 pK_a values that correspond with the three tertiary amines and three carboxylic acids. The C2E2 molecule exhibits resistance to pH change over the region of 1.8 – 2.7 where the molecules overlapping pK_a's lie. In order to determine these overlapping values a Bjerrum plot was used where the average number of protons (n_H) bound to the ligand at each pH was calculated and the pK_a values occur at half integer values of n_H. These plots have previously been utilized to establish the 6-pKa molecule, vancomycin or even the 30-pK_a molecule, apo-metallothionein[129]. The values determined from the Bjerrum plots were used as seed values for refinement in HYPERQUAD. The calculated ionization constants are shown in Table 4.2. The pKa's determined for C2E2 are consistent with DTPA analogues previously investigated for use as MRI contrast agents with two carboxylic acids are functionalized such as DTPA-BMA[130] and DTPA-BBA[131].

4.3.2 Solvent Extraction Studies

The formation constants of the Am(III)/ligand complex were determined using a solvent extraction method. Tracer levels of ^{241}Am were combined with HDEHP diluted in the dodecane organic phase. Distribution ratios, D, were determined as a function of ligand concentration at constant pH (1.3). As expected when the concentration of competing ligand is increased the ^{241}Am is chelated from the organic phase into the aqueous phase (Figure 4.2). The results indicate that C2E2 retains the ability to chelate ^{241}Am albeit at higher concentrations than those of DTPA. In order to achieve the same degree of binding C2E2 concentrations need to be approximately 20-fold higher than those of DTPA. In comparison

C2E1 retains a higher affinity for ^{241}Am with only 5-fold increases in concentration required to match DTPA.

In order to quantify these differences in affinity for ^{241}Am the conditional stability constants (lg β^{cond}) of the complexes were determined by linear fitting of the variations of (D/D_o - 1) versus C_{Ligand} as described by Leguay et al.[127] (where D_o stands for the D value in the absence of ligand). The lg β^{cond} for C2E2, C2E1 and DTPA were 1.36, 2.46 and 2.70 respectively. From the pKa values it is known that at pH 1.3, the predominant ligand species is in the protonated form and therefore the calculated formation constants are 19.6 ± 0.2, 21.7 ± 0.5 and 25.4 ± 0.3 for C2E2, C2E1 and DTPA, respectively. These values indicate that the stability of the C2E2-^{241}Am complex is 6 log units lower than that of the DTPA-^{241}Am complex. The solvent extraction data determined for DTPA and the corresponding formation constant is consistent with that previously determined[127].

4.3.3 Competitive Binding Studies

The suitability of this method for separation of both protein-bound ^{241}Am and DTPA-bound ^{241}Am was previously determined by Sueda et al.[119] At physiological pH ^{241}Am in solution is present as the +3 oxidation state and DTPA exists in the -5 oxidation state. Therefore, DTPA-bound ^{241}Am is results in a negatively charged complex, which is able to pass through the cationic filter. If the same logic is followed for C2E1 and C2E2 then the complexes with ^{241}Am are expected to be negatively and neutrally charged. As with DTPA, preliminary studies were conducted to confirm C2E1/2-bound ^{241}Am passed through the cation filter further confirming that the protein, ligand and free fractions could be adequately separated. In the previously performed studies the ^{241}Am stock was a citrate salt however, for

the present work ^{241}Am nitrate was used to ensure consistency between the *in vitro* characterization and planned *in vivo* studies. Reevaluation of DTPA binding with ^{241}Am nitrate was therefore performed to help further characterize the model and enable comparisons between each ligand. The dose response curves for C2E2, C2E1 in rat, beagle and human plasma are shown in figure 4.3a-f. The binding of ^{241}Am to either the ligand or plasma proteins follows a sigmoidal profile characteristic of a competitive binding relationship. The parameters obtained from the logistic regression analysis are show in Table 4.3. There were minor losses of activity during transfer steps however there was > 95% recovery for all samples. A minor fraction < 10% of each sample was considered to be associated with either small organic or inorganic ligands present in the plasma with affinity for ^{241}Am such as carbonates and citrates. Similarly to the solvent extraction study, C2E2 and C2E1 were able to chelate ^{241}Am from the plasma, albeit at higher concentrations than required with DTPA. The efficiency with which each ligand bound ^{241}Am varied between species. To permit comparisons between the different species and ligands the EC_{50} values for the each condition have been calculated. The maximum percentage of ^{241}Am capable of being bound is 45-65 % in all species. The EC_{50} value represents the concentration of ligand at which 50 % of maximal chelation occurs. The concentrations of ligand to achieve similar levels of chelation were highest in rat plasma and lowest in beagle plasma. The concentrations required in human plasma lay between those of each species. This suggests that if used for approval via the animal rule, efficacy in the rat model may underestimate the human response whilst beagle efficacy studies may result in an overestimation.

The EC_{50} for each ligand can be used to determine the dose of each ligand required to obtain chelation. In addition incorporation of the relative binding numbers into a

pharmacokinetic model will help to determine the contribution of each ligand to the overall efficacy of C2E2.

4.3.4 Pharmacokinetics

The homogeneity and concentration of the dosing solution was confirmed by HPLC by taking two samples form the top, middle and bottom of prepared solution. The solution was homogeneous and was found to be 97.8 ± 0.5 % of the target concentration. The concentration-time profile for C2E2 and its metabolites in beagle dogs is shown in Figure 4.4. There was no significant difference between the AUC_{0-24} for each sex, so the data was combined into a single profile, though the lack of significance may be due to the small the size of each group. The data was modeled using a two-compartment model with elimination from the central compartment, along with first-order absorption. The use of a two compartment model is consistent with the elimination of DTPA from dogs[132]. C2E2 is rapidly absorbed reaching its peak concentration 1 hour after administration. Elimination occurs from the first compartment with a half-life of 0.69 hours and from the second compartment with a half-life of 6.66 hrs. Only a small fraction (~2%) of the absorbed dose was hydrolyzed to C2E1, or DTPA and the concentrations of both of these ligands are below those required to effectively chelate ^{241}Am. By combining the *in vitro* competitive binding concentrations with the pharmacokinetic profile time for which plasma concentrations of C2E2 are above those necessary to achieve both 50 and 90 % of the maximal binding can be determined. The concentration of C2E2 is above the 90 % threshold for 3.8 hrs and above the 50% threshold for 4.7 hrs. These results suggest that any efficacy observed in dogs at 100 mg/kg doses is due to C2E2 and not its metabolites.

Table 4.1 The precursor → product ion transitions and retention times for C2E2, C2E1 and DTPA used during LC/MS/MS quantification.

Analyte	Precursor → product	Approx. retention time (min)
Fe-DTPA	m/z 445 → m/z 313	3.7
Fe-DTPA-IS	m/z 450 → m/z 350	3.7
C2E1	m/z 442 → m/z 160,188	1.0
C2E2	m/z 450 → m/z 188, 216	3.1
C2E5-IS	m/z 539 → m/z 218	6.3

Table 4.2 Acid dissociation constants determined for C2E2 and C2E1 compared to those known for DTPA.

Ligand	**pKa_7**	**pKa_6**	**pKa_5**	**pKa_4**	**pKa_3**	**pKa_2**	**pKa_1**
DTPA	1.45 ± 0.15	1.60 ± 0.15	1.80 ± 0.05	2.55 ± 0.05	4.31 ± 0.02	8.54 ± 0.02	10.51 ± 0.01
C2E1	< 1.5	< 1.5	1.18 ± 0.45	1.72 ± 1.5	2.99 ± 0.05	6.35 ± 0.38	9.63 ± 0.03
C2E2	-	1.45 ± 0.09	1.76 ± 0.05	1.87 ± 0.05	3.52 ± 0.03	4.68 ± 0.02	9.40 ± 0.02

Table 4.3 Logistic equation parameters and calculated EC_{50} and EC_{90} values for C2E2, C2E1 and DTPA binding with ^{241}Am.

Ligand	Plasma	Parameters for logistic equation (Estimate ± 95 % Conf. Interval)			EC_{50} (μM)	EC_{90} (μM)
		a	*b*	*c*		
C2E2	Rat	0.04 ± 0.00	46.17 ± 3.43	54.62 ± 1.58	46.17	101.10
	Beagle	0.37 ± 0.02	4.91 ± 0.18	64.79 ± 0.50	4.91	10.85
	Human	0.23 ± 0.04	8.87 ± 0.80	60.29 ± 1.25	8.87	18.42
C2E1	Rat	0.06 ± 0.01	25.50 ± 3.36	45.74 ± 1.51	25.50	60.12
	Beagle	0.57 ± 0.13	2.02 ± 0.38	61.69 ± 1.4	2.02	5.87
	Human	0.16 ± 0.03	5.26 ± 0.74	55.22 ± 1.06	5.26	18.99
DTPA	Rat	0.31 ± 0.03	5.36 ± 0.43	35.69 ± 0.85	5.36	12.44
	Beagle	1.87 ± 0.69	1.08 ± 0.18	54.75 ± 2.16	1.08	2.25
	Human	0.98 ± 0.13	2.82 ± 0.30	55.88 ± 1.40	2.82	5.05

Figure 4.1 Titration of DTPA di-ethyl ester with 1 N potassium hydroxide.

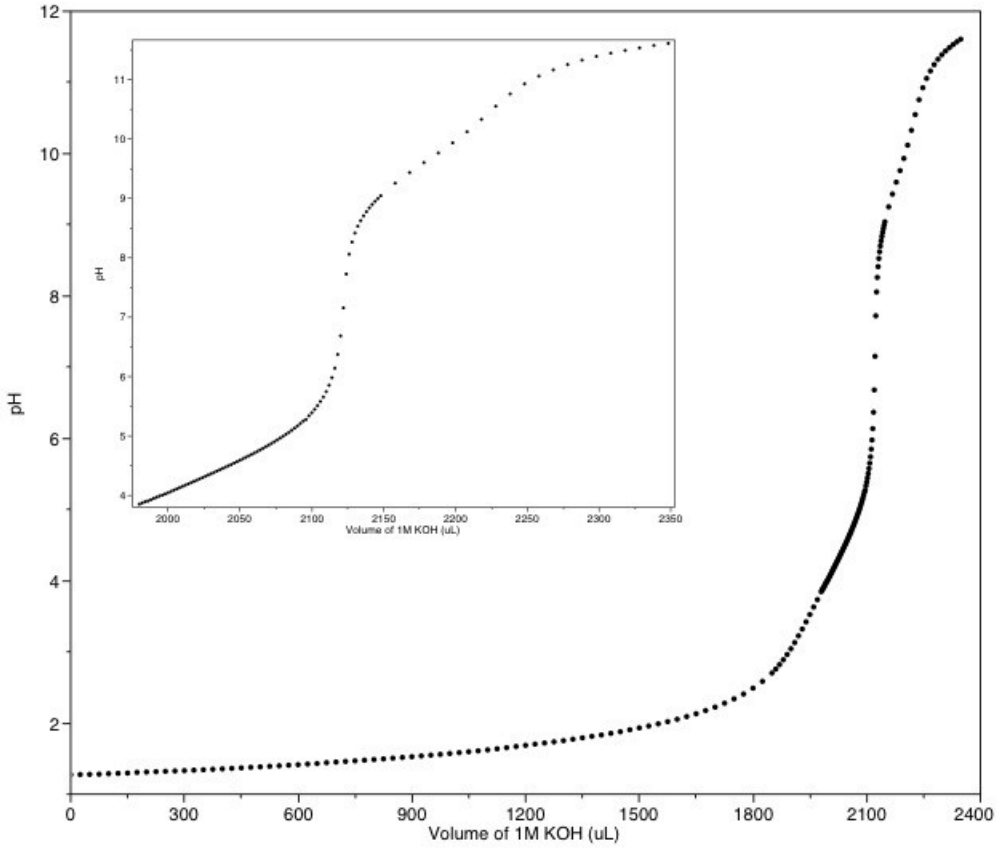


Figure 4.2 Distribution of ^{241}Am as a function of total ligand concentration at I = 0.1 M (NaCl), T = 25 ^{o}C, pH = 1.3, and C_{HDEHP} = 0.1 M in dodecane.

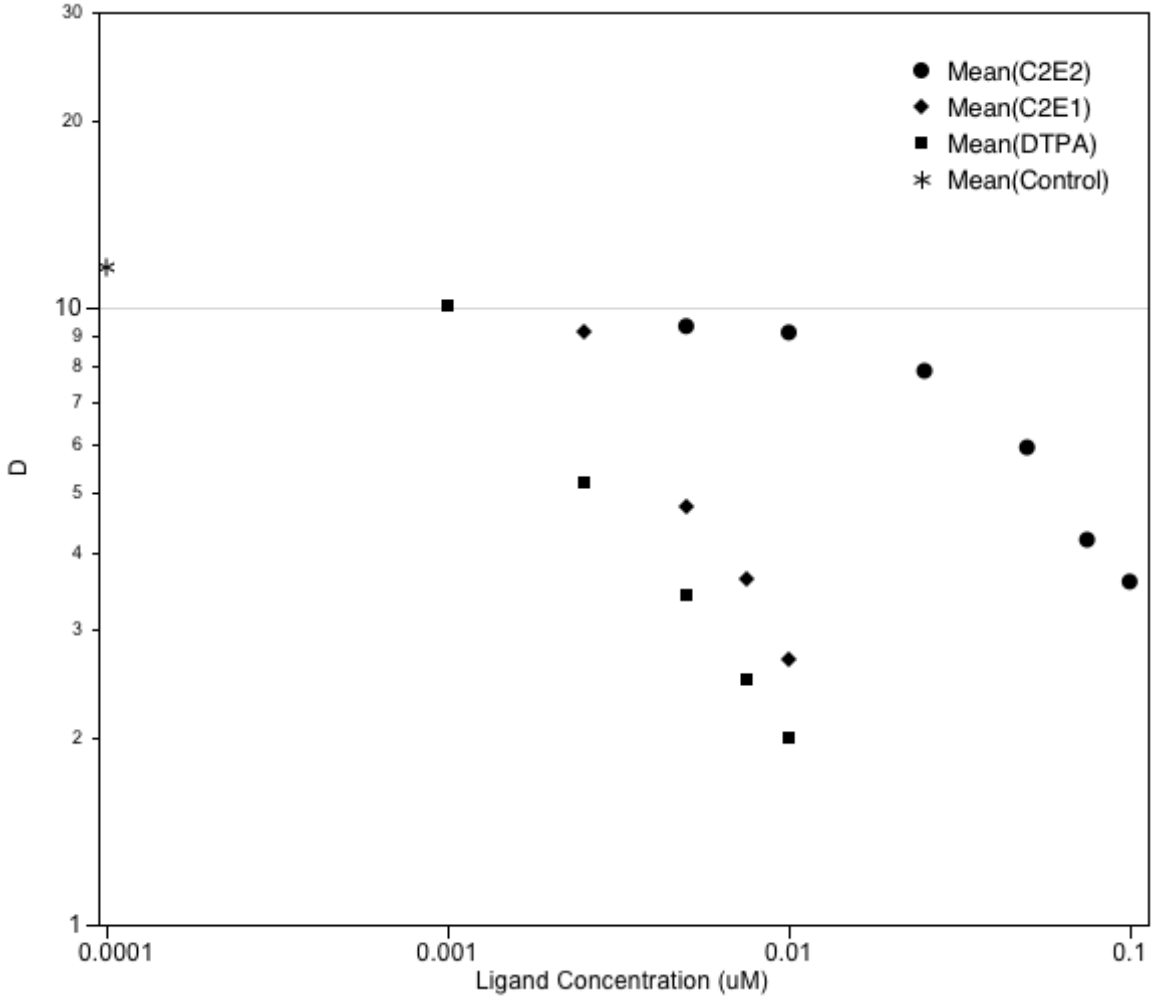


Figure 4.3 The competitive binding of 3nM ^{241}Am by C2E2 (a-c) and C2E1 (d-f) (solid line) and plasma proteins (dotted line) after 0.5 h incubation at 37 ^{o}C in rat (a, d), beagle(b, e) and human plasma (c, f).

