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Research Article

Significance of Single Variables in Defining Adequate Animal Models to Assess the Efficacy of New Radionuclide Decorporation Agents: Using the Contamination Dose as an Example

Erin E. Jarvis, Dahlia D. An, Birgitta Kullgren, and Rebecca J. Abergel*

Chemical Sciences Division, Glenn T. Seaborg Center, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

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ABSTRACT Internal radioactive contamination can arise in different ways, from accidents affecting nuclear sites and industrial or medical sources, to the possible terrorist use of radiological dispersal devices. In all cases, internalized radionuclides may pose significant health risks and must be removed from the body. The need for safe, practical, and efficacious actinide chelation therapy options has motivated the current preclinical development of new hydroxypyridinone-based actinide decorporation agents. In order to seek regulatory approval for such new decorporation agents, a number of efficacy studies must be performed and, in principle, must respond to selective criteria of the Animal Efficacy Rule from the US Food and Drug Administration (FDA). While many *in vivo* decorporation efficacy studies have been conducted over the past few decades using different animal species and contamination procedures, there is no designated standard animal model for the evaluation of new actinide chelation drugs. This study aims at probing the influence of a single variable such as the contamination dose on the efficacy of the chelators 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) at removing plutonium-238 administered as a soluble citrate complex. The dependence of body burden on contamination dose observed in untreated animals was reduced with the hydroxypyridinonate chelators, more so than with the commercially available diethylenetriaminepentaacetic acid chelator. This study demonstrates how a slight change in the design of decorporation experiments can remarkably impact the efficacy profile of the tested drug, and thus the path a sponsor might take to gain FDA approval. Drug Dev Res 73 : 281–289, 2012.

Key words: actinides; internal contamination; decorporation; nuclear medical countermeasure; chelation therapy

INTRODUCTION

The first decade of the 21st century has been marked by an increased public awareness and concern that radionuclides may be released after an accident affecting nuclear sites or as a consequence of the possible terrorist use of radiological dispersal devices [Pellmar and Rockwell, 2005; Cassatt et al., 2008; Bunn and Heinonen, 2011; Miller et al., 2011]. In addition to the hazards posed by external ionizing radiation, exposure to disseminated radionuclides could result in

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*Correspondence to: Rebecca J. Abergel, Lawrence Berkeley National Laboratory, One Cyclotron Road, MS 70A-1150, Berkeley, CA 94720, USA.
E-mail: rjabergel@lbl.gov

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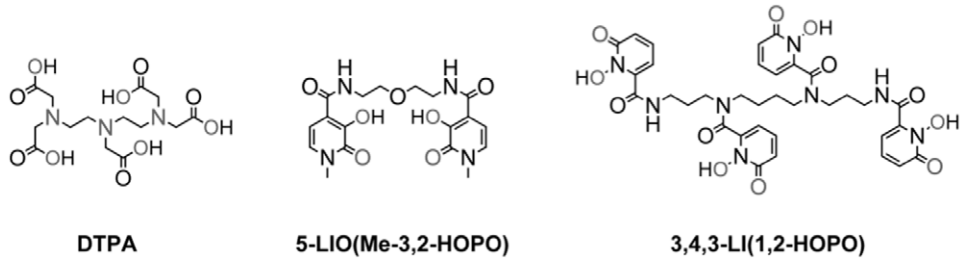

Fig. 1. Structures of the actinide chelating agents discussed in this study: diethylenetriaminepentaacetic (DTPA), 5-LIO(Me-3,2-HOPO) and 3,4,3-LI(1,2-HOPO); metal-binding atoms are highlighted in gray.

internal contamination of individuals. The currently available treatments in the event of a radionuclide incident vary and are dependent on the intake pathway, the level of contamination, the chemical form of the radioisotope, as well as the treatment time after the contamination [Battacharyya et al., 1992; Fisher, 2000; Wood et al., 2000; Ansoborlo et al., 2007a]. For internalization of radionuclide aerosols by inhalation, treatments include lung lavage and chelation therapy [National Council on Radiation Protection and Measurements (NCRP), 2008]. Such treatments aim at increasing the solubility of the actinides deposited in the human respiratory tract, removing the actinide mechanically, and allowing subsequent chelation of the absorbed blood fraction. For contamination by ingestion, treatments include gastric lavage, use of precipitating agents and purgatives, and chelation therapy [NCRP, 2008]. For wound contamination, treatments include wound irrigation, surgical excision, as well as local and systemic chelation therapy [NCRP, 2008]. In all cases, the use of decorporation agents that form stable and excretable complexes with the targeted metal ions is suggested, and current chelation treatment recommendations have been reviewed extensively [Battacharyya et al., 1992; Fisher, 2000; Wood et al., 2000; US Food and Drug Administration, 2004].