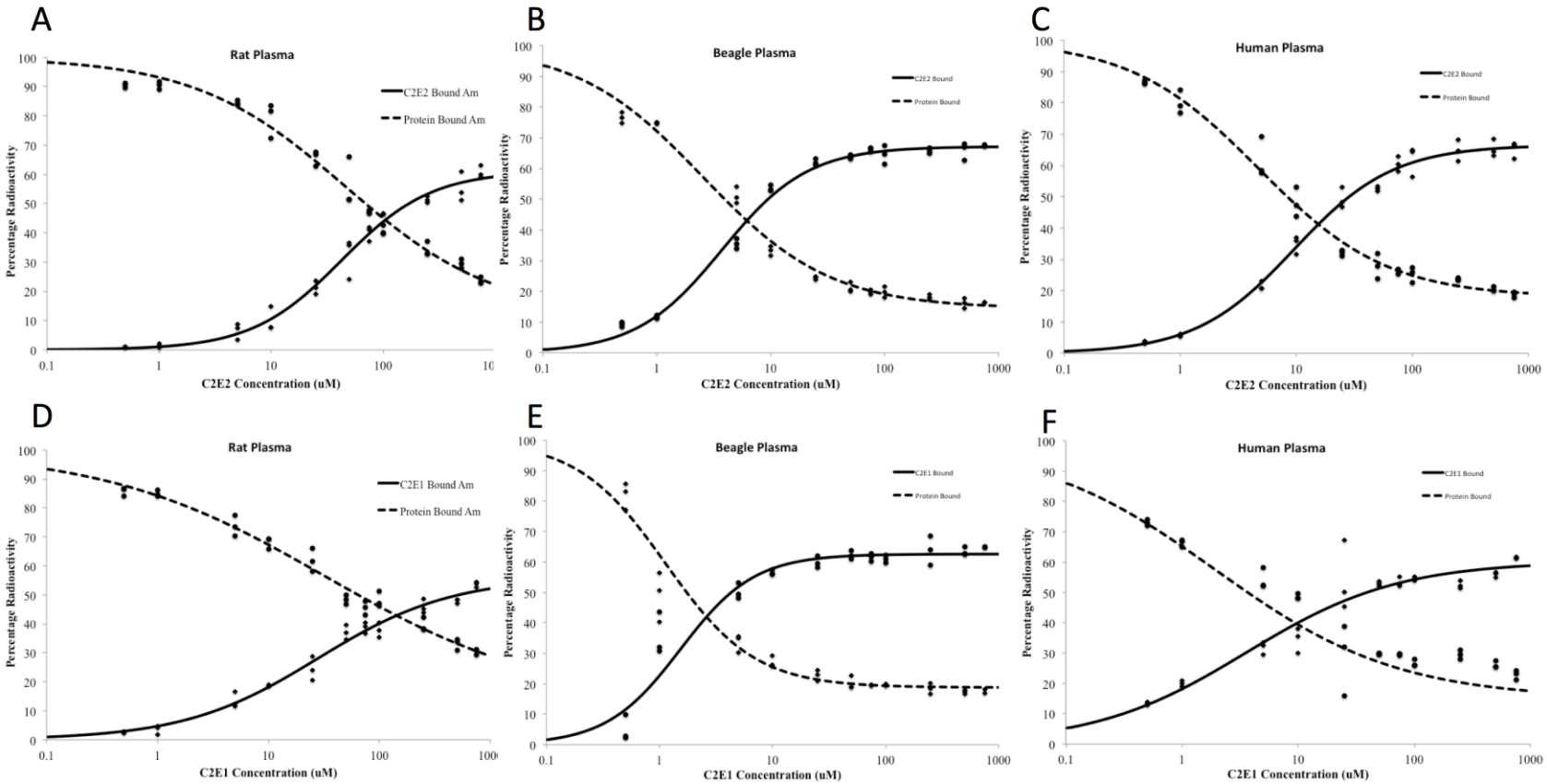
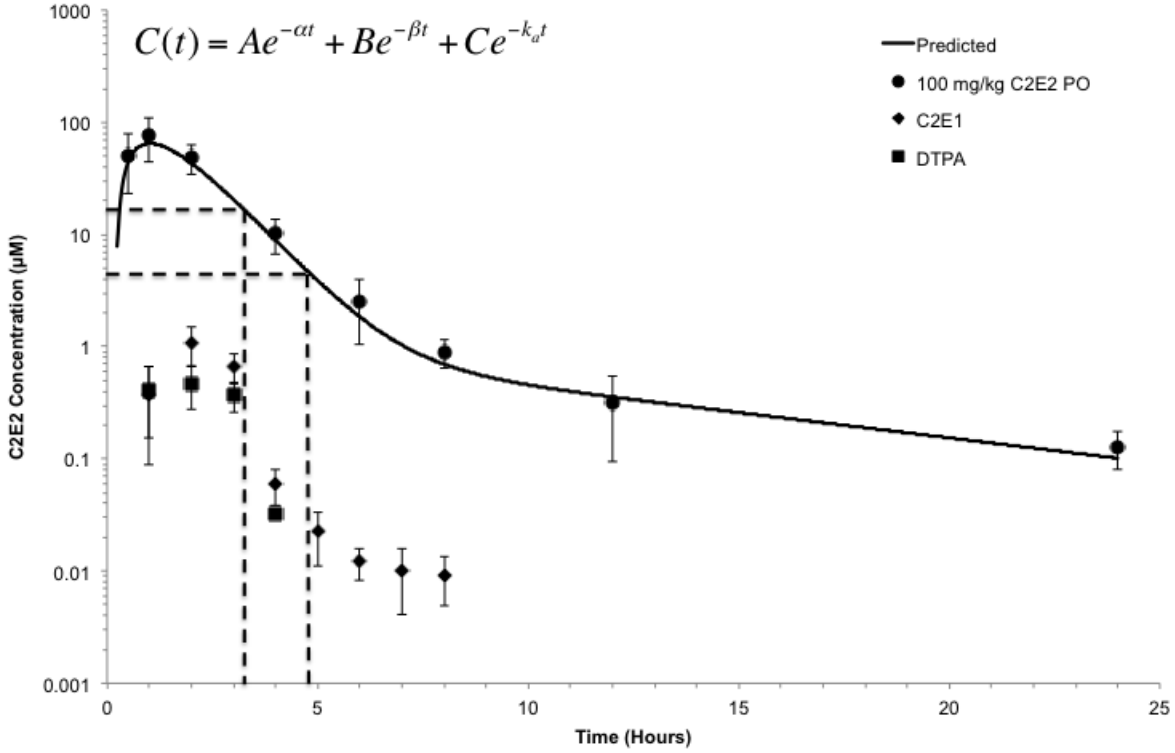


Figure 4.4 The plasma concentrations of C2E2, C2E1 and DTPA after administration of a 100 mg kg^{-1} oral solution. The effective duration based on EC_{50} and EC_{90} values are indicated by dotted lines.



4.4. Discussion

Due to the nature of internal radioactive contamination FDA approval of decorporation agents may proceed under the animal rule, whereby animal studies can be used to provide evidence for effectiveness. Under this rule the mechanism of action must be known and efficacy must be shown in more than one species and both should be shown to react with a response to similar to humans[133]. Exposure to americium is most likely to occur through either inhalation or wound sites from where redistribution to the primary sites of deposition such as the liver and bone will occur. This rate of distribution is based on many factors such as the site of deposition and the rate of solubilization and absorption of the internalized form into the plasma[134].

The primary site of action for C2E2 is the plasma during the redistribution of americium. Once in the plasma ~30 % of the total ^{241}Am is bound to transferrin, ~30 % is associated with additional plasma proteins such as albumin and globulins and the remainder is bound to low molecular weight complexes such as citrate[34]. The americium-transferrin complex has a stability constant (log K_1) of 10.4 and that of the citrate complex is 8.6[35b]. Thus even after removal of two of DTPA's coordination sites, C2E2 retains the ability to bind americium at a strength nine orders of magnitude higher than that of the strongest ligand in the plasma.

In addition to determining the affinity of C2E2 for ^{241}Am, it is useful to determine the concentration required for effective chelation in plasma to aid justification of a specific dose. The data presented here confirms previous research suggesting that there are species differences in the effective concentrations required for chelation of ^{241}Am between species[119]. This has implications for the choice of animal models used to demonstrate efficacy of decorporation agents. For the three ligands examined the concentrations required for chelation in rat plasma were higher than those needed in beagles and humans suggesting that

the efficacious human doses may be better approximated from beagle data. Plasma is a complex solution and the source of the species differences has not been determined. The concentration of transferrin, albumin and other serum globulins which are responsible for binding around 60 % of the ^{241}Am are at similar concentrations across all three species, however the concentration of serum iron is higher in rat than beagle or human plasma. In the case of DTPA iron binds with a higher stability constant (Log K 28.0[135]) and will compete with ^{241}Am, the extra iron present in rat plasma could be one cause for the higher concentrations required.

Although predominantly in the plasma both C2E2 and ^{241}Am also distribute into the interstitial fluid where the plasma protein concentration is lower (only 42 % of the plasma transferrin[136]). In this environment it is likely that the concentrations of C2E2 required to achieve binding are lower than those determined for plasma, therefore the duration of action may be longer than that predicted using effective ligand concentrations derived from plasma.

The americium nitrate salt was used throughout these studies; this is in contrast to the previous studies with DTPA where the citrate salt was used. Comparison of concentrations required for effective DTPA chelation identified a difference between each salt. Differences in the plasma clearance of the citrate and nitrate complexes were reported[123] with americium citrate clearing the plasma faster than the nitrate complex. This may be due to the low affinity of nitrate for americium (log K 1.3[137]) resulting in rapid binding to transferrin as opposed to citrate where the binding to transferrin may occur more slowly.

The administration of chelation therapy via the oral route provides fast, economical and noninvasive access to treatment. In addition oral administration results in a lower C_{Max} than IV treatments but causes an increase in the elimination half-life of the drug due to continued

absorption. Although, C2E2 was designed as a pro-drug there was limited hydrolysis of the esters and therefore low concentrations of C2E1 and DTPA. Though the lack of metabolism should not limit efficacy as after a 100 mg/kg oral dose the effective duration in dogs was increased by 2 hours (doubled) compared to the standard IV treatment. The administration of higher doses of C2E2 may result in further extension of the effective duration and increased concentrations of C2E1 and DTPA that may yield further increases in efficacy.

4.5 Conclusion

Although the number of coordination sites available on the DTPA pro-drugs have been reduced compared with DTPA, C2E2, and its metabolite, C2E1, have maintained high affinity for ^{241}Am. In addition to determining their binding constants relative to DTPA, the concentrations required to achieve binding in plasma have been investigated *in vitro*. As expected higher concentrations of C2E2 are required to achieve the same effect as DTPA. Species differences were noted in the concentrations of ligand required to achieve chelation with beagle plasma most representative of a human response. Oral administration of a 100 mg/kg C2E2 dose to beagles demonstrated that the effective plasma concentrations could be reached. The binding constants and pharmacokinetic parameters determined as part of these studies can be combined with efficacy data into a pharmacokinetic-pharmacodynamic model with the aim of predicting decorporation efficacy from different dosages and dosing regimes.

CHAPTER V

C2E2 EFFICACY, TOXICITY & PHARMACOKINETICS IN RAT

5.1. Introduction

The following two chapters report on the efficacy of C2E2 in rats (Chapter V) and dogs (Chapter VI). Due to the nature of internal radionuclide contamination it is unethical to conduct human clinical efficacy trials. Furthermore, human contaminations are sporadic and inconsistent by nature so the patient population is too small to conduct well-controlled clinical trials. In order to overcome this problem a new pathway for drug approval was established by the FDA, known as the "animal rule". This pathway allows drug approval in the absence of human efficacy trials if a sufficiently well characterized animal model for predicting human response.

It is therefore imperative that the animal models used for demonstrating efficacy are carefully selected. The animal models chosen for this research were based on a range of criteria. Firstly the similarities in uptake, retention and clearance of radionuclides were considered. Unfortunately for americium, no single model accurately predicts the human biokinetics and two species can help to increase the confidence in the animal models response[101]. Therefore rat and dog models were chosen due to the availability of well-established radionuclide pharmacokinetics studies in these species.

Utilizing two animal models also serves an additional benefit, as efficacy can be investigated after different routes of radionuclide exposure. Both inhalation and wound site contaminations were selected as they are the most likely to occur in a mass casualty situation[138]. The choice of which species to contaminate via each route was based on literature precedent and ease of conducting the studies. On initiation of the project there were more than five published studies utilizing a rat inhalation contamination model or a rat wound model. For dogs, there were more than five articles using an inhalation model and only one using a wound model.

Radionuclide clearance from the lung can be considered to occur via two competing pathways, either mechanical clearance or absorption into the blood[139]. The mechanical clearance of particles from the lung is species-dependent, in contrast dissolution and absorption to the blood is relatively independent[140]. The clearance half-life from the lungs of mice and rats, are typically on the order of 30-60 days. In comparison, long-term clearance halftimes of insoluble particles from the lungs of dogs, monkeys and humans are on the order of 900-1500 days[141]. Therefore, the biokinetics of inhaled aerosol particles in the dog's respiratory tract would be most similar to that seen in humans.

The National Council on Radiation Protection and Measurements (NCRP) has developed a biokinetic model based on experimental animal data for wound contamination[142]. The model was based predominantly on data obtained from studies in rats with smaller contributions from mice, rabbits and dogs. NCRP Report 156 concluded that the most important factors affecting the biokinetics of radionuclides in a wound site were the radionuclide and its chemical form, the deposited mass of radionuclide, the tendency of the radionuclide to hydrolyze at neutral physiological pH, and the type and magnitude of wound

injury (puncture, laceration, cut, burn)[143]. The effect of the species on the biokinetics was secondary to these factors, and as such, most experimental animal models were considered appropriate for interpreting human wound-site biokinetics and absorption rates to blood and lymph[142]. Therefore, for C2E2 studies the rat wound model and dog inhalation model was selected. This choice also allowed rat efficacy studies to be conducted at UNC, where contamination via inhalation is not possible.

Another important consideration during the design of efficacy trials was the treatment delay after contamination. For all the studies a delay of 24 hours was chosen, as this is a realistic time frame for delivery of the drug during a mass casualty situation. This also provides a conservative estimate of efficacy, with administration at earlier time-points likely to increase total decorporation and lower tissue retention.

Overall the rat wound and beagle inhalation models are those that most closely resemble human contamination and have the largest volume of supporting data. Decorporation efficacy has been determined after administration of a single dose in both species and for rats multiple daily dosing was investigated for up to ten days. Additionally the bioavailability and pharmacokinetics have been investigated in rats. For both species the maximum tolerated dose has been determined for both a single dose and ten daily doses. The genetic toxicity potential of C2E2 was also evaluated using a standard battery approach including *in vitro* bacterial mutation assay, chromosomal aberration assay and micronucleus test (Chapter VI).

5.2 Methods

5.2.1 Rat Am-Nitrate I.M. Contamination Model

To prepare the injection solutions a 200 μL aliquot was withdrawn from a stock solution of ^{241}Am nitrate (0.1 mCi in 5 mL 1 M HNO_3)(Eckert & Ziegler Isotope Products, Valencia, CA), evaporated to dryness, dissolved in concentrated HNO_3 (15 M, 5 mL) then evaporated to dryness and dissolved in dilute HNO_3 (0.1 M, 8 mL) to form the injection solution. During efficacy studies each rat received 250 nCi of ^{241}Am nitrate by intramuscular injection (0.1 mL, 0.1 M HNO_3) into the right hind leg, under isoflurane anesthesia. In all studies there was a 24-hour delay from contamination to initiation of treatment in order to mimic a real life response time. The delay also allows the consistency of contamination between groups to be determined by comparison of the ^{241}Am excreted within the first 24-hours between each animal.

5.2.2 C2E2 Multiple Dose Decorporation Efficacy in Rats

Groups of 4 female Sprague-Dawley rats contaminated with 250 nCi of $^{241}Am(NO_3)_3$ by IM injection were administered 7 daily oral doses of C2E2 by gavage at 84 or 200 mg/kg under *ad lib* feeding conditions utilizing a 10 % w/w solution of C2E2 in sterile water. Control groups with *ad lib* access to food and water were given 7 daily doses of medium chain triglyceride (MCT) (2 mL/kg) by oral gavage (MCT was the vehicle for another compound in the study). Treatment was initiated 1 day after contamination and was administered daily for 7 days. Animals were euthanized 12 days after contamination and the following tissues were collected: liver, both kidneys, both femurs as well as the ipsilateral

and the contralateral hind leg muscle tissue and pelt from the injection site. Urine and feces were collected from the time of contamination through euthanasia. Cage washings were also collected 24 hours after the last dose (Day 8) and at the end of the study (Day 12). Statistical analysis of endpoint data (total decorporation, liver burden, wound site retention and estimated skeletal burden) was by one-way ANOVA with Tukey's post test. Effects were considered significant at $p < 0.05$.

5.2.3 C2E2 Multiple Dose Decorporation Efficacy in Rats: 5 vs 10 Daily Treatments

Sprague-Dawley rats (N = 8/gender/group) were contaminated with 250 nCi of $^{241}Am(NO_3)_3$ by intramuscular injection. Daily oral gavage treatment with C2E2 (600 mg/kg in water) was started 24 hours after contamination and continued for five (qd x 5 group) or ten days (qd x 10 group); a third group of untreated animals was included as a control. Excreta (urine, feces and cage washes) were collected daily until euthanasia 12 days after contamination at which time select organs were harvested. Statistical analysis of endpoint data (total decorporation, liver burden, wound site retention and estimated skeletal burden) was by one-way ANOVA with Tukey's post test, and analysis of the effect of treatment on daily decorporation was by repeated- measures ANOVA with Tukey's post test, in all analysis effects were considered significant at $p < 0.05$.

5.2.4 Effect of Dosing Schedule on C2E2 Multiple Dose Efficacy in Rats

Sprague-Dawley rats (N = 8/gender/group) were contaminated with 250 nCi of $^{241}Am(NO_3)_3$ by intramuscular injection. Immediately after injection, body weights were recorded (Day 0 weight) and animals placed in individual metabolic cages. The rats were

assigned to four groups; untreated control, 5 daily oral gavage doses of 600 mg/kg C2E2 once daily, 5 twice daily oral gavage doses of 300 mg/kg C2E2 or 5 daily intravenous doses of Zn-DTPA solution (13.3 mg/kg) via a jugular vein catheter. The C2E2 dosing solution utilized was a 10% w/w solution in water. All animals were observed at least once daily for morbidity, mortality, and general appearance. Excreted urine and feces were collected at 2, 4, 8, 10, 12, 14, 18, 20 and 24 hours after the first dose and daily for the remainder of the study. Statistical analysis of endpoint data (total decorporation, liver burden, wound site retention and estimated skeletal burden) was by one-way ANOVA with Tukey's post test. Effects were considered significant at $p < 0.05$.

5.2.5 Efficacy Studies: Tissue Collection and Processing

All excreta were transferred to 20 mL scintillation vials, weighed and placed in a gamma counter (Wizard2 2480, Perkin Elmer) for detection of ^{241}Am gamma activity. In addition following necropsy selected tissues were removed, weighed and placed in a gamma counter (Wizard2 2480, Perkin Elmer) for detection of ^{241}Am gamma activity. The total amount of ^{241}Am administered to the animals was determined by quantifying the 59.7 keV photons emitted by ^{241}Am in duplicate aliquots (100 µL) of the injection solution using a gamma counter (wizard series, Perkin-Elmer). A counting window from 40 – 80 keV and a 60-second counting time were used for acquisition and each reading was corrected for background at acquisition. All experimental tissues and samples were counted using the same gamma counter and protocol. For all the samples, ^{241}Am content was expressed as a percentage of the initial dose. The femur from the leg opposite to the injection site was scaled by a factor of 20 to estimate total skeletal ^{241}Am burden.

5.2.6 Acute Maximum Tolerated Dose of C2E2 in Rats

Fifty Sprague-Dawley rats (Charles River Laboratories; 10–12 weeks) were housed individually with *ad libitum* access to food and water under a 12-hour light/dark cycle. The weights were 267 ± 23 mg and 188 ± 13 mg for the males and females respectively. C2E2 was dissolved in DI water and each rat received 8 mL/kg of dosing solution. Rats (n = 5/sex/dose) were administered vehicle control or C2E2 at 200, 600, 1000 or 2000 mg/kg by oral gavage. The dose was escalated depending upon tolerability of the previous dose based upon clinical signs and clinical chemistry. Clinical observations were conducted pre-dose, approximately 0.5, 1, 2 and 4 hours post dose and twice daily on non-dosing days. Four days after receiving C2E2, rats were euthanized by CO_2 asphyxiation followed by thoracotomy. Blood was collected at necropsy via cardiac puncture and serum chemistry (alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine) was evaluated. Study endpoints were evaluated for normality followed by an ANOVA ($p<0.05$) and, if significant, groups were compared using the Holm-Sidak test. If treatment groups failed the normality test, a Kruskal-Wallis ANOVA ($p<0.05$) on ranks with post hoc Dunn's comparison to the control group was made.

5.2.7 Ten Day Oral Dose Range Finding Study of C2E2 in Rats

Male and female Sprague-Dawley rats (200-400 g; 10-11 weeks of age) were divided into groups of 5 rats/sex each and administered 0 (reverse osmosis water) or 200, 400, 800, or 2000 mg/kg C2E2 utilizing a dose volume of 8.3 mL/kg for up to 10 consecutive days. Parameters evaluated included clinical observations, body weight, food consumption, toxicokinetics, hematology, clinical chemistry, coagulation, urinalysis, trace elements

(blood), gross pathology, organ weights, and histopathology of selected tissues. Satellite rats (n = 3 for control animals; n = 6 per test article group) were used for toxicokinetic (TK) evaluations. The TK animals had blood collected via the jugular vein on Day 1 and Day 10 at 0.25, 1, 3, 6, 12, and 24 hours post dose. A pre-dose sample was collected on Day 10 only. The TK time points for the two cohorts of C2E2-treated rats and the time points selected for the vehicle control animals are summarized in Table 5.1. Blood samples were rapidly transferred to vacutainer tubes (containing 5 mg sodium fluoride; 4 mg potassium oxalate). These tubes were inverted several times to ensure proper mixing and were maintained on wet ice or chilled cryorack. Samples were then centrifuged at 1,300 x g for 10 minutes at 4 °C within 15 minutes of collection. Aliquots (125 µL) of plasma were split into two chilled 2.0 mL microcentrifuge tubes containing 125 µL 20% formic acid and were frozen on dry ice and stored at -80 °C until analysis. Analytical evaluation was performed by the UNC GLP Bioanalytical Facility as described in Chapter III (Calculated concentrations: C2E2, DTPA; Estimated concentrations: C2E3, C2E1). Pharmacokinetic parameters were derived by non-compartmental methods for C2E2, C2E1, and DTPA using WinNonlin® Professional Version 6.2.1 (Pharsight Corp.).

5.2.8 Determination of Oral Bioavailability in Rats

A single dose pharmacokinetic study of C2E2 in Female Sprague-Dawley rats was performed. Jugular-cannulated Sprague-Dawley rats (226-250 g; n = 3 per time point) were divided into two groups and administered either a single IV (16.9 mg/kg; caudal vein) or PO (84 mg/kg) dose of C2E2 (water) under *ad lib* conditions. All rats had a pre-dose sample drawn on the day of dosing. Blood was collected from the jugular catheter (0.7 mL per draw; 5 blood draws/animal) at 0.083, 0.25, 0.5, 1, 3, and 8 h post dose. Blood samples were

collected by syringe and immediately transferred to chilled vacutainer tubes containing 5 mg sodium fluoride and 4 mg potassium oxalate. These tubes were inverted to ensure proper mixing and placed on ice until centrifugation. Tubes were centrifuged at 1,300 x g for 10 minutes at 4 °C within 5 minutes of collection. Aliquots of plasma were then divided into chilled 1.7 mL microcentrifuge tubes within 5 minutes of centrifugation. Analysis for C2E2, C2E1, and DTPA in plasma was performed at the UNC GLP Bioanalytical Facility. Pharmacokinetic parameters in plasma were determined from concentration-time profiles by established non-compartmental methods using WinNonlin® Version 5.2.

5.2.9 Pharmacokinetics of ^{14}C-C2E2 in Rats

In order to further investigate the pharmacokinetics of C2E2, its metabolites and determine the fraction absorbed an additional single dose pharmacokinetic study in Male Sprague Dawley rats was performed using ^{14}C-C2E2. Jugular- and portal-cannulated Sprague-Dawley rats (263-298 g; n = 3 per time point) were divided into four groups. Either a single IV (16.9 mg/kg; jugular or portal vein) or PO (600 mg/kg) dose of C2E2 (water) under *ad lib* conditions or fasted conditions. Blood was collected from the jugular catheter (0.7 mL per draw) at 0.083, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post dose. Blood samples were collected by syringe and immediately transferred to chilled vacutainer tubes containing 5 mg sodium fluoride and 4 mg potassium oxalate. These tubes were inverted to ensure proper mixing and placed on ice until centrifugation. Tubes were centrifuged at 1,300 x g for 10 minutes at 4°C within 5 minutes of collection. Urine was collected at 2, 4, 8, 12 and 24 hours and feces were collected at 4, 8, 12 and 24 hours.

Analysis for C2E2, C2E1, and DTPA in plasma and urine was performed using a HPLC (Prominence UFLC; Shimadzu, Columbia, Maryland) with a flow-through radiochemical detector (Radiomatic 650TR; PerkinElmer). The HPLC method was the same as previously described for C2E2 analysis except detection and quantification occurred in a 120 μL flow cell using standard measurement parameters for ^{14}C rather than using the CAD detector.

In addition total ^{14}C content was analyzed by taking aliquots of plasma and urine, adding directly to 10 mL of scintillation cocktail (Ultima Gold; PerkinElmer, Waltham, Massachusetts) and analyzing by liquid scintillation counting (LSC; Tri-Carb 3110 TR, PerkinElmer). The LSC data were corrected for quenching using an external standard. Feces, were diluted with a 1:1 mixture of acetonitrile and pH 4, 25 mM citrate buffer and homogenized in polycarbonate vials containing steel grinding balls. The homogenization was performed by shaking the vials on the high throughput homogenizer (PowerGen; Thermo Fisher Scientific, Waltham, Massachusetts) for 5–8 min at high speeds. Approximately 200 mg of each homogenate was sampled and combined with 1 mL of solvable (PerkinElmer) and heated in an oven at 60°C for 1–3 h until digestion was visibly complete. Once the samples were cooled, hydrogen peroxide (30% in water) was added in two aliquots of 100 μL to decolorize the sample. The samples were returned to the 60°C oven for an additional 1 h. After cooling, each sample was diluted with 10 mL of scintillation cocktail and analyzed by LSC. The radioactivity measured in each sample was weight-corrected to determine the total radioactivity in each whole tissue.

Table 5.1 Toxicokinetic Blood Collection Schedule.

Group	Set	Day of Dosing	Time Points
1	Three/sex	1, 10	1, 6, and 24 hours postdose
2, 3, 4, 5	1st three/sex/group	1, 10	0.25, 3, and 12 hours postdose
2, 3, 4, 5	2nd three/sex/group	1	1, 6, and 24 hours postdose
		10	Predose, 1, and 6 hours postdose

5.3. Results

5.3.1 C2E2 Multiple Dose Decorporation Efficacy in Rats

Extending the administration of C2E2 from a single dose to seven daily doses resulted in a significant enhancement in total ^{241}Am elimination. Table 5.2 and Figure 5.1 show total decorporation and residual liver burden at euthanasia after 7 daily oral doses of C2E2 compared to MCT control. Administration of seven daily doses of either 84 or 200 mg/kg C2E2 to female rats, resulted in the total decorporation of 21.6 ± 1.3 or 32.3 ± 3.7% of the total injected dose, respectively. These results are similar to those achieved with a single 600 mg/kg or 1000 mg/kg dose where total decorporation is 24.2 ± 3.3% and 31.3 ± 5.5%, respectively. Therefore, after 7 daily administrations, a 5-fold reduction in daily dose can achieve similar decorporation efficacy as a single dose. As with DTPA the enhancement in elimination was predominantly due to increased urinary clearance.

Liver retention of ^{241}Am was significantly reduced ($p<0.01$) after multiple doses. The repeated administration of 84 mg/kg/day was as effective as a single 1000 mg/kg dose resulting in a liver burdens of 14.2 ± 2.1%, and 13.7 ± 1.3%, respectively. Skeletal burden was not significantly reduced by either dose.

Although increased decorporation was observed over control at both treatment levels, only the 200 mg/kg dose was significant ($p <0.001$) and administration of multiple IV DTPA doses further enhanced total decorporation and reduced liver burden compared to C2E2 (Table 5.2 and Figure 5.1).

Table 5.2 Total ^{241}Am Decorporation (Mean ± SD), represented as percent of the administered ^{241}Am dose, after treatment with increasing doses of orally administered C2E2 or intravenously administered DTPA. Daily dosing began 24 h after contamination and continued for a total of 7 days. n = 4 for all groups.

Drug Dose (mg/kg)	Urine (%)	Feces (%)	Cage wash (%)	Total Decorporation (% Injected)	Liver burden (% Injected)	Skeletal burden (% Injected)
0	3.5 ± 0.9	10.2 ± 3.4	1.7 ± 0.8	15.3 ± 4.7	16.0 ± 3.1	11.9 ± 3.5
84	8.8 ± 1.9	9.1 ± 1.1	3.8 ± 1.3	21.6 ± 1.3	14.2 ± 2.1	13.0 ± 1.7
200	14.1 ± 2.3	13.4 ± 3.3	4.7 ± 1.9	32.3 ± 3.7	7.9 ± 2.1	9.6 ± 1.7
14 (DTPA)	31.9 ± 4.6	13.5 ± 2.7	8.6 ± 4.2	53.9 ± 8.6	2.7 ± 0.6	6.9 ± 0.9

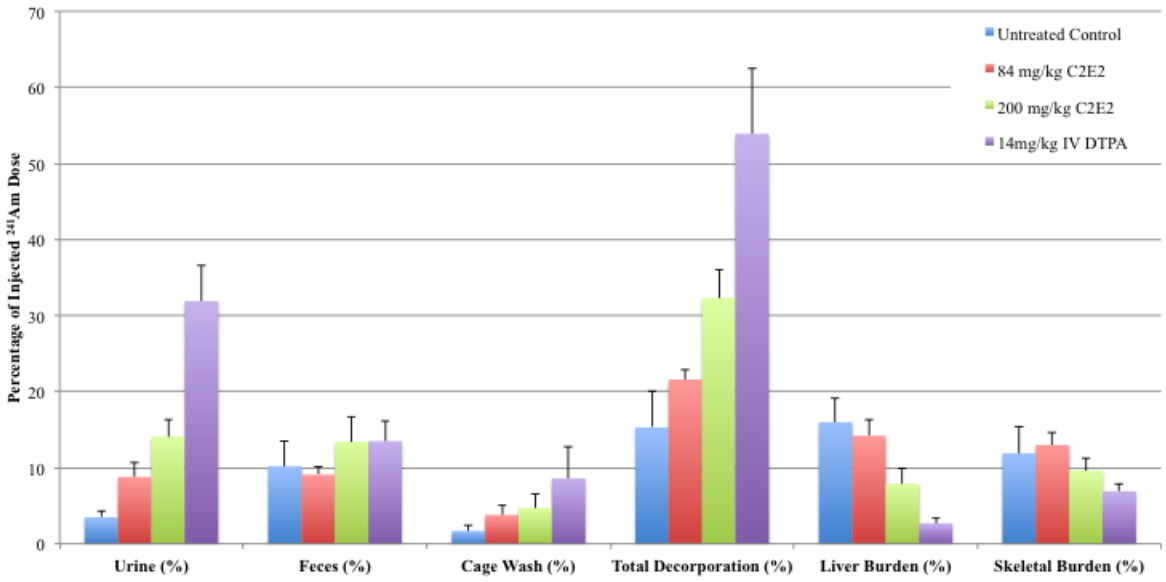


Figure 5.1 Total ^{241}Am Decorporation (Mean ± SD), represented as percent of the administered ^{241}Am dose, after treatment with increasing doses of orally administered C2E2 or intravenously administered DTPA. Daily dosing began 24 h after contamination and continued for a total of 7 days. n = 4 for all groups.

5.3.2 Effect of Dosing Schedule on C2E2 Multiple Dose Efficacy in Rats

The data obtained for male and female rats during this study have been separated due to differences previously observed in americium biokinetics (Chapter III), though the trends observed for C2E2 in this study are similar for both genders. In addition data from two rats (one qd x 5 and one qd x 10, both male), which died due to gavage error, were excluded from analyses. The radionuclide clearance was the same across the three treatment groups on Day 1 before treatment commenced, indicating the contaminations were consistent across each group (Figure 5.2).

Treatment with C2E2 at 600 mg/kg significantly increased decorporation in both male and female rats compared with untreated control animals ($p<0.001$) (Table 5.3). In both genders total decorporation was higher in the qd x 10 group compared to the qd x 5 group, although the increase was only statistically significant in the female animals. Figure 5.2 clearly demonstrates that the first dose is most effective at enhancing elimination, with subsequent doses resulting in diminishing daily enhancement of ^{241}Am elimination. During the first five days of C2E2 treatment, equivalent decorporation was obtained in animals in the qd x 5 and qd x 10, indicating consistent dose administration and additional treatment beyond five days resulted in a significant but decreasing benefit. In male rats, every dose of C2E2 in both the qd x 5 and qd x 10 arms resulted in a significant increase in decorporation compared with untreated controls. In female rats all doses of C2E2 except the ninth and tenth doses in the qd x 10 treatment group resulted in a significant increase in decorporation compared with untreated controls. In both sexes decorporation returned to levels equivalent to or lower than in untreated controls within 48 hours of the last administered dose.

Both the liver (Table 5.4) and skeletal (Table 5.5) burdens were statistically lower following treatment with C2E2 at 600 mg/kg compared to control animals ($p<0.001$). Following ten daily treatments the mean liver burden appears lower than five daily treatments, however as with total decorporation the difference was only significant in female animals ($p<0.05$). Similarly, the skeletal burden is only reduced to a significant extent in female rats after when treatment is increased from five to ten daily doses ($p<0.001$).

There is a clear benefit of repeated daily treatment with C2E2. This study suggests that there is an additional benefit to continuing C2E2 treatment beyond five days. The return, expressed as the additional radionuclide decorporation achieved for each additional dose administered, appears to decrease approximately linearly from the third dose onward and the data suggest little or no additional benefit would be observed for treatment beyond ten daily doses.