The only currently available chelation drugs designated for decorporation of transuranic actinides plutonium, americium, and curium are the diethylenetriaminepentaacetic acid (DTPA) trisodium calcium and trisodium zinc salts, Ca-DTPA and Zn-DTPA (Fig. 1) [US Food and Drug Administration, 2004, 2006]. While DTPA drug products have been used investigationally for over 40 years, the US Food and Drug Administration (FDA) approved the corresponding New Drug Applications (Hameln Pharmaceuticals, Hameln, Germany) only, in 2004, to increase the rates of elimination of these materials from the body after internal contamination [US Food and Drug Administration, 2004]. The use of DTPA salts for actinide decorporation presents some significant limitations: these chelation treatments are not effective for the decorporation of other actinides such as uranium and neptunium [Cerveny, 1989], and they can only be administered intravenously or through a nebulizer, which is a considerable deterrent for the treatment of a very large population of contaminated individuals in a crisis setting [Cassatt et al., 2008].

A considerable effort has been undertaken at the Lawrence Berkeley National Laboratory over the past three decades to design new synthetic actinide decorporation agents [Gorden et al., 2003; Durbin, 2008]. As a result, two hydroxypyridinone-based multidentate chelators, 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) (Fig. 1), emerged as lead candidates for actinide decorporation therapy and are currently being evaluated in a preclinical development program [Abergel et al., 2010; Abergel and Raymond, 2011; Chang et al., 2012]. While reasonable efficacy and safety profiles have been established for both ligands [Abergel et al., 2010], a number of additional nonclinical *in vivo* pharmacology, toxicology, and efficacy studies are still essential in order to seek regulatory approval. These studies must be carefully designed to meet the criteria described in the FDA's "Animal Efficacy Rule" [US Food and Drug Administration, 2009, 2011], which allows for approval of new drug products based on animal efficacy data when adequate and well-controlled efficacy trials in humans cannot be ethically conducted. Such studies cannot be conducted ethically because they would involve administering a potentially lethal or permanently disabling toxic substance or organism to healthy human volunteers.

There is no precedent for regulatory approval of new decorporation treatments under the Animal Efficacy Rule, and no particular models have been established for decorporation experiments in animal species. In addition to the treatment regimen, several parameters can be varied in such experiments, from the choice of the animal species to the route of contamination, the isotopic ratio, and chemical form of the contaminant, or the amount of contaminant and the associated radiation dose administered [Taylor et al., 2000; Stradling et al., 2000a, 2000b; Ansoborlo et al.,

2007a; Durbin, 2008]. A large majority of decorporation efficacy studies performed over the past three decades with experimental chelators was conducted on laboratory mice, rats, and larger mammalian species such as dogs and baboons. In view of the available literature, all of these species seem suitable to establish the efficacy profiles of new chelating agents. Similarly, various routes of contamination have been investigated, including intravenous (IV) injection, inhalation, and wound simulation through subcutaneous or intramuscular injections. An important difference in these contamination modes is the unit mass of contaminant deposited in specific tissues such as the lungs and respiratory tract for inhalation studies. The particular actinide isotopes chosen for decorporation experiments will depend on the desired indication, and the chemical form used for the contaminant will also largely affect its solubility properties and biokinetic distribution [Ansoborlo et al., 2006, 2007b; Durbin, 2006].

The amount of administered contaminant is a parameter that is rarely varied in a single study. The work presented herein seeks to explore the effect of contamination dose variation on the decorporation efficacy of two experimental agents and one comparator, namely 3,4,3-LI(1,2-HOPO), 5-LIO(Me-3,2-HOPO), and Ca-DTPA. To ensure consistency between this and previously reported studies [Gorden et al., 2003; Durbin, 2008; Abergel et al., 2010], the chosen animal model was the young adult female Swiss-Webster mouse (Simonsen Laboratories, Gilroy, CA, USA), and contamination was performed through a single IV injection of soluble ^{238}Pu-citrate. While only slight differences were observed in the relative ^{238}Pu distribution profiles resulting from contamination doses ranging from 12.5 to 100 nCi per mouse, the absolute content of radionuclide retained in the body and specific tissues was significantly altered. However, this contamination dose-dependent effect was reduced with the hydroxypyridinonate chelators that exhibited higher potency. This study demonstrates how a slight change in the design of decorporation experiments can remarkably impact the efficacy profile of a tested drug. Such effects should be taken into account prior to considering more obvious parameter changes such as the animal species.