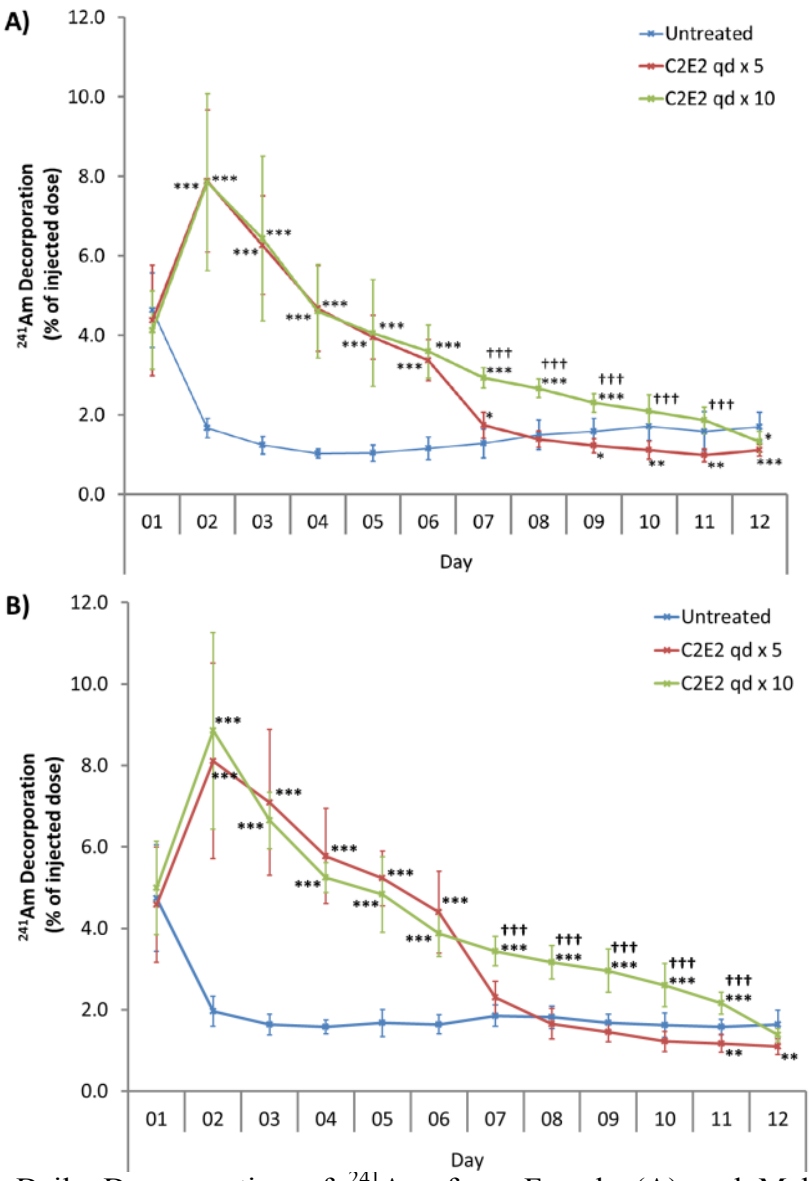


Figure 5.2 Daily Decorporation of ^{241}Am from Female (A) and Male (B) Sprague-Dawley Rats as a Percentage of the Injected Dose. The effects of treatment with C2E2 (600 mg/kg) administered by daily oral gavage for five (C2E2 qd x 5; red) or ten (C2E2 qd x 10; green) days are compared with each other and with untreated controls (blue). Excreta were collected daily from animals contaminated with ^{241}Am (250 nCi in 0.1 M HNO3) by intramuscular injection (0.1 mL). Animals were contaminated at Day 0 and C2E2 administration began on Day 1. Therefore, the excreta collected in the 24 hours following the last C2E2 dose is reported on Day 6 for the qd x 5 group and on Day 11 for the qd x 10 group. Data shown are the means ± standard deviation from 7 – 8 animals per group. A repeated-measures ANOVA with Tukey's post test was performed to probe differences between the treatments at each time point with differences considered significant at $p < 0.05$. * indicates a comparison with the untreated group and † indicates a comparison between the qd x 5 group and qd x 10 groups. The level of confidence for the comparisons is indicated by the number of symbols, e.g. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 5.3 Total ^{241}Am decorporation 12 days after contamination[1]

Group	Male			Female		
	Mean ± SD	n	p	Mean ± SD	n	p
Untreated	23.4 ± 3.1	8	-	20.1 ±3.0	8	-
C2E2 600 mg.kg qd x 5	44.1 ± 6.2	7	<0.001	38.1 ± 4.0	8	<0.001
C2E2 600 mg.kg qd x 10	50.1 ± 4.2	7	<0.001	43.9 ± 5.8	8	<0.001*

[1]As a percentage of the administerd dose. Statistical analysis was by one-way ANOVA with Tukey's post test. Differences considered significant ($p < 0.05$) are reported for comparison with untreated control groups.
*indicates a significant difference also between the qd x 5 and qd x 10 groups ($p < 0.05$).

Table 5.4 Total ^{241}Am liver burden 12 days after contamination[1]

Group	Male			Female		
	Mean ± SD	n	p	Mean ± SD	n	p
Untreated	12.5 ± 3.0	8	-	19.5 ± 1.9	8	-
C2E2 600 mg.kg qd x 5	5.9 ± 1.1	7	<0.001	12.0 ± 2.0	8	<0.001
C2E2 600 mg.kg qd x 10	3.9 ± 1.1	7	<0.001	9.2 ± 2.4	8	<0.001*

[1]As a percentage of the administerd dose. Statistical analysis was by one-way ANOVA with Tukey's post test. Differences considered significant ($p < 0.05$) are reported for comparison with untreated control groups.
*indicates a significant difference also between the qd x 5 and qd x 10 groups ($p < 0.05$).

Table 5.5 Total ^{241}Am skeletal burden 12 days after contamination[12]

Group	Male			Female		
	Mean ± SD	n	p	Mean ± SD	n	p
Untreated	27.7 ± 4.4	8	-	23.4 ± 2.5	8	-
C2E2 600 mg.kg qd x 5	13.4 ± 1.7	7	<0.001	14.5 ± 1.1	8	<0.001
C2E2 600 mg.kg qd x 10	11.6 ± 1.5	7	<0.001	10.8 ± 1.1	8	<0.001***

[1]As a percentage of the administerd dose. Skeletal burden is estimated by multiplying the burden detected in the femur of the leg contralateral to the americium injection by a factor of 20.
[2]Statistical analysis was by one-way ANOVA with Tukey's post test. Differences considered significant ($p < 0.05$) are reported for comparison with untreated control groups.
***indicates a significant difference also between the qd x 5 and qd x 10 groups ($p < 0.001$).

5.3.3 C2E2 Multiple Dose Decorporation Efficacy in Rats: QD vs BID Administration

All sixteen male and female rats completed the study. The elimination of americium was significantly enhanced in all treatment groups compared to the untreated control ($p<0.001$). Dividing the C2E2 dose in half and administering twelve-hourly did not significantly alter total decorporation (Table 5.6). The americium tissue burden in select tissues is shown in Figure 5.3 and Figure 5.4 for male and female rats, respectively.

In addition to examining total decorporation and tissue burden, the urinary elimination profile of americium over time was obtained. Figure 5.5 shows the profiles of urinary decorporation after once and twice daily treatment with C2E2 in male rats. By looking at the first 24-hours after administration of the initial C2E2 dose, the increase in urinary americium elimination over control is clear. When a divided dose was given every 12 hours, americium elimination was initially enhanced the effect was smaller than that of the single dose. A second elimination peak was observed after 14 hours following administration of the second daily dose. This peak was also larger than the first, which suggests that C2E2 was still present from first dose. However, as urine was not manually expressed in this study the time of day and the activity of the animals may also affect the profile. Over each 24-hour period of the study, the QD and BID groups result in very similar profiles of daily urinary elimination; consistent with the hypothesis that drug AUC is the best indicator of efficacy in the rat.

Division of the C2E2 dose into two equal doses resulted in no significant difference in efficacy ($p<0.05$) compared to a single dose for both male and female rats. This approach may therefore be used to lower the dose required for each administration and increase the therapeutic window for C2E2.

Table 5.6 Total ^{241}Am decorporation 12 days after contamination following five days of a 600 mg/kg C2E2 dose administered either once daily or divided between two equal doses[1].

Group	Male			Female		
	Mean ± SD	n	p	Mean ± SD	n	p
Untreated	17.8 ± 2.4	4	-	15.9 ± 0.8	3	-
Daily C2E2	39.7 ± 3.0	4	$p < 0.01$	34.8 ± 6.0	4	$p < 0.01$
Twice Daily C2E2	43.6 ± 1.8	4	$p < 0.01$	28.9 ± 1.5	4	$p < 0.01$
IV DTPA	54.0 ± 4.5	4	$p < 0.01$	51.3 ± 2.3	4	$p < 0.01$

[1]As a percentage of the administered dose. Statistical analysis was performed by one-way ANOVA with Tukey's post test. Differences considered significant ($p < 0.05$) are reported for comparison with untreated control groups.

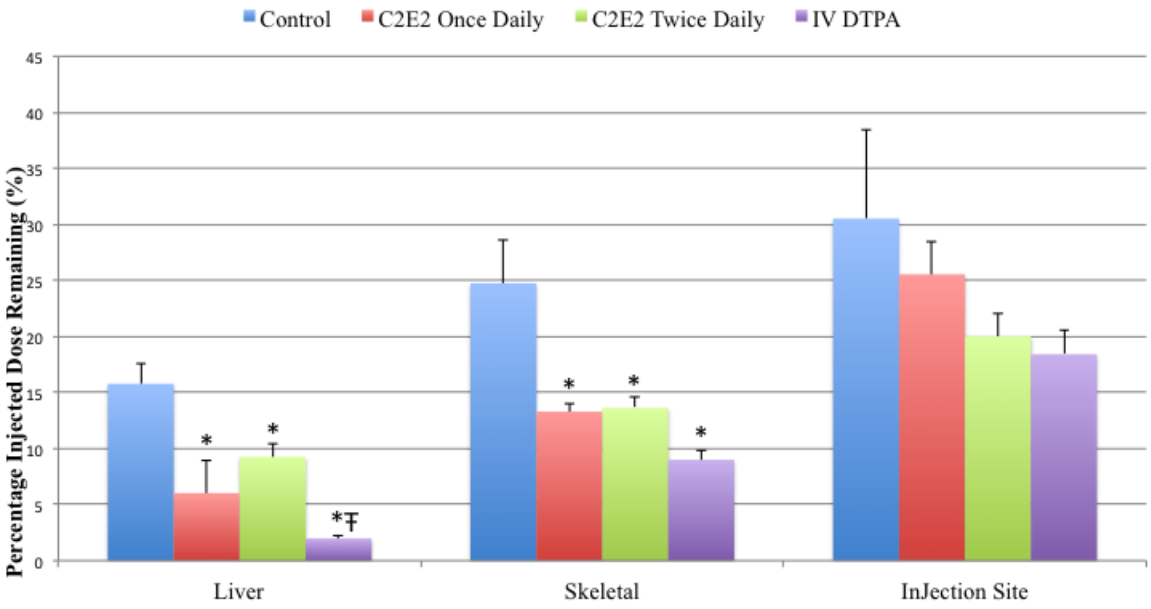


Figure 5.3 Total ^{241}Am Decorporation (Mean ± SD), represented as percent of the administered ^{241}Am dose, after treatment with either once or twice daily orally administered C2E2 or intravenously administered DTPA to male SD rats. Daily dosing began 24 h after contamination and continued for a total of 12 days. n = 4 for all groups. Statistical analysis was performed by one-way ANOVA with Tukey's post test. * ($p < 0.01$ compared to control), Ŧ ($p < 0.01$ compared to C2E2 BD)

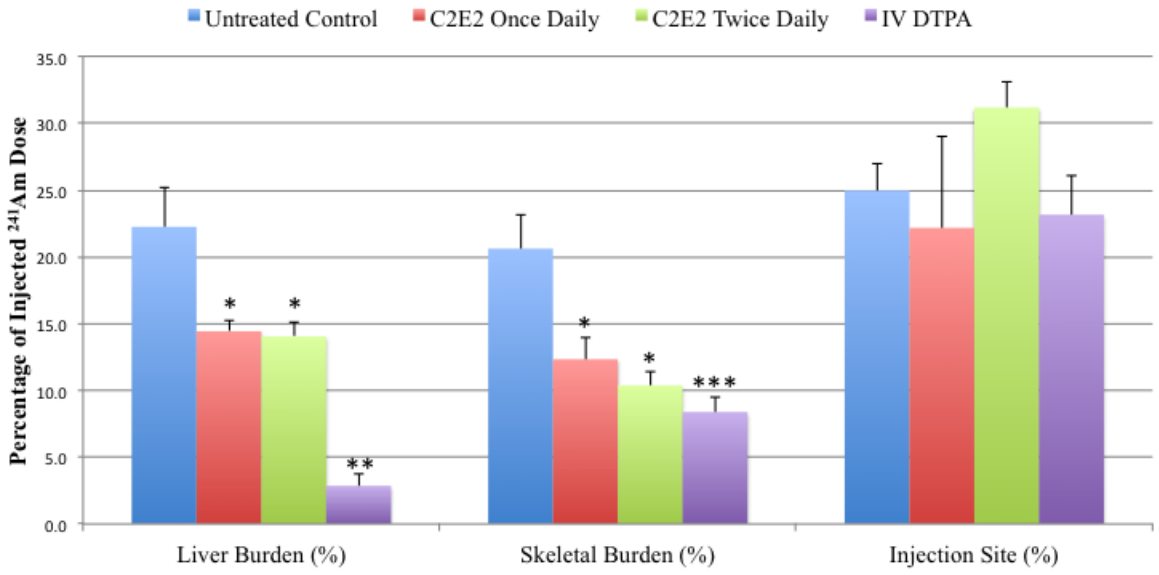


Figure 5.4 Total ^{241}Am Decorporation (Mean ± SD), represented as percent of the administered ^{241}Am dose, after treatment with either once or twice daily orally administered C2E2 or intravenously administered DTPA to female SD rats. Daily dosing began 24 h after contamination and continued for a total of 12 days. n = 4 for all groups. Statistical analysis was performed by one-way ANOVA with Tukey's post test. * ($p < 0.01$ compared to control), ** ($p < 0.01$ compared to C2E2 Once and Twice Daily), *** ($p < 0.05$ compared to C2E2 Once Daily)

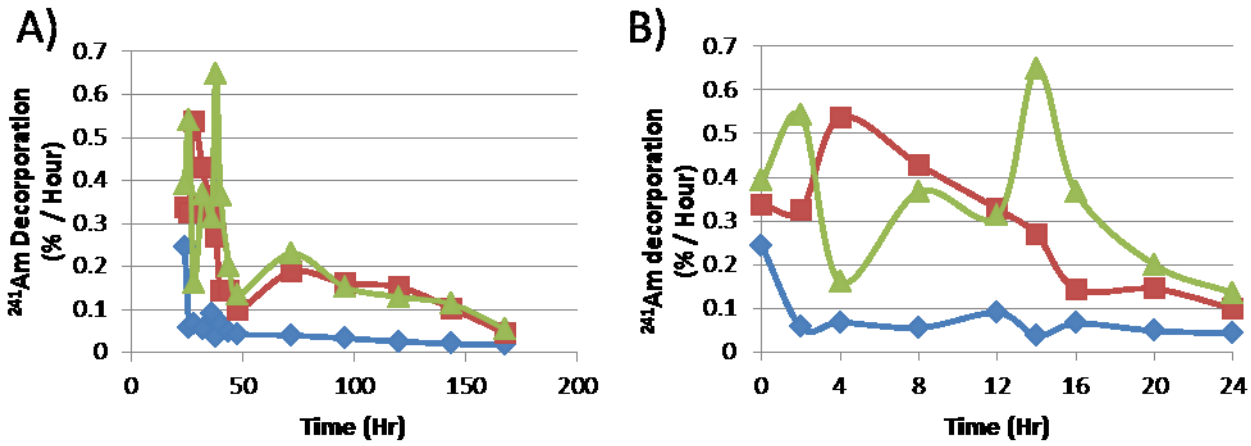


Figure 5.5 Percentage of initial americium injection eliminated in the urine per hour in male rats. A) ^{241}Am concentration in urine excreted over the duration of the study. B) Expanded view starting 24 hours after americium contamination as the first C2E2 dose was administered. The percent decorporation per hour at time zero reflects the average hourly decorporation over the first 24 hours of the study. Blue - Untreated control, Red – C2E2 600 mg/kg QD, Green - C2E2 300 mg/kg BID.

5.3.4 Acute Maximum Tolerated Dose of C2E2 in Rats

After a single dose of C2E2, no mortality or test article-related clinical observations for male or female rats were identified at any dose level. Additionally, there were no significant changes in the clinical chemistry endpoints at any dose levels. No abnormal gross pathological findings were identified following macroscopic examinations performed at necropsy in rats receiving doses ≤ 600 mg/kg. At ≥ 1000 mg/kg, there was a small incidence of pale lesions in the jejunum, stomach or spleen, however, due to the lack of dose-response for each observation, they were not considered to be test-article related. The single-dose no-observed-adverse-effect-level (NOAEL) was determined to be 2000 mg/kg in rats.

5.3.5 10 Day Maximum Tolerated Dose of C2E2 in Rats

No test article-related clinical observations occurred in rats given ≤ 800 mg/kg/day. Rats in the highest dose group, receiving 2000 mg/kg/day were sacrificed on day 4 of the dosing phase due to clinical observations indicating declining health characterized by fecal abnormalities (few, liquid, mucoid, or nonformed), poor food consumption, and hypoactivity. Based on microscopic examination, the cause of morbidity was gastrointestinal lesions present in 4/5 males and 1/5 females. Specifically, elevation/loss of the keratinized layer of the nonglandular stomach, blunting/fusion of duodenal villi, attenuated cecal mucosa, and congested cecal mucosa was present in some rats given 2000 mg/kg. Microscopic individual cell degeneration in the mucosal epithelium of gastrointestinal tract was also noted in females given ≥ 800 mg/kg and males given ≥ 400 mg/kg however, these findings were not considered adverse due to the minimal severity of changes, which were only slightly greater than a normal background observation, and the absence of clinical signs. In addition to

gastrointestinal lesions rats given 2000 mg/kg/day also had degeneration of acinar cells of the pancreas and tubules of the kidneys. C2E2-related clinical pathology effects for animals administered 2000 mg/kg were small and did not indicate specific target organ toxicity. Notable effects included: minimally higher absolute red blood cell count, hemoglobin, and hematocrit (males only); mildly lower absolute reticulocyte and lymphocyte counts; and mildly higher glucose, albumin and total protein levels. The findings for red blood cell mass and serum proteins were consistent with hemoconcentration and likely related to the fecal abnormalities observed clinically. Other findings were likely associated with poor condition, poor food consumption, or a stress (endogenous corticosteroid) response. Below 2000 mg/kg/day C2E2-related clinical pathology effects were limited to mildly lower urea nitrogen in animals administered 800 mg/kg/day and mildly higher albumin in females administered >400 mg/kg/day. The exact mechanism for these minor effects was not determined; however they were not considered toxicologically important or indicative of specific target organ toxicity. A preliminary toxicokinetic (TK) analysis was performed on mean C2E2, C2E1, and DTPA concentration-time data determined at each time point for each sex. A plasma sample was not collected at 24 hours post dose on Day 10; for TK analysis, concentrations at this time point were assigned the Day 10 pre-dose concentration, based on an assumption that steady-state conditions had been achieved. TK parameters for all analytes are reported in Tables 5.7 to 5.8. C2E2 was the predominant compound observed in the systemic circulation. In general, C2E2 C_{max} and AUC values increased with C2E2 dose in both sexes. C2E2 exposures at 400 mg/kg and above were greater in female than in male rats on Day 1. On Day 10, there was little difference between sexes in C2E2 exposures at any dose level except for the 400 mg/kg group, in which C2E2 C_{max} in males was about one-fourth that in females.