MATERIALS AND METHODS

Contaminant and Test Article Solutions

A stock solution of ^{238}Pu-nitrate in 4 M HNO_3 was purchased from Eckert and Ziegler Isotope Products (Valencia, CA, USA) and used to prepare injection solutions. Plutonium contamination doses were composed of 0.2 ml aliquots of solutions containing the following radioactivities:100 nCi (3.70 kBq, 5.78 ng), 50 nCi (1.85 kBq, 2.89 ng), 25 nCi (0.93 kBq, 1.45 ng), or 12.5 nCi (0.46 kBq, 0.72 ng) in 0.008 M sodium citrate and 0.14 M NaCl, pH 4. The test articles 3,4,3-LI (1,2-HOPO) and 5-LIO(Me-3,2-HOPO) were prepared by Synthetech, Inc. (Albany, OR, USA) and Albany Molecular Research, Inc. (Albany, NY, USA), respectively, as described previously [Abergel et al., 2010]; DTPA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Test article solutions were prepared such that the selected dosage (30 μmol/kg for Ca-DTPA and 3,4,3-LI[1,2-HOPO]; 100 μmol/kg for 5-LIO[Me-3,2-HOPO]) was contained in 0.5 ml of 0.14 M NaCl, the pH being adjusted to 7.4–8.4 with 1 N NaOH. All solutions were filter-sterilized (0.22 μm) prior to administration.

Animals and General Procedures

All procedures and protocols used in the described *in vivo* studies were reviewed and approved by the Institutional Animal Care and Use Committee of Lawrence Berkeley National Laboratory and were performed in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facilities. The animals used were young adult female Swiss-Webster mice (104 ± 1 days old and 32.3 ± 2.4 g). Gross body and tissue compositions, plasma, extracellular fluid, and red cell volumes of the whole body, major tissues and organs of these mice (intact or bled 25% to 40% of their total blood volume) have been determined previously [Durbin et al., 1992]. The mice were kept under a 12-h light cycle and controlled temperature (18–22°C) and relative humidity (30–70%), and were given water and food *ad libitum*. Each five-mouse group was housed together in a plastic stock cage lined with a 0.5-cm layer of highly absorbent low-ash pelleted cellulose bedding (ALPHA-dri; Shepherd Specialty Papers, Redwood City, CA) for separation of urine and feces. IV injections into a warmed lateral tail vein were performed under isoflurane anesthesia. Groups of five mice were injected IV with a single dose of ^{238}Pu-citrate, and ligand or control saline solutions were administered by intraperitoneal (IP) injection at 1 h postcontamination. The mice were euthanized at 24 h postcontamination by cervical dislocation over their cages to collect the urine expelled at death and immediately wrapped in plastic and quick-frozen for later dissection.

Tissue Sampling and Processing

After partial thawing of the frozen mice, livers and kidneys were dissected, and the abdominal tissue remainder (ATR, including intact gastrointestinal [GI]

tract, reproductive organs, spleen, urinary bladder, abdominal fat) was removed. The livers, kidneys, ATR, and partially eviscerated carcasses were managed as individual samples, while separated urine and feces were pooled for each group. The samples were dried at 100°C and dry-ashed at 575°C. The ashed samples were dissolved in dilute HNO_3. The small tissue samples that were dried, ashed, and dissolved in their glass counting vials and weight aliquots of the dissolved large samples (bulk soft tissue, skeleton, urine, feces) were mixed with Ultimagold (Perkin Elmer Corporation, Shelton, CT) for detection of the radiotracers by liquid scintillation counting (Packard Tri-Carb model B4430; Perkin Elmer).

Data Management and Analysis

All experiments used radioactive ^{238}Pu as a tracer and were therefore managed as metabolic balance studies, in which blood, all tissues, and excreta were radioanalyzed; material recoveries were higher than 97% of the amount injected and data were normalized to 100% recovery. The corrected experimental data are expressed as percent of injected dose (% injected dose [ID]), values are arithmetic means ± standard deviation. Described methods were used to calculate total soft tissue ^{238}Pu (in all soft tissues except liver and kidneys) and to partition the ^{238}Pu measured in the ATR samples between tissues and GI contents [Durbin et al., 1997b]. When comparing values between groups in the decorporation study, the term "significant" is used in the statistical sense, indicating $P < 0.01$ by one-way analysis of variance (ANOVA) followed by adequate post hoc analysis. The Dunnett's multiple comparison test was used to compare the groups of animals treated with a chelating agent to the corresponding control group that was administered saline, while the Tukey's honestly significant difference multiple comparison test was used to perform pairwise comparisons between all the groups treated with a chelating agent. Both tests were set at the 99% confidence interval level. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Removal data reported in total activity (and not %ID) as a function of the ID were fitted by linear regression analysis using Origin 6.1 (OriginLab, Northampton, MA, USA).