Table 5.7 C2E2 toxicokinetic parameters in Sprague-Dawley rats after oral gavage doses of C2E2 for 10 days (based on mean concentrations from 3 animals/sex at each time point).

Day	Dose (mg/kg)	Sex	C_{max} (ng/mL)	C_{max}/Dose (ng/mL)/(mg/kg)	T_{max} (hr)	$AUC_{(0\text{-}tlast)}$ (hr*ng/mL)	$AUC_{(0\text{-}tlast)}$/Dose (hr*ng/mL)/(mg/kg)
1	200	F	906	4.53	0.25	2280	11.4
		M	857	4.29	1.00	3230	16.2
	400	F	17,500	43.8	0.25	22,000	55.0
		M	1820	4.55	0.25	7740	19.4
	800	F	14,800	18.5	1.00	38,100	47.6
		M	5670	7.09	1.00	18,300	22.9
	2000	F	43,000	21.5	0.25	139,000	69.5
		M	24,100	12.1	1.00	94,000	47.0
10	200	F	2770	13.9	0.25	3580	17.9
		M	2970	14.9	0.25	4140	20.7
	400	F	6320	15.8	0.25	10,300	25.8
		M	1550	3.88	0.25	6240	15.6
	800	F	23,700	29.6	0.25	28,000	35.0
		M	18,600	23.3	0.25	35,100	43.9
	2000	F	ND	ND	ND	ND	ND
		M	ND	ND	ND	ND	ND

NA – Not applicable
ND – No data

Table 5.8 C2E1 toxicokinetic parameters in Sprague-Dawley rats after oral gavage doses of C2E2 for 10 days (based on mean concentrations from 3 animals/sex at each time point).

Day	Dose (mg/kg)	Sex	C_{max} (ng/mL)	C_{max}/Dose (ng/mL)/(mg/kg)	T_{max} (hr)	$AUC_{(0\text{-}tlast)}$ (hr*ng/mL)	$AUC_{(0\text{-}tlast)}$/Dose (hr*ng/mL)/(mg/kg)
1	200	F	0	0.0000	NA	0	0.0000
		M	0	0.0000	NA	0	0.0000
	400	F	127	0.3175	0.25	81.3	0.2033
		M	19.2	0.0480	0.25	15.9	0.0398
	800	F	65.7	0.0821	1.00	204	0.2550
		M	57.6	0.0720	1.00	184	0.2300
	2000	F	364	0.1820	0.25	1250	0.6250
		M	235	0.1175	1.00	1280	0.6400
10	200	F	18.3	0.0915	0.25	NC	NC
		M	20.8	0.1040	0.25	NC	NC
	400	F	66.4	0.1660	0.25	56.0	0.1400
		M	36.0	0.0900	0.25	30.9	0.0773
	800	F	124	0.1550	0.25	223	0.2788
		M	253	0.3163	0.25	464	0.5800
	2000	F	ND	ND	ND	ND	ND
		M	ND	ND	ND	ND	ND

NA – Not applicable
NC – Could not be calculated; only one measurable data point in curve
ND – No data

5.3.6 Determination of Oral Bioavailability in Rats

Following both oral and IV administration, the parent molecule C2E2 was the most abundant circulating analyte in rats. C2E1 and DTPA were observed in plasma at substantially lower levels than C2E2. The synthetic impurity, C2E3, was also observed in plasma of rats at very low levels after an IV dose of C2E2 (C2E3 was not detected after the oral C2E2 dose). Peak concentrations were attained at 0.033 hours (t_{max}) for C2E1, C2E2, C2E3, and DTPA following IV administration and 0.25 hours for C2E1, C2E2, and DTPA following oral administration. The terminal half-life for C2E2 was estimated to be 0.35 and 1.1 hours following the IV and oral doses, respectively, although these may underestimate the true half-life values, as sampling was only performed through 8 hours post dose. For example, C2E2 concentrations were measurable through 24 hours post dose in rats after a single dose and after 10 days of once-daily oral administration during the dose-range finding toxicity study. During the toxicity study oral doses had terminal half-lives of 0.25-1 hour.

Oral bioavailability was calculated from the area under the plasma concentration vs. time curve (AUC) values of C2E2 and under these experimental conditions the absolute oral bioavailability of C2E2 in female Sprague-Dawley rats was 29%.

4.3.7 Pharmacokinetics of ^{14}C-C2E2 in Rats

All sixteen rats successfully completed the study. The inability to synthesize large quantities of ^{14}C-C2E2 resulted in low plasma ^{14}C concentrations and HPLC could not be used to determine the species present. The samples were therefore analyzed by liquid scintillation counting, however the plasma ^{14}C concentrations were still too low to determine concentration time profiles over 24 hours. High ^{14}C concentrations were observed in the

urine and the ^{14}C content must arise from C2E2 absorbed prior to elimination. During the study oral groups were administered a 600 mg dose containing 50 mg of ^{14}C-C2E2 and IV groups received 50 mg of pure ^{14}C-C2E2. Assuming that 99% of the dose is eliminated within 24 hours, then a comparison between the total quantity of ^{14}C observed in the urine of IV and oral groups can be used to approximate the fraction of C2E2 absorbed. For fed and *ad lib* rats the fraction of ^{14}C material seen in the urine compared to the IV group was 0.42 and 0.48, respectively. This suggests that oral absorption was greater than 40% after administration of a 600 mg dose. This is higher that the fraction noted in the previous pharmacokinetic study that utilized an oral dose of 84 mg/mL and supports evidence of non-linear exposure seen with C2E2 in toxicokinetic studies.

5.4 Discussion

The efficacy of orally administered C2E2 after a single dose was previously discussed in Chapter 1. The study demonstrated that compared to untreated controls ^{241}Am retention could be significantly reduced orally using C2E2 in a dose dependent manner (Tables 5.9 and 5.10). The increased elimination of ^{241}Am was primarily due to enhanced urinary excretion consistent with other highly charged hydrophilic compounds such as DTPA. Although americium elimination was enhanced over control, large doses were required and IV DTPA therapy remained superior. In addition to demonstrating oral efficacy, no statistical difference in americium burden was observed between rats with *ad libitum* access to food and rats fasted overnight prior to C2E2 treatment. Therefore food effects were not investigated during future studies.

Oral decorporation therapy, in addition to increasing the promptness at which therapy can be given in a mass casualty situation, is also more conveniently administered over multiple doses than IV therapy. Administration of multiple daily IV DTPA doses has previously been used to enhance decorporation. We hypothesized that radionuclide decorporation provided by C2E2 could similarly be enhanced with multiple administrations and that the daily dose could be lowered reducing the pill burden. Therefore a multiple dose study was conducted over twelve days with C2E2 administered for seven consecutive days.

C2E2 was administered at doses of 84 and 200 mg/kg with total decorporation significantly higher than untreated control animals at 200 mg/kg with 32.3 ± 3.7 % of the injected dose eliminated. In comparison total decorporation from a single 200 mg/kg C2E2 dose in female rats after 7 days was 14.4 ± 0.7 %. This confirms that the additional doses further enhance americium elimination. Although mainly due to C2E2, the multiple dose

study was conducted over an additional five days, which also contributed to the increased decorporation as control animals eliminated 11.2 ± 2.1 % and 15.3 ± 4.7 % for 7 and 12 days, respectively. Furthermore, after 7 daily 200 mg/kg doses of C2E2, total decorporation begins to approach that of a single 14 mg/kg IV DTPA treatment (36.1 ± 4.6 %) and liver burden is reduced from 12.3 ± 1.2 % to 7.9 ± 2.1 % suggesting that an extended oral regimen may be able to replace the single IV DTPA dose in a mass casualty situation. By comparison, total decorporation following administration of C2E5 was 8.4 ± 1.0 %, 14.8 ± 4.2 % and 18.5 ± 4.4 % following single doses of 40, 100 and 200 mg/kg to rats contaminated by IM injection of ^{241}Am-nitrate[125]. These decorporation values are lower than expected, with the control animals eliminating only 7.1 ± 1.4 % of the injected americium after 7 days. This may be due to use of a slightly different injection site in early studies conducted in our lab. Therefore to more accurately compare single dose C2E2 to C2E5 the percentage increase in elimination over control was considered (Table 5.11). This demonstrates that C2E2 is less effective that C2E5, with approximately a six fold increase in dose required to achieve the same effect. Further increases in efficacy are obtained with multiple doses of C2E5 and to achieve the same effect, the C2E2 dose must be approximately 2.5-3.5 fold higher than that of C2E5 (Table 5.12).

While, the multiple dose study successfully demonstrated that repeated dosing enhances decorporation, it also promoted further questions regarding the duration of therapy. In the majority of cases DTPA has been given as a single dose; in severe cases multiple doses have been administered. In these cases the urinary americium levels are monitored to ensure therapy continues to provide a benefit. In order to investigate the effects of treatment duration on C2E2 efficacy the study protocols were modified to include daily cage washes in

place of every three days. This minimized the variation observed in daily excretion data and allowed inter-day comparisons of americium elimination to be made.

The 600 mg/kg dose was selected for additional efficacy studies in rats based on data obtained during the single dose decorporation and toxicology studies. Following administration of five daily doses total decorporation was 44.1 ± 6.2 % and 38.1 ± 4.0 % in male and female rats, respectively. Similarly to the previous multiple dose study, repeat dosing showed a clear benefit over a single dose in which total decorporation was 26.4 ± 1.4 % and 23.6 ± 2.3 % for male and female rats, respectively. Prolonging the duration from five to ten days further increased the total decorporation to 50.1 ± 4.2 % and 43.9 ± 5.8 % in male and female rats, however this increase was not statistically significant.

Figure 5.2 clearly shows that the greatest elimination is achieved after the first dose with subsequent doses yielding successively smaller returns. Upon contamination, a fraction of the radionuclide administered rapidly distributes from the wound site into the plasma resulting in initially high plasma levels. This can be seen in control animals excretion profiles, where approximately 4.5% of the administered dose is eliminated within the first 24-hours. Thereafter, only a small percentage (~2%) is released each day. Consequently, initially there is a large fraction of translocating radionuclide available for chelation and elimination as shown by the large percentage of decorporation in the initial days (7-8 %/day). After wound contamination in rats, americium rapidly distributes to the liver over 12 hours followed by slower uptake until day 4 when constant elimination begins to occur[138]. Distribution to the skeleton is also fast with the majority of deposition occurring within 4 hours, however translocation continues to deposit material within the skeleton and steady state is not reached until 4 days[138]. Therefore, as each day progresses not only does the total

amount of americium remaining decrease, the percentage deposited within the skeleton increases, thereby lowering the fraction available for elimination.

Although increasing the duration from five to ten days did not result in a significant increase in total decorporation overall, each additional day resulted in a significant increase in decorporation over control. Therefore extending therapy for ten days is beneficial and as ionizing radiation causes cancer through stochastic effects, any enhancement in elimination is beneficial and thus therapy should be given until no significant increase in daily decorporation is seen over control.

Ultimately treatment duration should be determined by a number of factors, including route of contamination, amount of radionuclide deposited and age of the patient at time of contamination. A balance must be struck between the risk posed from additional chelation treatment and the benefit of removing another fraction of radionuclide. Furthermore as the duration of therapy is extended, the daily dose must be reduced to minimize toxicity, thereby reducing the benefit of treatment at early time-points where tissue disposition is greatest. The use of a shorter time course is therefore preferable.

The pharmacokinetics of C2E2 was determined in female rats after administration of C2E2 via both the oral and IV routes. Oral absorption was fast with t_{max} achieved in 15 minutes. With a half-life of 1.1 hours, the C2E2 duration of action is longer than with IV DTPA, as expected of an oral product, although the majority of the drug will be cleared within 6 hours. During toxicokinetic studies C2E2 exposure increased with increasing dose level from 200 to 2000 mg/kg. The increase in C_{max} and AUC_{0-24} however was greater than dose proportional. There were no significant differences between genders and no accumulation of the dose was observed after multiple dosing. The bioavailability of C2E2

was calculated from the dose normalized AUCs of both the oral and IV doses. The oral bioavailability of DTPA was increased from 5% to 29% through the addition of two ethyl esters. Although oral absorption of greater than 90% is required to classify a drug as highly permeable, C2E2 has sufficient bioavailability to be delivered orally and further enhancement maybe possible through modification of the formulation.

In order to further investigate the oral absorption of C2E2 a second pharmacokinetic study was performed using ^{14}C-C2E2. The purpose of the study was two fold; to determine the oral bioavailability at the higher dose of 600 mg/kg and to determine if C2E2 or its metabolites were the predominant species in urine. C2E2 was administered orally, via the jugular vein and via the portal vein in order to determine the total oral bioavailability, the fraction absorbed and fraction metabolized during the first pass. Unfortunately the ^{14}C-C2E2 sample concentrations were insufficient to enable speciation via HPLC and so the total ^{14}C content was determined by scintillation counting. Although unable to provide exact speciation data an approximation of the fraction absorbed can be determined by comparing the total ^{14}C content excreted in the urine over the duration of the study. The results suggested that the fraction absorbed was > 40% supporting toxicokinetic studies in which larger doses result in greater exposure.

In hope of further enhancing decorporation or lowering the pill burden the daily dose was split evenly into two 12-hourly doses. The aim was to help maintain plasma levels of C2E2 so that chelation could occur for longer over each 24-hour period. After five days treatment there was no significant difference in total decorporation between each group in either male of female rats. However liver burden was lower in the single daily dose group than those receiving the split dose. As the AUCs for both treatments are equal, these results

suggest that decorporation is linked to AUC and not C_{max}. Though, a high C_{max} may contribute to lower ^{241}Am liver burden as both a single dose, and IV DTPA treatments result in enhanced decorporation. In addition to assessing decorporation, the rate of americium elimination in urine were studied during the first 24 hours to determine differences between each group. The urinary excretion profiles (Figure 5.5) showed that the majority of the effect of C2E2 occurs within the first 2-4 hours after administration. For the twice-daily dose, a second peak in excreted americium is observed after administration of the second dose, interestingly this peak is higher than the first, suggesting that urinary elimination is yet to reach background levels 12 hours after administration of the first dose. After 24 hours no C2E2 accumulation was observed in the 10 day toxicokinetic analysis; accumulation may be possible with dose intervals of 12 hours. For DTPA even though approximately 60% of the dose is eliminated in 1.4 min, a small fraction (~0.5%) remains in the plasma after 24 hours with small amounts continuing to be excreted over 7 days[64]. Although small, this fraction may be responsible for the increase in americium elimination seen for a number or days following a single dose. For C2E2 a small faction may also be present but not detectable.

Urine was also collected for the 24 hours after contamination prior to treatment delivery. The urinary elimination profile for untreated controls follows second order kinetics with rapid elimination of ^{241}Am occurring over the first 4-8 hours, followed by a gradual decline over the remainder of the study. Four days after contamination natural excretion is less than 0.02 % ID/hour. If the ^{241}Am elimination profile is assumed to be proportional to free circulating ^{241}Am it is easy to see why chelation efficacy is reduced over time, and why early access to treatment will enhance decorporation.

In addition to demonstrating efficacy in well-characterized animal models, radionuclide decorporation agents must still comply with all the standard FDA toxicological requirements. In order to conduct human clinical trials, non-clinical safety studies must initially be performed. These non-clinical studies generally encompass toxicity studies, nonclinical pharmacokinetics, reproduction toxicity and genotoxic studies. For toxicity studies both MTD and repeated dose studies are conducted in two mammalian species (one nonrodent). The duration of the repeated dose study relates to the duration of proposed clinical trials. For C2E2, toxicity was assessed in both rats and dogs (Chapter VI). For both species in addition to determining the MTD, multiple dose toxicity was assessed for 10 days based on the fact that little additional therapeutic benefit is likely to be achieved with additional doses.

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 rats indicates that large doses of C2E2 are well tolerated. After single doses no adverse effects were noted and the maximum tolerated dose was therefore set at the highest dose tested (2000 mg/kg). The toxicity of C2E2 was then evaluated for 10 days. Again no organs were identified as the targets of toxicity, however gastrointestinal lesions caused morbidity at doses of 2000 mg/kg/day. After 10 consecutive daily doses the NOAEL for C2E2 was 800 mg/kg. This is higher than the doses used during all multiple dose studies.