RESULTS

A series of plutonium decorporation efficacy studies was performed with the two hydroxypyridinonate ligands under development, 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO). In these studies, the contamination protocol was established to span a range of single ^{238}Pu-exposure doses, from low radioactivity at detectable levels to high radioactivity resulting in observed animal discomfort. The groups of mice were therefore given a single IV injection of ^{238}Pu-citrate at one of the following activities: 12.5, 25, 50, and 100 nCi. These activities correspond to respective metal doses of 0.08, 0.19, 0.37, and 0.74 nmol/kg for mice with an average body weight of 32.3 g. All protocols then included one prompt ligand IP injection at 1 h after the contamination, with mice euthanized 24 h later. Ligand doses were based on the ligand denticity and previous decorporation studies with plutonium and other actinides [Durbin et al., 1989; Durbin, 2008; Abergel et al., 2010]: 30 μmol/kg for 3,4,3-LI(1,2-HOPO) and Ca-DTPA, and 100 μmol/kg for 5-LIO(Me-3,2-HOPO). Such doses ensure at least a 10^5-fold molar excess of ligand as compared with the plutonium contaminant. The excretion and distribution of retained plutonium in mice given the ligands were expressed in %ID and are shown in Table 1, while total body retention is illustrated in Figure 2. Ligand potency is evaluated by comparing plutonium retention and distribution in ligand-treated mice with corresponding plutonium-injected controls (absolute efficacy) and mice similarly treated with Ca-DTPA (relative efficacy). Treatment at 1 h postcontamination ensured that the results of these studies would reflect the potential efficacy of the ligands at removing deposited Pu(IV) from target tissues such as the skeleton, liver, and kidneys [Durbin et al., 1997a].

Independent of the contamination dose, about 90% of the injected ^{238}Pu remained in the body of control animals after 24 h. However, slight differences were seen in the distribution pattern of the contaminant: after contamination with one of the three higher doses (25, 50, and 100 nCi), ^{238}Pu was mainly found in the skeleton and the liver, with respective averages of 41% and 44% of the ID, little was found in the soft tissues and kidneys (~5.6%ID and 1.1%ID, respectively), and excretion was predominantly urinary. In contrast, for the lower contamination dose (12.5 nCi), a higher ^{238}Pu liver content (62%ID) and a lower skeleton content (16%ID) were observed, together with an increased fecal excretion. Similarly, the high decorporation efficacy of the tested hydroxypyridinonate ligands resulted in comparable ^{238}Pu body contents at the three higher contamination doses [~15%ID and ~17%ID for 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO), respectively] that were significantly lower than for both the control mice and the Ca-DTPA-treated mice (~41%ID). In the case of the lower contamination dose, both ligands still significantly reduced the total body ^{238}Pu content [19%ID and ~33%ID for 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO), respectively] but

TABLE 1. Removal of ^{238}Pu from Mice by Injected Hydroxypyridinonate Ligands and Ca-DTPA[a]

		Percent of injected actinide ± SD at 24 h[b]					
		Tissues				Excreta[c]	
^{238}Pu contamination dose (nCi)	Treatment	Skeleton	Liver	Soft tissue	Kidneys	Urine	Feces and GI content
12.5	3,4,3-LI(1,2-HOPO)	14 ± 1.5	4.7 ± 2.1[d,e]	0.7 ± 0.5[d]	0.2 ± 0.1[d]	23	58
	5-LIO(Me-3,2-HOPO)	29 ± 4.1	2.1 ± 1.0[d,e]	1.0 ± 0.5[d]	0.2 ± 0.1[d]	15	53
	Ca-DTPA	8.6 ± 1.1[d]	16 ± 1.1[d]	2.0 ± 0.4[d]	0.5 ± 0.1[d]	67	6.1
	Controls	16 ± 1.5	62 ± 5.5	7.2 ± 1.5	2.4 ± 1.2	6.9	5.4
25	3,4,3-LI(1,2-HOPO)	8.1 ± 0.8[d,e]	4.9 ± 1.0[d,e]	1.4 ± 0.4[d,e]	0.3 ± 0.3[d]	22	63
	5-LIO(Me-3,2-HOPO)	13 ± 0.8[d]	3.7 ± 2.0[d,e]	1.7 ± 0.3[d]	0.3 ± 0.1[d]	20	61
	Ca-DTPA	16 ± 5.0[d]	24 ± 6.7[d]	2.5 ± 0.4[d]	0.7 ± 0.3	50	6.9
	Controls	45 ± 1.6	42 ± 2.5	5.0 ± 0.5	1.0 ± 0.3	4.4	3.2
50	3,4,3-LI(1,2-HOPO)	7.4 ± 0.8[d,e]	4.9 ± 1.2[d,e]	0.8 ± 0.2[d,e]	0.2 ± 0.1[d,e]	26	61
	5-LIO(Me-3,2-HOPO)	11 ± 1.2[d]	3.0 ± 0.5[d,e]	1.4 ± 0.2[d,e]	0.2 ± 0.1[d]	20	64
	Ca-DTPA	16 ± 2.2[d]	18 ± 4.8[d]	2.7 ± 0.3[d]	0.5 ± 0.1[d]	58	5.5
	Controls	42 ± 4.6	43 ± 5.8	6.1 ± 0.8	1.2 ± 0.3	5.1	2.9
100	3,4,3-LI(1,2-HOPO)	8.7 ± 0.4[d,e]	6.4 ± 2.1[d,e]	1.4 ± 0.7[d]	0.2 ± 0.1[d]	24	59
	5-LIO(Me-3,2-HOPO)	12 ± 1.4[d]	2.9 ± 0.7[d,e]	1.3 ± 0.2[d]	0.2 ± 0.1[d]	20	64
	Ca-DTPA	15 ± 2.1[d]	24 ± 5.8[d]	2.5 ± 0.5[d]	0.5 ± 0.2[d]	52	6.5
	Controls	37 ± 4.8	46 ± 3.8	5.8 ± 0.7	1.1 ± 0.3	7.4	2.7

[a]Ligands were given to groups of five mice by IP injection [30 μmol/kg for Ca-DTPA and 3,4,3-LI(1,2-HOPO), 100 μmol/kg for 5-LIO(Me-3,2-HOPO)] at 1 h after IV injection of Pu-citrate (0.2 ml injection volume, injection dosage varying from 12.5 to 100 nCi), and the mice were euthanized at 24 h.

[b]Data, expressed as percent of injected ^{238}Pu (%injected dose (%ID), mean ± SD), were normalized to 100% material recovery for each five-mouse group. Discrepancies are due to rounding. SD = $(\Sigma \text{ dev}^2\ [n-1]^{-1})^{1/2}$; for tissues, n = number of mice (five mice, one group for each protocol).

[c]Excreta of each five-mouse group were pooled and SD is not available.

[d]Mean is significantly less than for appropriate injected controls ($P < 0.01$, one-way ANOVA followed by post hoc analysis with Dunnett's multiple comparison test).

[e]Mean is significantly less than for mice similarly treated with Ca-DTPA ($P < 0.01$, one-way ANOVA followed by post hoc analysis with Tukey's honestly significant difference (HSD) multiple comparison test).

ANOVA, analysis of variance; DTPA, diethylenetriaminepentaacetic acid; GI, gastrointestinal; IP, intraperitoneal; IV, intravenous; SD, standard deviation.

at levels closer to that observed with Ca-DTPA (~27%). In all contamination protocols, both hydroxypyridinonate compounds markedly increased fecal excretion [~70% and ~75% of the total excretion for 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO), respectively], whereas Ca-DTPA promoted solely urinary excretion (~90% of total excretion). An important characteristic of hydroxypyridinonate ligands is that they significantly reduced the ^{238}Pu liver content compared with both control mice and Ca-DTPA-treated mice at all levels of contamination. Moreover, at the three higher contamination levels, the two test compounds significantly reduced the ^{238}Pu skeleton content in comparison with the control mice, with 3,4,3-LI(1,2-HOPO) being significantly more efficacious than Ca-DTPA. Finally, at all contamination levels, treatment with 3,4,3-LI(1,2-HOPO), 5-LIO(Me-3,2-HOPO), or Ca-DTPA all resulted in significantly decreased soft tissue and kidney ^{238}Pu content, compared with controls, with significantly lower levels for 3,4,3-LI(1,2-HOPO) than for Ca-DTPA in some cases.

In decorporation studies, the percentage of injected radionuclide dose found in excreta or in specific organs is the typical parameter used to evaluate the efficacy of chelating agents [Wood et al., 2000; Stradling et al., 2000a; Ansoborlo et al., 2007a] as reported earlier. While this parameter is a direct indication of the rate of elimination enhancement capacity of a decorporation agent, when compared with control animals or other standard chelating treatments, it is less obviously linked to the absolute amount of radionuclide and resulting radioactivity found in the different pools. Figure 3 depicts the total plutonium activities retained in the body and different organs, displaying linear relationships with the ID. These data were fitted by linear regression analysis, resulting in remarkable slope differences between the chelating treatments. In all cases, the dose-dependence is stronger for the control groups (body retention slope of 0.904 ± 0.005) than for those treated with a chelator, and is stronger for the groups treated with Ca-DTPA (body retention slope of 0.409 ± 0.015) than for those treated with the hydroxypyridinonate