In comparison, a dose escalation study performed with C2E5 used doses of 0, 20, 200, 600 or 1000 mg/kg in 300 mM acetic acid, pH 4.3. Mortality occurred at doses of 1000 mg/kg and weight loss (up to 16%) was noted at doses of 600 mg/kg. Following 10 days

studies abnormal posture, urine staining, mortality was seen at doses $\geq$ 250 mg/kg. In addition increased ALT, AST, total bilirubin and creatinine levels were also seen at doses $\geq$ 250 mg/kg. The NOAEL was determined to be 114 mg/kg/day for the 10 day study.

Overall, orally administered C2E2 is not as efficacious as IV DTPA therapy in rats. Though after administration of seven daily 200 mg/kg doses total decorporation approaches that of a single IV dose of DTPA. Furthermore, after five days of C2E2 treatment at 600 mg/kg/day total decorporation is higher than a single dose of C2E2. As the majority of patients contaminated receive only a single dose of DTPA, a short course of C2E2 may be a viable substitute in a mass casualty situation.

In order to achieve effective decorporation in rats large doses are required. Based on the differences in plasma binding data described in chapter IV, the rat may not be the best model for predicting human dose levels. For both C2E2 and DTPA higher concentrations of ligand are required in rat plasma to achieve the same degree of ^{241}Am chelation as human plasma. The data also suggests that C2E2 efficacy may be further underestimated compared to that of DTPA. For DTPA the ligand concentration must be 1.9 times higher in rats to achieve the same effect as in humans, whereas for C2E2 a 5.2 fold increase is required. Therefore C2E2 efficacy is expected to be greater in the beagle model and the doses required more representative of those required in humans.

Table 5.9 Mean (± SD) ^{241}Am Decorporation 7 Days after IM Contamination in Male SD Rats: Treatment with a Single Oral Dose of C2E2 24 h after Contamination

C2E2 Dose (mg/kg)	Urine (%)	Feces (%)	Cage Wash (%)	Total Decorporation (%)	Liver Burden (%)
0	5.8 ± 1.4	6.6 ± 2.4	2.3 ± 0.8	14.6 ± 2.1	17.2 ± 2.8
200	11.3 ± 2.3	6.9 ± 0.6	2.8 ± 0.2	20.9 ± 2.7	10.6 ±0.8
200[1]	11.8 ± 1.3	6.1 ± 1.2	2.7 ± 1.1	20.6 ± 3.2	12.8 ± 3.9
600	14.3 ± 3.5	8.8 ± 1.9	3.5 ± 1.5	26.4 ± 1.4	13.2 ± 1.3
600[1]	16.6 ± 1.1	7.9 ± 1.7	3.5 ± 1.6	27.9 ± 2.5	10.4 ± 0.8
1000	21.9 ± 5.3	8.5 ± 2.2	3.7 ± 1.2	34.1 ± 8.0	8.7 ± 2.0
1000[1]	20.7 ± 3.7	8.5 ± 1.9	3.5 ± 1.5	32.8 ± 6.6	10.1 ± 1.9
DTPA Dose (mg/kg)	**Urine (%)**	**Feces (%)**	**Cage Wash (%)**	**Total Decorporation (%)**	**Liver Burden (%)**
14	22.2 ± 2.8	12.1 ± 2.0	4.4 ± 1.2	38.7 ± 4.5	6.1 ± 0.7

[1]Fasted Condition; other groups allowed access to food *ad libitum*

Table 5.10 Mean (± SD) ^{241}Am Decorporation 7 Days after IM Contamination in Female SD Rats: Treatment with a Single Oral Dose of C2E2 24 h after Contamination

C2E2 Dose (mg/kg)	Urine (%)	Feces (%)	Cage Wash (%)	Total Decorporation (%)	Liver Burden (%)
0	4.7 ± 1.0	4.9 ± 1.0	1.6 ± 0.5	11.2 ± 2.1	21.4 ± 2.5
200	6.7 ± 1.5	5.9 ± 0.8	1.8 ± 0.5	14.4 ± 0.7	17.7 ± 2.1
200[1]	8.4 ± 1.3	4.7 ± 2.1	1.3 ± 0.3	14.3 ± 3.2	20.9 ± 3.5
600[2]	13.1 ± 0.4	8.4 ± 1.7	2.1 ± 1.0	23.6 ± 2.3	19.1 ± 5.4
600[1]	14.3 ± 2.9	7.3 ± 1.5	2.6 ± 0.8	24.2 ± 3.3	18.9 ± 2.3
1000[3]	14.8 ± 1.3	7.7 ± 2.4	3.1 ± 0.1	25.7 ± 3.5	15.7 ± 0.9
1000[1]	19.8 ± 3.9	7.1 ± 1.3	4.5 ± 0.7	31.3 ± 5.5	13.7 ± 3.1
DTPA Dose (mg/kg)	**Urine (%)**	**Feces (%)**	**Cage Wash (%)**	**Total Decorporation (%)**	**Liver Burden (%)**
14	22.1 ± 2.6	10.3 ± 1.7	3.6 ± 0.8	36.1 ± 4.6	12.3 ± 1.2

[1]Fasted Condition; other groups allowed access to food *ad libitum*
[2]Group size = 3
[3]Group size = 2

Table 5.11 Percentage increase in ^{241}Am decorporation over control, after treatment with single doses of orally-administered C2E2 or C2E5. Dosing began 1 day after contamination and continued the study was terminated after 7 days. n = 4 for all groups

	^{41}Am Decorporation (% of Administered Dose)	
Drug Dose (mg/kg)	**C2E5**	**C2E2**
40	18.3 %	-
100	108.5 %	-
200	160.5 %	37.2 %
600	-	93.7 %
1000	-	131.8 %

Table 5.12 Total ^{241}Am decorporation, represented as percent of the administered ^{241}Am dose, after treatment with increasing doses of orally-administered C2E2 or C2E5. Daily dosing began 1 day after contamination and continued for a total of 7 days. n = 4 for C2E5 and n=6-8 for C2E2 groups.

	^{41}Am Decorporation (% of Administered Dose)	
Drug Dose (mg/kg)	**C2E5**	**C2E2**
0	7.1 ± 1.4	12.9 ± 2.1
40	8.4 ± 1.0	-
100	14.8 ± 4.2	-
200	18.5 ± 4.4	17.7 ± 2.0
600	-	25.0 ± 1.9
1000	-	29.9 ± 6.2

CHAPTER VI

ORALLY ADMINISTERED DTPA DI-ETHYL ESTER FOR DECORPORATION OF ^{241}AM: EFFICACY IN A DOG INHALATION-CONTAMINATION MODEL AND SAFETY ASSESSMENT IN DOGS

6.1 Introduction

This increasing terrorist threat has spurred the development of radionuclide decorporation agents for treatment of individuals contaminated with various radioactive materials, notably isotopes of the transuranic elements Am, Pu and Cm. In 2004, pentetate calcium trisodium injection (Ca-DTPA) and pentetate zinc trisodium injection (Zn-DTPA) received 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, 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[144]. 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[145]. Esterification is often used to overcome barriers in drug delivery such as poor solubility[87] or absorption[146]. The carboxylic acids present on DTPA provide multiple sites for esterification that we hypothesized could be used for prodrug formation to 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 as prodrugs for oral bioavailability enhancement[88, 90, 147]. 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[148].

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[94-95], 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. 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.

6.2 Materials and Methods

6.2.1 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[90] (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 AAALAC accredited facilities.

6.2.2 Beagle Inhalation Contamination Model for ^{241}Am

Inhalation contamination was performed in a method similar to that previously described[138]. The beagles were fasted, sedated (acepromazine i.m.; 0.05 mg/kg.) and anesthetized with Isoflurate (5%) 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 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 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 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.

6.2.3 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. Twenty-four hours after contamination, C2E2 solutions were administered by oral gavage. Dogs received either DI water (vehicle control) or C2E2 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, 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.

6.2.4 Acute Maximum Tolerated Dose of C2E2 in Dogs

Four beagles (Covance Research Products Inc.), (n = 2/sex/dose), 8 to 10 months old 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 food 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. Toxicokinetic analyses for C2E2, the mono ethyl-ester of DTPA (C2E1) and DTPA were performed using WinNonlin Professional Edition (Pharsight Corporation, Version 5.2).

6.2.5 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 food 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.

6.2.6 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 ICH S2(R1)[149]. 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[150, 151]. 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 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 (PCEs) per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) observed while scoring at least 500 erythrocytes per animal.

6.3 Results

6.3.1 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 (500 mg/kg) producing significant increases in decorporation over the 300 mg/kg dose ($p<0.01$). As shown in Figure 6.1, urinary excretion of ^{241}Am increased in a dose-dependent manner, which persisted for the duration of the study. Urinary excretion was 8-fold higher than control for 100 and 300 mg/kg doses and 13-fold higher at 500 mg/kg. Fecal elimination showed modest enhancement over control with all dose levels for three days after treatment before returning to control levels. Treatment with doses ≥ 300 mg/kg resulted in a 50 % increase in fecal elimination of ^{241}Am, significantly higher than control ($p<0.05$).

In addition to evaluating enhancements in urine and fecal ^{241}Am elimination, assessing the ability of a decorporation agent to prevent uptake of ^{241}Am into body tissues, particularly the liver, kidney and bone, is important. Significant reductions in liver, kidney and lung ^{241}Am burden were observed at all dose levels ($p<0.05$). Figure 6.2 demonstrates that a dose-dependent decrease in tissue burden was only observed in both 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.

6.3.2 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. In some cases, transient emesis, excessive salivation and fecal abnormalities occurred that did not affect the overall health of the dog and, therefore, were not considered 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 dogs exhibited increased alanine and aspartate aminotransferase (ALT and AST) activities indicating hepatocellular injury, though this did not uniformly affect all dogs. As evident in Table 6.1, systemic exposure to C2E2 (AUC_{0-24}) generally increased in a dose proportional manner, and there was no greater than a 2-fold difference in mean C2E2 C_{max} and AUC_{0-24} values between sexes. A reduction in C2E2 exposure was observed under fed conditions compared to the fasted state. Specifically, 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. Overall, a single dose of C2E2 by oral gavage to male and female dogs at escalating doses resulted in minimal, non-adverse clinical observations at all dose levels, and the maximum tolerated dose was identified as 750 mg/kg.

6.3.3 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 was observed after administration of multiple doses. As in the acute maximum tolerated dose study, transient emesis and/or fecal abnormalities 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 NOAEL of 200 mg/kg/day for 10 days of oral dosing in beagles was established.

6.3.4 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 PCEs.

The mutagenicity of C2E2 was evaluated in five bacterial tester strains at doses from 5 to 5000 μg/plate in the presence and absence of 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 in the absence or presence of S9 metabolic activation (Table 6.2). All vehicle and positive control values were within acceptable ranges. These results indicate that C2E2 was not mutagenic in the Ames assay for bacterial gene mutation up to 5000 μg/plate in the presence and absence of 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. The male rat vehicle control values had micronucleated PCE means of 0.05 ± 0.6% and the positive control, cyclophosphamide, induced statistically significant increases in micronucleated PCEs (2.14 ± 0.60%). Thus, it was concluded that there were no statistically significant or treatment-related increases in micronucleated PCEs at any C2E2 dose level examined.

Table 6.1 Systemic C2E2 exposure following oral administration of single escalating doses in dogs (n = 2/sex).

Dose Level (mg/kg/day)	Sex	C_{max} (ng/mL)	AUC_{0-24} (ng.hr/mL)
100	M	39900	91950
	F	30700	79780
300	M	74650	230600
	F	108800	263100
750	M	267000	530400
	F	245500	498900
300*	M	3335	32860
	F	3555	25930

*Indicates dosed under fed conditions

Table 6.2 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)

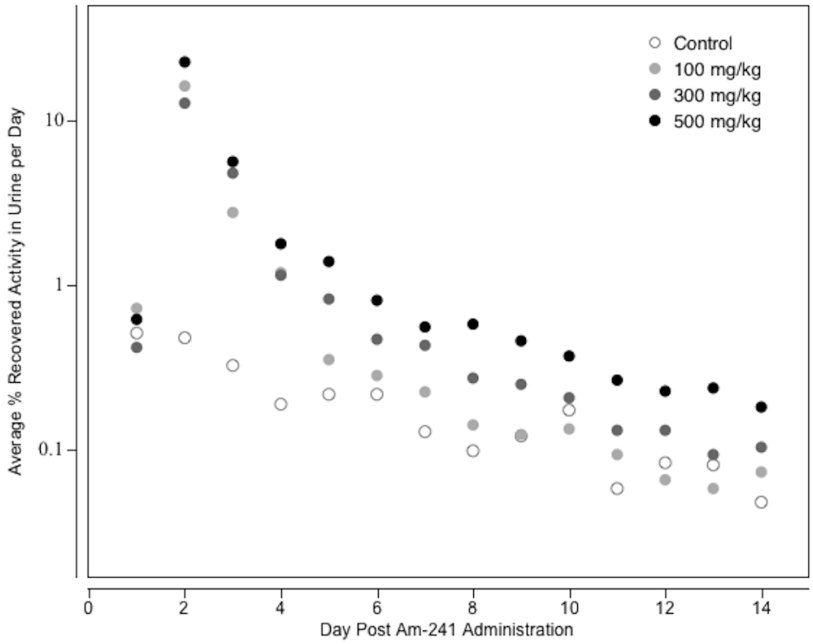


Figure 6.1 Daily urinary excretion after a single oral administration of C2E2 to dogs 24 hours after inhalation contamination with ^{241}Am nitrate.

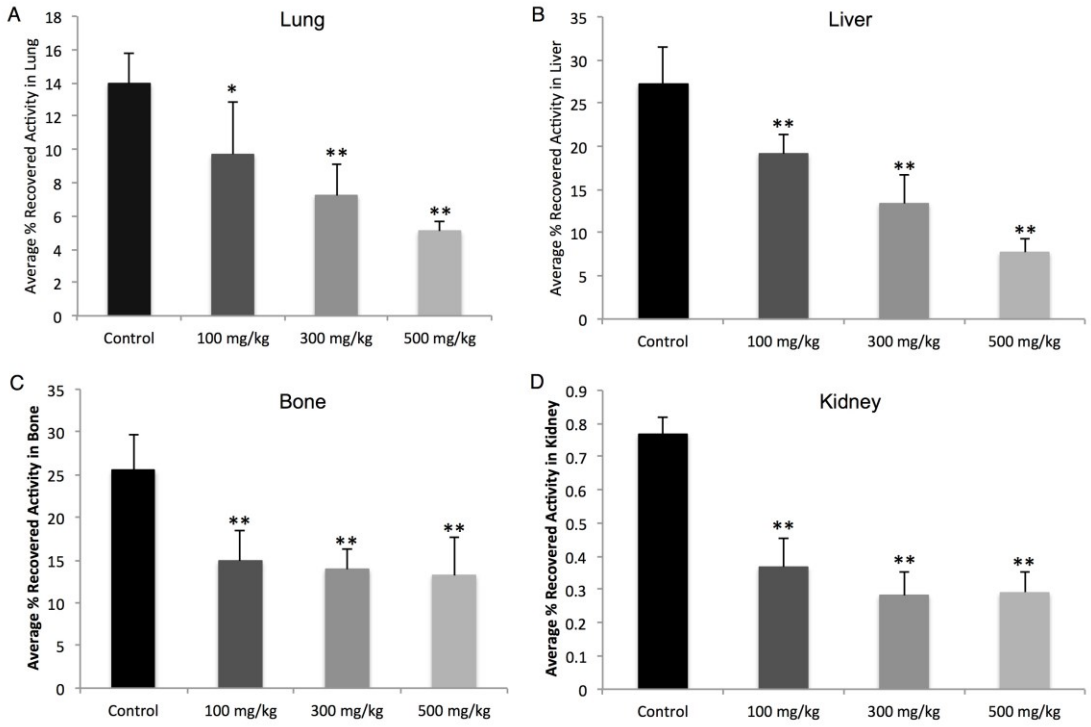


Figure 6.2 Percent of respirable ^{241}Am dose remaining in lung (A), liver (B), total bone (C) and kidney (D) in dogs administered different doses of C2E2 at 24 h after inhalation contamination with ^{241}Am nitrate (*$p<0.05$ against control group, **$p<0.01$ against control group).

6.4 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[119]. 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[105]. 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[96-97].

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-h 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[141]. 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[140]. The rate of particle clearance in dogs and monkeys are a better approximation of human clearance than rodents[141]. For these non-rodent species, human ^{241}Am biokinetics in other compartments are better approximated in dogs[148], 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[152]. 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)[153] 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[154.] 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 that, although may delay release, will 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. Although only twice as high as the lowest effective single dose, it is presumed that lower repeat doses would be required to achieve the same effect. The therapeutic index is determined by the ratio of a drug's ED_{50} and LD_{50}. Unlike traditional diseases in which the ED_{50} 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[71]. Consequently a balance must be struck between decorporation efficacy and toxicity. In order 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[149]. 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.

6.5 Conclusion

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 NOAELs were identified. 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 an effective orally-administered medical countermeasure for treating 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.