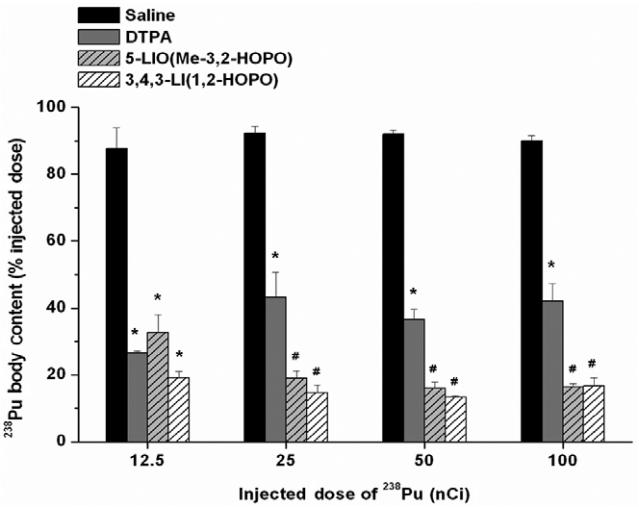

Fig. 2. Body retention of ^{238}Pu after contamination at different dose levels and treatment with hydroxypyridinonate ligands or Ca-DTPA. Ligands were given to groups of five mice by intraperitoneal injection [30 µmol/kg for Ca-DTPA and 3,4,3-LI(1,2-HOPO), 100 µmol/kg for 5-LIO(Me-3,2-HOPO)] at 1 h after intravenous injection of Pu-citrate (0.2 ml injection volume, injection dosage varying from 12.5 to 100 nCi), and mice were euthanized at 24 h. Data, expressed as percent of injected ^{238}Pu (% ID, mean ± standard deviation), were normalized to 100% material recovery for each five-mouse group. Groups with significantly lower retention than for control mice are indicated by * ($P < 0.01$, one-way analysis of variance [ANOVA] followed by post hoc analysis with Dunnett's multiple comparison test), while groups with significantly lower retention than for Ca-DTPA-treated mice are indicated by # ($P < 0.01$, one-way ANOVA followed by post hoc analysis with Tukey's HSD multiple comparison test).

compounds [body retention slopes of 0.160 ± 0.008 and 0.167 ± 0.011 for 3,4,3-LI(1,2-HOPO) and 5-LIO (Me-3,2-HOPO), respectively]. Subtle differences can be noted between the two experimental ligands: the dose-dependence in ^{238}Pu removal efficacy from the liver is stronger for 3,4,3-LI(1,2-HOPO) [slopes of 0.060 ± 0.004 and 0.030 ± 0.001 for 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO), respectively], while it is stronger for 5-LIO(Me-3,2-HOPO) [slopes of 0.085 ± 0.004 and 0.121 ± 0.009 for 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO), respectively] when removing ^{238}Pu from the skeleton. This graphical analysis of the retained plutonium activities displays the increase in potency of the hydroxypyridinonate ligands as the contamination level is raised.

DISCUSSION

The main goal of decorporation treatments after internal contamination with actinides is to reduce the risks of cancer and other chronic debilitating conditions associated with internal radiation dose and dose rate by enhancing the rate of actinide elimination from the body. It is therefore accepted that *in vivo* evaluation of efficacy for new decorporation agents can be based on direct measurement of the residual body burden and of the elimination of the contaminant [US Food and Drug Administration, 2006]. In most biokinetic profiles and decorporation studies reported in the literature, the measured actinide body or discrete organ burden and excretion levels are expressed as %ID, with dose also referring to activity. Such reporting methods are usually valid, as most studies compare the efficacy of different treatments with control groups, when all groups are given the same contaminant dose. While it has been shown repeatedly in the past that the experimental hydroxypyridinonate ligands 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) display remarkable Pu decorporation properties in several animal species and for different Pu species [Durbin et al., 1989, 1997b; Poncy et al., 1993; Stradling et al., 2000a; Ansoborlo et al., 2007a; Durbin, 2008; Abergel et al., 2010], the initial purpose of this work was to assess whether the efficacy of these decorporation treatments depends on the Pu contamination dose. In the presented decorporation study, the mice were contaminated with ^{238}Pu-citrate solutions through single doses ranging from 12.5 nCi to 100 nCi, with all other experimental parameters (animals, contamination route, treatment route and regimen, sample collection, and analysis) kept constant. Table 1 and Figure 2 display the results of this experiment by comparing Pu body/organ burdens and excretion levels, expressed as %ID, between the different treatment groups. As described in the previous section, the three higher contamination dose levels (25, 50, and 100 nCi) result in similar outcomes, with both hydroxypyridinonate compounds being significantly more efficacious at promoting the elimination of ^{238}Pu than Ca-DTPA, and all three chelation treatments significantly enhancing the excretion of ^{238}Pu as compared with placebo treatment. However, at the lowest contamination level of 12.5 nCi, the Pu body distribution in control animals was different from the pattern observed at higher doses, and the respective efficacies of all three chelating treatments were comparable with each other. Interestingly, the proportion of ^{238}Pu in the liver of the control animals is higher at the lower contamination dose than for higher doses. This effect could be due to the possible saturation at higher contamination levels of the pathways participating in the transfer of Pu from blood to hepatocytes and intracellular spaces of other nonparenchymal cells, involving cellular membrane-associated proteins and receptor-mediated endocytosis [Ansoborlo et al., 2006; Durbin, 2006; Jensen et al., 2011]. In addition, while the ^{238}Pu skeleton deposits seem reduced in control animals injected with low doses, they appear difficult to remove through chelation, which may be due to

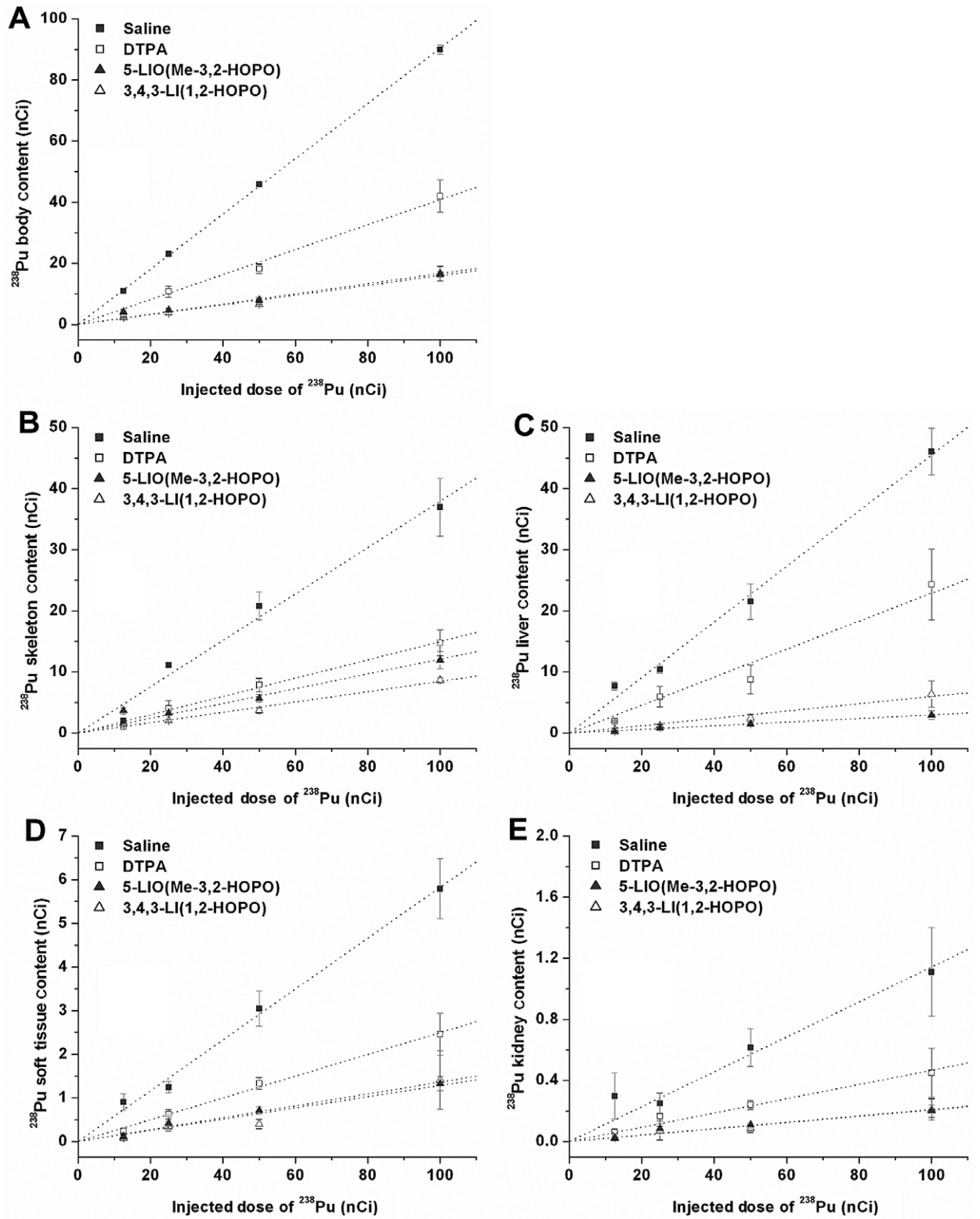

Fig. 3. (**A**) Body, (**B**) skeleton, (**C**) liver, (**D**) soft tissue, and (**E**) kidney ^{238}Pu content after contamination at different dose levels and treatment with hydroxypyridinonate ligands or Ca-DTPA. Ligands were given to groups of five mice by intraperitoneal injection [30 μmol/kg for Ca-DTPA and 3,4,3-LI(1,2-HOPO), 100 μmol/kg for 5-LIO(Me-3,2-HOPO)] at 1 h after intravenous injection of Pu-citrate (0.2 ml injection volume, injection dosage varying from 12.5 to 100 nCi), and mice were euthanized at 24 h. Data expressed as total ^{238}Pu activity (nCi, mean ± standard deviation) through solid points, and linear regression fits ($r > 0.99$) are given as dashed lines.

concentration-dependent Pu speciation and sorption equilibrium at the bone surface [Guilmette et al., 1998; Ansoborlo et al., 2006; Durbin, 2006].

Independent of the contamination dose, the experimental compounds 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) are efficacious Pu chelating agents as they enhance the rates of Pu elimination. However, important differences in the outcome of the chelation treatments must be noted in terms of internal burden and radiation dose. Based on the analysis of total Pu amount and activity presented in the previous section, there are clear linear correlations between the injected activity and the resulting body/organ burdens, with or without chelation treatments. These dependence patterns are more pronounced in untreated and Ca-DTPA-treated groups than in groups treated with hydroxypyridinonate chelators. At higher contamination doses, both 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) show enhanced efficacy profiles resulting in larger displacement and removal of internalized Pu. This result is particularly important, as the long-term effects of Pu intake are associated with the amount of internalized radionuclide. Several studies have shown in the past that the risk of osteosarcoma and incidence of bone cancer in mice increase with the contamination dose and can be reduced with chelation therapy [Rosenthal and Lindenbaum, 1967; Taylor et al., 1981; Jones et al., 1986]. The contamination range used in the present work (12.5 to 100 nCi per mouse or 0.39 to 3.1 μCi/kg) was chosen based on these previous studies that examined the incidence of bone tumors in contaminated mice. In a study by Taylor et al. [1981], osteosarcomas were found in 90% of adult female C57BL/Do mice injected with a single 2.9 μCi/kg dose of (^{238}Pu)-citrate and in 56% of those contaminated at the 0.9 μCi/kg level during