CHAPTER VII

PERSPECTIVE AND CONCLUSIONS

7.1 Discussion

Since transuranic elements were first discovered research has been conducted to understand their distribution[37] and health effects after internalization while simultaneously methods to enhance elimination were developed incase of accidental contamination[33]. Due to differences in each radionuclides physiochemical properties, pharmacokinetics and atomic structure the most efficient therapies are specific to each radionuclide[63]. For elimination of plutonium and americium, IV DTPA is the only FDA approved treatment available[58]. Although effective, IV therapy is not suited for use during a mass contamination scenario. Following the success of DTPA a number of approaches were investigated in hope of further increase radionuclide elimination. As DTPA resulted improved efficacy over EDTA addition of a further ethylene bridge produced triethylenetetramine hexaacetic acid (TTHA)[155]. TTHA had higher metal binding affinities though this did not translate to improved efficacy[156]. Esterified DTPA was also utilized in hope of improving plutonium decorporation from tissues however due to limited efficacy and toxicity this approach was abandoned[90].

In 2005 the NIH and NIAID evaluated the ability to respond to a number of terrorist threats and recognized that there was an unmet need for oral decorporation agents[98]. This led to the funding of three approaches for the development of an oral alternative to DTPA, including SRI International (Menlo Park, CA), Nanotherapeutics Incorporated (Alachua, FL) and the lab of Dr. Jay at the University of Kentucky[106].

Under the BCS, DTPA is can be categorized as a class III compound. Traditionally for such compounds permeability can be enhanced through the use of efflux inhibitors, formulation approaches or prodrugs. The oral bioavailability of DTPA is limited due to its charge. Masking the charge through prodrug formation results in permeability enhancement and this approach was shown to be effective with the DTPA-pentaethyl ester, C2E5[95] and with partially esterified TTHA analogues[157]. However the increased lipophilicity of these compounds resulted in toxicity. This is likely due to increased cellular absorption and chelation of essential metals[88]. Formation of a partially esterified DTPA molecule, such as C2E2, lowers the net charge while increasing lipophilicity. The log D values for C2E5, C2E2 and DTPA were 2.4[94], -2.1 and -2.9[117], respectively. Although only modestly enhanced over DTPA, bioavailability was improved from 3[110] to 29 % in rats, higher than either competing product as discussed later.

One of the major concerns with the use of prodrugs is their stability[158]. Limited hydrolysis was observed in simulated fluids and so both esters on C2E2 will remain intact prior to absorption. Ideally once absorbed the esters should be metabolized to reveal the parent compound DTPA. Unfortunately this was not the case for C2E2, however as six chelation sites were available the chelation of americium was preserved. Thus, C2E2 is itself an active molecule and cannot be considered a prodrug, though any ester liberation in the plasma will yield a more effective chelator.

The ionization constants determined for C2E2 are similar to other bi-functionalized DTPA analogues[130]. Due to the presence of three acid and three basic groups with pKas spanning the pH range, C2E2 will always be ionized, except for pH 2.7 where the net charge is neutral and a fraction of the compound will be unionized. The six coordination sites, allow

C2E2 maintain the ability to chelate americium albeit at lower concentrations than DTPA. The Log K value for americium binding is 19.6, this is similar to that of EDTA (log K 18.2[135]).

Although differences in radionuclide binding to plasma proteins had previously been recognized[34], the effect on DTPA concentrations required for chelation was only recently investigated[119]. As efficacy must be demonstrated in animal models these differences can help to select the appropriate species. In the present work the americium nitrate salt was used in place of citrate to ensure consistency across *in vitro* and *in vivo* data. Differences were noted between the binding of americium citrate and nitrate salts by DTPA. These differences could stem from the higher association of citrate for americium than nitrate (Log K 8.6 vs 1.8). The nitrate salt may bind to transferrin more readily increasing the concentration of DTPA required compared with the citrate salt. The *in vitro* binding data shows that the dog model is the most appropriate for establishing efficacy of C2E2 in humans as although efficacy may be slightly overestimated when using the dog model, the difference is much smaller than the underestimation observed in rats. In rats a 5-fold increase in C2E2 is required to achieve the same effect as in humans, where as only a 2-fold increase is required for DTPA. This means that the rat model unfairly discriminates against C2E2 compared to DTPA when used to predict human doses. Furthermore the difference in glomerular filtration rate (GFR) between species causes further underestimation of efficacy in the rat model. The dog GFR is similar to humans but still high, any differences in binding efficacy may be more than compensated for due to increased exposure. While the dog model offers a better representation of efficacy in humans, investigations in rats still have value especially when

compared to DTPA. The rats also offer a cost effective way to evaluate different dosing regimens, prior to conducting expensive dog decorporation studies.

The approaches of Nanotherapeutics and SRI are based on enhanced DTPA formulations. Briefly, Nanotherapeutics has prepared nanoDTPA by micronizing DTPA and zinc acetate (approximately 2:1 ratio) in a jet mill until a size of < 100 microns was obtained. Enteric-coated capsules were then filled with 470 mg of the micronized powder (300 mg DTPA)[159]. Similarly, SRI has formulated DTPA into enteric coated capsules containing a permeation enhancer that caused bioavailability to increase to 12 % in rats[119].

The efficacy of C2E2 in rats has been established after administration of both single and multiple doses. Maximum decorporation was seen after administration of 10 daily doses of 600 mg/kg with 50.1 and 43.9 % of the injected dose eliminated over 12 days for both male and female rats, respectively. Under all conditions tested the efficacy of C2E2 was lower than that achieved with DTPA, though as the duration of therapy increases the efficacy gap is reduced. In rats five daily 600 mg/kg doses of C2E2 were as effective as a single IV DTPA administration in lowering total decorporation, though liver and skeletal burdens were not as effectively reduced. As the majority of people contaminated with americium are given a single dose of DTPA[160], a short oral course of C2E2 could be used as alternative treatment. The DTPA controls used during the experiments conducted at UNC were given as bolus injections as appose to infusions, the high C_{max} associated with these injections may be the reason for the high liver burden reductions and increase in americium elimination via the fecal route seen with DTPA treatments.

For C2E2 greater efficacy is seen in dogs than rats. Single doses of 100 mg/kg led to significant increases in total decorporation over control. There was no DTPA control for this

study and the all published DTPA data uses multiple injections. There is also no comparable data for the efficacy of competing products in either rats or dogs as IV contamination models and different durations were used during efficacy studies limiting the accuracy of comparisons. However, the pharmacokinetics of NanoDTPA and the toxicity of both competing products have been reported. A Nanotherapeutics pharmacokinetic study in male dogs, showed that when given orally a Zn-DTPA solution has a bioavailability of 3 %. The NanoDTPA formulation increased oral DTPA exposure 1.5-3 fold (based on AUC_{0-24})[105] however the doses were 3-6 times larger, consequently the formulation reduced bioavailability to 1.5 % in dogs.

The toxicity of DTPA is relatively low and large cumulative doses (580 g) have safely been administered to humans[161]. The target organs for DTPA toxicity are the kidneys, liver and GI tract, though doses 200 fold higher than those used in humans were required to observe these toxicities[160]. As the NanoDTPA product is mainly composed of DTPA its toxicity was low with 700 mg/day/dog (~70 mg/kg/day) doses tolerated for 14 days with no adverse effects observed aside from emesis. The maximum tolerated dose for the SRI product was 7560 mg/day and the NOAEL 1325 mg/kg/day in dogs and 1000 mg/kg/day (highest dose tested) in rats for 7 daily doses[119]. Again emesis was noted in dogs and there were no significant toxicological findings, confirming the low toxicity of DTPA. The disadvantage of using permeation enhancers is that absorption of ingested radionuclides may be increased in contaminated individuals. Also for those heavily contaminated the gastrointestinal mucosal may be damaged by radiation and so further disruption by permeation enhances is a concern.

The NOAELs were identified as 800 and 200 mg/kg/day in rats and dogs, respectively after 10 daily administrations. These are lower than those obtained for the DTPA

products, though this was expected as C2E2's bioavailability and consequently exposure is higher. Previous studies with lipophilic chelators have also shown that toxicity increases in proportion to the number of esters[90]. However, the NOAELs determined for C2E2 are higher than the doses likely to be recommended for treatment in humans.

Although successful at enhancing decorporation of americium a variety of radionuclides may be responsible for contamination and those that occur with multiple radionuclides the hydroxypyridinonate (HOPO) group of compounds are favorable. These chelators have been designed to chelate a wide range of radionuclides with high affinity[162]. However, to achieve enhanced chelation oral absorption was not a priority and the compounds have poor oral bioavailability in rats (<5 %)[163]. The efficacy of two of these compounds 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) for decorporation of ^{238}Pu, ^{241}Am and $^{233}U(VI)O_2$ has been demonstrated in mice after i.p and oral administration with promising results[164]. The NOAELs for 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) are also above their presumed target oral efficacy doses. Although additional work is needed, the information currently available for these two HOPO agents highlights their potential as alternatives to intravenous DTPA therapy. However, their high molecular weight and likely complex metabolic profile may add challenges to their regulatory approval.

This work has demonstrated the efficacy of C2E2 for oral decontamination of americium. Only a handful of experiments must be conducted prior to submission of an investigational new drug (IND) application. Protein binding data in the species used for repeated dose toxicity studies should be evaluated[165] and the remaining safety pharmacology studies to assess cardiovascular, CNS and respiratory effects must be completed. Lastly the

manual patch clamp hERG assay for assessment of drug interactions with the hERG potassium channel and QT prolongation.

Assessing the binding affinity of C2E2 for these metals in plasma may lead to additional indications enabling turnover of stock in the national stockpile. In addition to transuranic elements other than Am, such as Pu and Cm, radioisotopes of non-transuranic elements (^{60}Co) and non-radioactive metals associated with metal overload syndromes (Pb, Fe) should be assessed. The binding constants for EDTA can be used as a guide to determine those metals for which C2E2 is likely to have a high affinity.

For BCS III compounds such as DTPA chemical modification is the most successful method of overcoming permeation-limited bioavailability. Though successful the C2E2 molecule could be further optimize to maximize absorption while retaining the ability to chelate ^{241}Am. Increasing the length of the ester promoeities may result in further enhancement in absorption as seen with partial esterified triethylenetetraminepentaacetic acids[91], though with increased permeation toxicity is likely to increase. A balance must therefore be struck between ester length, affinity for the metal, and toxicity. Ultimately the ideal prodrug for DTPA would have all the carboxylic acids masked as with C2E5 and after absorption would be completely metabolized to DTPA. For traditional pro-drugs with one or two carboxylic acids there are a diverse set of alcohols and phenols available for conjugation allowing absorption and enzymatic conversion to be tailored[166]. Although the ethyl esters proved to be stable *in vivo*, utilization of alternative groups may result in more complete liberation. Investigation into the reason C2E5 ester hydrolysis slows after reaching liberation of the first two esters and is ineffective for C2E2, may aid in the design of more effective DTPA prodrugs in the future.

Although less effective for BCS class III compounds than class II, further improvements in absorption could be obtained through formulation[167]. Permeation enhancers are commonly for poorly permeable compounds but are not ideal for decorporation products. Either a gastro-retentive drug delivery system that prolongs the residence time in the stomach such as floating tablets[168] or a muccoadhesive system[169], which can increase local concentrations at the intestinal mucosal membrane, may help to increase absorption of C2E2.

7.2 Closing Remarks

There is currently no treatment available in the strategic national stockpile enabling fast and efficient administration in the event of a mass contamination scenario involving the radionuclides of americium, plutonium and curium. Although effective, IV administration of DTPA requires health care professionals and is therefore not amenable to a mass casualty situation where any delay in treatment resulting in lower efficacy. On the contrary an oral chelation agent such as C2E2 can be rapidly distributed and administered limiting tissue disposition of internalized radionuclides. This doctoral research demonstrates that C2E2 is an effective orally administered medical countermeasure with low toxicity. Providing the human pharmacokinetics follow a similar trend as that seen with DTPA, C2E2 is a promising alternative for treating individuals contaminated with transuranic elements.

APPENDIX I

PHARMACOKINETIC PHARMACODYNAMIC MODEL OF C2E2 AND PREDICTION OF MRSD IN HUMANS

Introduction

The effectiveness of a drug depends on the relationship between the administered dose, the resulting drug concentration in the body and the pharmacological effect caused by these concentrations[170]. These properties are described by both the pharmacokinetics and pharmacodynamics of the drug. Pharmacokinetics defines the concentration time profile of a drug obtained from a particular dose and is based on absorption, distribution, metabolism and excretion. Pharmacodynamics describes the magnitude of the drugs effect relative to its concentration. A mathematical model that encompasses both the pharmacokinetics and pharmacodynamics (PK-PD model) can be used to determine the dose required to produce a specific response[171]. Due to the inability to assess the clinic efficacy of decorporation agents in humans the development of a PK-PD model is of particular importance in supporting selection of an effective human dose and must be used in order to meet the fourth requirement of the FDA animal rule[100].

To enable prediction of the human dose, the pharmacokinetics must be determined in both animals and humans. The pharmacokinetics of C2E2 has been investigated in both

Sprague-Dawley rats (Chapter V) and beagle dogs (Chapter VI). Without human pharmacokinetic data it is not possible to select an effective dose, however using *in vitro* data, animal pharmacokinetic parameters and efficacy results an estimation can be made based on similarities with DTPA.

For C2E2, chelation of americium is achieved in a similar fashion as with DTPA. Target concentrations of DTPA were based on animal efficacy data. Americium decorporation is inversely related to DTPA dose and above concentrations of 30 μmol/kg (11.8 mg/kg) large increases in dose are required to gain additional efficacy in beagles[71]. Molar DTPA levels in plasma far exceed those of americium and recently target concentrations were determined *in vitro* using a competitive binding method[119]. Through use of this *in vitro* data it is possible to establish species differences in target concentrations and combined with pharmacokinetic data determine relative durations of action from a single dose. In chapter III the target plasma concentrations for C2E2 were identified and when combined with the pharmacokinetic data described in chapters V and VI can help guide dose selection. As with all new drugs human pharmacokinetics and toxicity must be determined in a phase I clinical trial. Prior to conducting a phase I trial it is important to determine the first-in-human (FIH) dose[172]. There are many methods used for prediction of the FIH dose[173], however current FDA guidance suggests that the maximum recommended starting dose (MRSD) should be calculated from NOAELs determined during animal toxicity studies[174]. The phase I trial is especially important for radionuclide decorporation agents as the pharmacokinetics established will help guide dose selection in place of formal efficacy trials.

Below the efficacious human dose is estimated and the MRSD is also calculated based on animal toxicity data described in Chapters V and VI.

Pharmacokinetic and Pharmacodynamic Models

The data obtained from rat and beagle toxicokinetic studies is presented in Tables A.4-10. For each dose area under the curve (AUC) values were determined using a two-compartment model. The results of the divided dose study in rats suggest that decorporation efficacy is dependent on cumulative AUC rather than C_{max}. Thus to enable efficacy prediction from a particular dosing regimen, the percentage decorporation was modeled against cumulative AUCs for each dose administered for each species using the following equation:

$$E = \frac{E_{\max} \cdot C}{EC_{50} + C}$$

There are several limitations to this approach. For rats both single and multiple dose studies have been conducted. The duration of the single and multiple dose studies were different, so there is potential for additional decorporation to occur over the extra time. In order to limit this effect the data was corrected for untreated decorporation using the control groups for each study. The majority of the multiple dose studies in rats were performed at doses of 600 mg/kg for which there is no PK data. Therefore, AUCs for the 600 mg/kg dose were calculated by normalizing the data obtained from the 200, 400, 800 and 2000 mg/kg doses (Tables A.4 – A.7) to 600 mg/kg (Table A.8) and calculating the median concentration by sex. The normalization process is based on the assumption that there is a linear relationship between dose and plasma concentration. For C2E2 this is not always the case however the differences are not consistent between doses and a superior alternative was not found.

There is limited data for dogs with efficacy studies only conducted using three single doses, though a pharmacodynamic model was created using the available data (Figure A.2). In order to improve the prediction of human response more data is required in beagles. Due

to the increased exposure observed in dogs, efficacy of smaller doses should be assessed to further characterize the lower range of the pharmacodynamic curve. As cumulative AUC is a good indicator of efficacy a multiple dose study in dogs may not be required, as the exposure may be similar to that established with the high single dose.

Both the PK tables and pharmacodynamic models demonstrate that higher AUCs are obtained in dogs with a single oral administration of 100 mg/kg achieving similar exposure to 5 daily 600 mg/kg doses in rats. As expected, based on the *in* vitro binding data, C2E2 causes a greater enhancement decorporation in dogs for any given cumulative AUC compared to that in rats. The increase in exposure and resulting efficacy are also likely due to differences in the pharmacokinetics of C2E2 across species. The pharmacokinetic behavior of C2E2 is similar to DTPA with elimination best modeled using a 2-compartment model. For DTPA elimination is inversely related to body size. This is due to differences in renal clearance, with both EDTA and DTPA metal complexes eliminated in a similar fashion to insulin, where the glomerular filtration rates are 5.9, 3.8 and 1.3 for rat, dog and humans, respectively[116]. As the same trend is seen with C2E2 it is expected that human doses are likely to be lower than those required in beagles. As renal clearance is inversely proportional to AUC a dose that is 1/3 that of the beagle dose may be expected to produce a similar exposure providing the bioavailability is constant between species. Based on the data obtained and similarities between C2E2 and DTPA, human efficacy may be observed at single doses in the range of 30-40 mg/kg (67 μmol/kg) with higher efficacy observed with larger or repeated dosage.