autopsy. In a subsequent report from Jones et al. [1986], different treatment regimens were investigated and chelation with Zn-DTPA could reduce the bone tumor incidence from 79% down to 44% after a single 3.51 μCi/kg ^{238}Pu-citrate injection and from 52% to 17% after a 1.08-μCi/kg contamination. It is therefore remarkable that chelation therapies with 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) have the capability to attenuate the dose-dependent risks of long term effects by promoting the excretion of larger amounts of Pu at higher contamination doses. This effect is most likely due to the different molecular mechanisms associated with Pu contamination since the ligand concentrations were kept constant and resulted in large ligand/Pu stoichiometric ratios for all contamination levels. Notably, both ligands appear more potent when compared with Ca-DTPA at higher contamination levels. Such results could influence the choice of parameters such as the contamination level in animal decorporation efficacy studies since comparison with existing and available treatments is one requirement set by the FDA's Animal Efficacy Rule in the drug development process [US Food and Drug Administration, 2006, 2009].

Several parameters may vary in animal decorporation efficacy studies, including the animal species, the route of contamination, and the isotopic and chemical composition of the contaminant. While these variables must all be taken into account as scenarios of human contamination with actinides that include many unknowns, studying the influence of each of them on the decorporation potential of new chelating agents represents a tremendous amount of work. The present work demonstrates that even the choice of the contamination level may change the outcome of a decorporation efficacy study and introduce some bias. It is remarkable that both new hydroxypyridinonate chelating agents under clinical development, 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO), promote significant removal of ^{238}Pu from mice independent of the contamination dose. In addition, they both display efficacies similar to that of Ca-DTPA at the lowest contamination dose tested but are significantly and increasingly more potent at the other three dose levels. While this study points out the need to carefully design animal models for the development of new actinide decorporation agents, it also confirms the high decorporation potential of 3,4,3-LI(1,2-HOPO) and 5-LIO(Me-3,2-HOPO) and supports the need for additional nonclinical investigations.

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