In contrast, efficacy with IV DTPA can be observed at doses as low as 1 mg/kg (3 μmol/kg)[71]. The selection of a 30 mg/kg efficacious dose can also be supported by *in vitro*

binding data. If the bioavailability in beagles is similar to that in rats (~30%) the oral dose is equivalent to a 20 μmol/kg IV dose. Furthermore, in beagles a five-fold increase in C2E2 plasma concentration is required to achieve the same chelation effect as DTPA to achieve the same effect. Multiplying the lowest DTPA concentration by both of these factors results in a starting dose similar to that obtained from efficacy data.

Selection of MRSD for Human Dosing

In order to select a human starting dose the NOAELs from animal toxicity studies must be known. This relates to the highest dose level that does not produce a significant increase in adverse effects in comparison to control. Toxicity studies with C2E2 identified the single dose NOAELs as 2000 and 750 mg/kg in rats and dogs, respectively. The 10-day NOAELs were lower at 800 and 200 mg/kg/day for rats and dogs, respectively, and will be used to calculate the human equivalent dose (HED).

Toxic doses have been shown to scale in relation to body surface area. Typically conversion to body surface area is based on the weight of animals used within the study however body surface area conversion factors (BSA-CF) outlined by the FDA can be used to provide reasonable estimates of the HED. For C2E2 the NOAELs are 129 and 111 mg/kg when normalized to body surface area. The HED established from the beagle model should be selected as the value is more conservative and because the pharmacokinetics are more predictive of human response for similar drugs such as EDTA and DTPA.

Finally a safety factor is applied to the HED to allow for variation in scaling and minimize the probability of the initial dose being toxic. For C2E2 the standard safety factor of 10 should be applied so that the MRSD is 11 mg/kg. However, as this dose is only three

fold lower than the estimated lowest efficacious dose and exposure in humans is likely to be higher than expected due to decreased glomerular filtration rate it may be necessary to further lower the MRSD to 1 mg/kg.

Although estimations of both the human efficacious dose and the MRSD can be made from the data presented, further beagle studies would help to improve confidence. Ultimately, human pharmacokinetic data is required to determine the optimal dose and regimen.

Table A.1 Summary of Pharmacokinetic and Efficacy Data for Male Sprague Dawley Rats

Dose	# of Doses	Study Duration	Cumulative AUC	Control Decorporation (%)	Total Decorporation (%)	Decorporation Increase (%)
200	1	7	3764	14.6	20.9	6.3
600	1	7	13288	14.6	26.4	11.8
600	5	12	68735	23.4	44.1	20.7
600	5 OD	12	68735	17.7	39.7	22
600	5 BD	12	68735	17.7	43.6	25.9
600	10	12	137470	23.4	50.1	26.7

Table A.2. Summary of Pharmacokinetic and Efficacy Data for Female Sprague Dawley Rats

Dose	# of Doses	Study Duration	Cumulative AUC	Control Decorporation (%)	Total Decorporation (%)	Decorporation Increase (%)
200	1	7	4011	11.2	14.4	3.2
600	1	7	18307	11.2	23.6	12.4
200	7	12	28077	15.3	32.3	17
600	5	12	91535	20.1	38.1	18
600	5 OD	12	91535	15.9	28.9	13
300	5 BD	12	91535	15.9	34.8	18.9
600	10	12	183070	20.1	43.9	23.8

Table A.3 Summary of Pharmacokinetic and Efficacy Data for Beagle Dog (Male and Female Combined).

Dose	# of Doses	Study Duration	Cumulative AUC	Control Decorporation (%)	Total Decorporation (%)	Decorporation Increase (%)
100	1	7	85865	29.24	54.13	24.89
300	1	7	246279	29.24	63.78	34.54
500	1	7	640563	29.24	72.16	42.92

Figure A.1 Prediction of increase in decorporation over control from cumulative AUC in rats

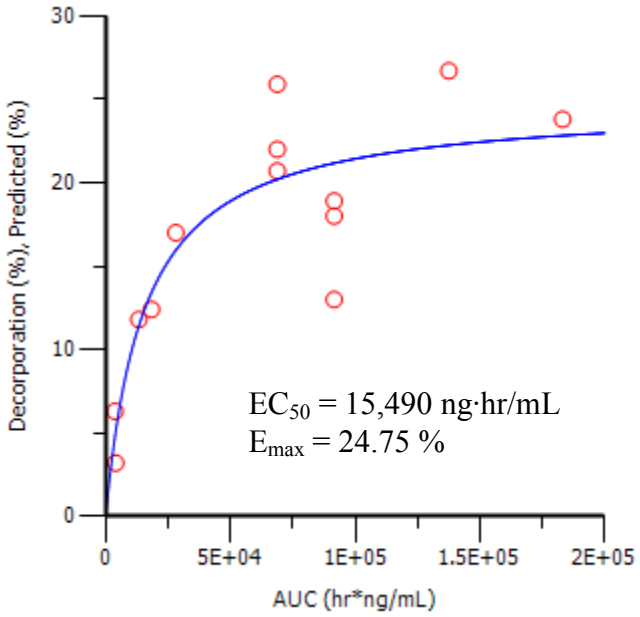


Figure A.2 Prediction of increase in decorporation over control from cumulative AUC in dogs

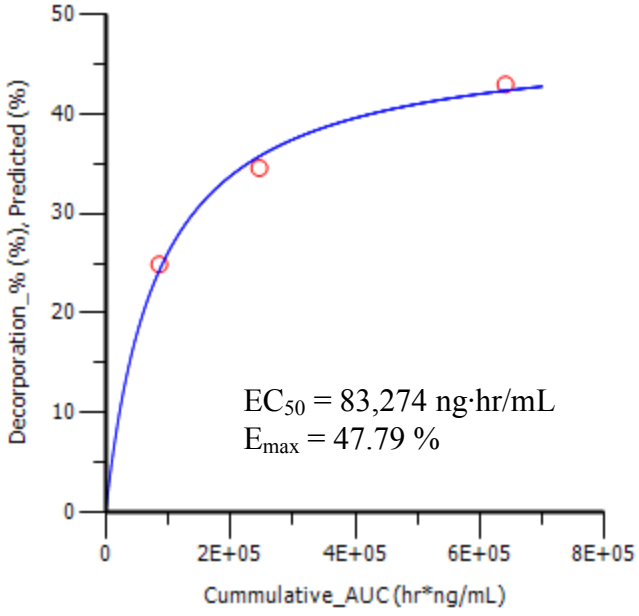


Table A.4 C2E2 plasma concentrations following administration of ten oral doses of 200 mg/kg in both male and female rats.

Male				Female			
ID	**Day**	**Time**	**C2E2 Conc.**	**ID**	**Day**	**Time**	**C2E2 Conc.**
B29700	1	0.25	1140	B29752	1	0.25	888
B29701	1	0.25	462	B29753	1	0.25	1000
B29702	1	0.25	889	B29754	1	0.25	830
B29703	1	1	740	B29755	1	1	550
B29704	1	1	1340	B29756	1	1	580
B29705	1	1	492	B29757	1	1	615
B29700	1	3	357	B29752	1	3	196
B29701	1	3	294	B29753	1	3	347
B29702	1	3	461	B29754	1	3	215
B29703	1	6	134	B29755	1	6	BQL < 100 ng/ml
B29704	1	6	BQL < 100 ng/ml	B29756	1	6	BQL < 100 ng/ml
B29705	1	6	167	B29757	1	6	BQL < 100 ng/ml
B29700	1	12	BQL < 100 ng/ml	B29752	1	12	242
B29701	1	12	137	B29753	1	12	149
B29702	1	12	127	B29754	1	12	BQL < 100 ng/ml
B29703	1	24	BQL < 100 ng/ml	B29755	1	24	BQL < 100 ng/ml
B29704	1	24	BQL < 100 ng/ml	B29756	1	24	BQL < 100 ng/ml
B29705	1	24	BQL < 100 ng/ml	B29757	1	24	BQL < 100 ng/ml
B29700	10	0.25	6590	B29752	10	0.25	856
B29701	10	0.25	1050	B29753	10	0.25	5640
B29702	10	0.25	1250	B29754	10	0.25	1820
B29703	10	1	663	B29755	10	1	639
B29704	10	1	507	B29756	10	1	602
B29705	10	1	468	B29757	10	1	1330
B29700	10	3	277	B29752	10	3	228
B29701	10	3	347	B29753	10	3	203
B29702	10	3	332	B29754	10	3	246
B29703	10	6	119	B29755	10	6	BQL < 100 ng/ml
B29704	10	6	104	B29756	10	6	BQL < 100 ng/ml
B29705	10	6	135	B29757	10	6	104
B29700	10	12	BQL < 100 ng/ml	B29752	10	12	134
B29701	10	12	464	B29753	10	12	BQL < 100 ng/ml
B29702	10	12	112	B29754	10	12	166

Table A.5 C2E2 plasma concentrations following administration of ten oral doses of 400 mg/kg in both male and female rats.

Male				Female			
ID	**Day**	**Time**	**C2E2 Conc.**	**ID**	**Day**	**Time**	**C2E2 Conc.**
B29700	1	0.25	1350	B29752	1	0.25	2580
B29701	1	0.25	1150	B29753	1	0.25	47600
B29702	1	0.25	2960	B29754	1	0.25	2270
B29703	1	1	1160	B29755	1	1	3290
B29704	1	1	912	B29756	1	1	2130
B29705	1	1	1490	B29757	1	1	9490
B29700	1	3	915	B29752	1	3	226
B29701	1	3	607	B29753	1	3	1740
B29702	1	3	686	B29754	1	3	592
B29703	1	6	150	B29755	1	6	243
B29704	1	6	204	B29756	1	6	154
B29705	1	6	196	B29757	1	6	387
B29700	1	12	147	B29752	1	12	162
B29701	1	12	118	B29753	1	12	396
B29702	1	12	201	B29754	1	12	224
B29703	1	24	BQL < 100 ng/ml	B29755	1	24	213
B29704	1	24	208	B29756	1	24	144
B29705	1	24	362	B29757	1	24	BQL < 100 ng/ml
B29700	10	0.25	1020	B29752	10	0.25	3670
B29701	10	0.25	1730	B29753	10	0.25	7280
B29702	10	0.25	1890	B29754	10	0.25	8000
B29703	10	1	1200	B29755	10	1	4040
B29704	10	1	957	B29756	10	1	3280
B29705	10	1	1650	B29757	10	1	2060
B29700	10	3	512	B29752	10	3	410
B29701	10	3	819	B29753	10	3	567
B29702	10	3	779	B29754	10	3	731
B29703	10	6	266	B29755	10	6	167
B29704	10	6	321	B29756	10	6	199
B29705	10	6	264	B29757	10	6	185
B29700	10	12	145	B29752	10	12	160
B29701	10	12	370	B29753	10	12	199
B29702	10	12	172	B29754	10	12	189

Table A.6 C2E2 plasma concentrations following administration of ten oral doses of 800 mg/kg in both male and female rats.

Male				Female			
ID	**Day**	**Time**	**C2E2 Conc.**	**ID**	**Day**	**Time**	**C2E2 Conc.**
B29711	1	0.25	5110	B29763	1	0.25	12500
B29712	1	0.25	3670	B29764	1	0.25	19500
B29713	1	0.25	4020	B29765	1	0.25	9140
B29714	1	1	4670	B29766	1	1	16000
B29715	1	1	6710	B29767	1	1	14000
B29716	1	1	5640	B29768	1	1	14300
B29711	1	3	2650	B29763	1	3	1900
B29712	1	3	989	B29764	1	3	4190
B29713	1	3	955	B29765	1	3	1290
B29714	1	6	478	B29766	1	6	419
B29715	1	6	589	B29767	1	6	459
B29716	1	6	322	B29768	1	6	280
B29711	1	12	219	B29763	1	12	226
B29712	1	12	210	B29764	1	12	191
B29713	1	12	251	B29765	1	12	179
B29714	1	24	112	B29766	1	24	167
B29715	1	24	106	B29767	1	24	280
B29716	1	24	BQL < 100 ng/ml	B29768	1	24	141
B29711	10	0.25	46800	B29763	10	0.25	33700
B29712	10	0.25	3880	B29764	10	0.25	32300
B29713	10	0.25	5250	B29765	10	0.25	5040
B29714	10	1	6770	B29766	10	1	2900
B29715	10	1	12100	B29767	10	1	3940
B29716	10	1	7360	B29768	10	1	8320
B29711	10	3	3930	B29763	10	3	2460
B29712	10	3	3210	B29764	10	3	1150
B29713	10	3	1980	B29765	10	3	1920
B29714	10	6	561	B29766	10	6	428
B29715	10	6	744	B29767	10	6	407
B29716	10	6	328	B29768	10	6	358
B29711	10	12	393	B29763	10	12	296
B29712	10	12	314	B29764	10	12	260
B29713	10	12	275	B29765	10	12	208

Table A.7 C2E2 plasma concentrations following administration of a single oral dose of 2000 mg/kg in both male and female rats.

Male				Female			
ID	**Day**	**Time**	**C2E2 Conc.**	**ID**	**Day**	**Time**	**C2E2 Conc.**
B29711	1	0.25	32900	B29763	1	0.25	25700
B29712	1	0.25	15500	B29764	1	0.25	51300
B29713	1	0.25	21600	B29765	1	0.25	52000
B29714	1	1	25100	B29766	1	1	37400
B29715	1	1	18600	B29767	1	1	19000
B29716	1	1	28600	B29768	1	1	17300
B29711	1	3	15000	B29763	1	3	43300
B29712	1	3	14100	B29764	1	3	16500
B29713	1	3	5160	B29765	1	3	13900
B29714	1	6	1360	B29766	1	6	1170
B29715	1	6	1960	B29767	1	6	3300
B29716	1	6	989	B29768	1	6	1410
B29711	1	12	592	B29763	1	12	2440
B29712	1	12	1200	B29764	1	12	710
B29713	1	12	653	B29765	1	12	601
B29714	1	24	1420	B29766	1	24	467
B29715	1	24	1350	B29767	1	24	383
B29716	1	24	683	B29768	1	24	303

Table A.8 C2E2 plasma concentrations calculated for a 600 mg/kg dose of C2E2 in rats. Data represents the median normalized plasma concentrations from 200, 400, 800 and 2000 mg/kg doses in Tables 6.4 – 6.7.

Male		Female	
Time	**C2E2 Conc.**	**Time**	**C2E2 Conc.**
0.25	3150	0.25	7710
1	2235	1	3990
3	1169	3	968
6	398	6	313
12	236	12	243
24	84	24	133

Table A.9 C2E2 plasma concentrations following administration of a single oral dose of 100 mg/kg in both male and female dogs.

Male			Female		
ID	**Time**	**C2E2 Conc.**	**ID**	**Time**	**C2E2 Conc.**
H04377	0.5	11800	H04379	0.5	12500
H04378	0.5	32200	H04380	0.5	35100
H04377	1	27900	H04379	1	18100
H04378	1	51900	H04380	1	41300
H04377	2	14400	H04379	2	20100
H04378	2	30300	H04380	2	23700
H04377	4	3520	H04379	4	2940
H04378	4	6380	H04380	4	5490
H04377	6	833	H04379	6	654
H04378	6	2110	H04380	6	926
H04377	8	337	H04379	8	377
H04378	8	580	H04380	8	325
H04377	12	BQL < 100 ng/ml	H04379	12	BQL < 100 ng/ml
H04378	12	141	H04380	12	284
H04377	24	BQL < 100 ng/ml	H04379	24	BQL < 100 ng/ml
H04378	24	BQL < 100 ng/ml	H04380	24	BQL < 100 ng/ml

Table A.10 C2E2 plasma concentrations following administration of a single oral dose of 300 mg/kg in both male and female dogs.

Male			Female		
ID	**Time**	**C2E2 Conc.**	**ID**	**Time**	**C2E2 Conc.**
H04377	0.5	16700	H04379	0.5	34400
H04378	0.5	90100	H04380	0.5	86100
H04377	1	23300	H04379	1	79600
H04378	1	126000	H04380	1	138000
H04377	2	21800	H04379	2	59700
H04378	2	122000	H04380	2	93400
H04377	4	5110	H04379	4	7090
H04378	4	21700	H04380	4	21200
H04377	6	1140	H04379	6	854
H04378	6	4150	H04380	6	4050
H04377	8	393	H04379	8	204
H04378	8	1190	H04380	8	947
H04377	12	238	H04379	12	129
H04378	12	990	H04380	12	180
H04377	24	BQL < 100 ng/ml	H04379	24	BQL < 100 ng/ml
H04378	24	223	H04380	24	123

Table A.11 C2E2 plasma concentrations calculated for a 500 mg/kg dose of C2E2 in dogs.

Male			Female		
ID	**Time**	**C2E2 Conc.**	**ID**	**Time**	**C2E2 Conc.**
H04377	0.5	83500	H04377	0.5	192000
H04378	0.5	199000	H04378	0.5	106000
H04377	1	78200	H04377	1	179000
H04378	1	285000	H04378	1	345000
H04377	2	113000	H04377	2	184000
H04378	2	171000	H04378	2	359000
H04377	4	24600	H04377	4	19800
H04378	4	24700	H04378	4	76800
H04377	6	3280	H04377	6	1770
H04378	6	3900	H04378	6	9280
H04377	8	785	H04377	8	344
H04378	8	1130	H04378	8	2140
H04377	12	1360	H04377	12	BQL < 100 ng/ml
H04378	12	281	H04378	12	657
H04377	24	BQL < 100 ng/ml	H04377	24	BQL < 100 ng/ml
H04378	24	160	H04378	24	189

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