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In Vitro Metabolism and Stability of the Actinide Chelating Agent 3,4,3-LI(1,2-HOPO)

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Abstract

The hydroxypyridinonate ligand 3,4,3-LI(1,2-HOPO) is currently under development for radionuclide chelation therapy. The preclinical characterization of this highly promising ligand comprised the evaluation of its *in vitro* properties, including microsomal, plasma, and gastrointestinal fluid stability, cytochrome P450 inhibition, plasma protein binding, and intestinal absorption using the Caco-2 cell line. When mixed with active human liver microsomes, no loss of parent compound was observed after 60 minutes, indicating compound stability in the presence of liver microsomal P450. At the tested concentrations, 3,4,3-LI(1,2-HOPO) did not significantly influence the activities of any of the cytochromal isoforms screened. Thus, 3,4,3-LI(1,2-HOPO) is unlikely to cause drug-drug interactions by inhibiting the metabolic clearance of co-administered drugs metabolized by these enzymes. Plasma protein binding assays revealed that the compound is protein-bound in dogs and less extensively in rats and humans. In the plasma stability study, the compound was stable after 1 h at 37°C in mouse, rat, dog, and human plasma samples. Finally, a bi-directional permeability assay demonstrated that 3,4,3-LI(1,2-HOPO) is not permeable across the Caco-2 monolayer, highlighting the need to further evaluate the effects of various compounds with known permeability enhancement properties on the permeability of the ligand in future studies.

INTRODUCTION

There is heightened concern regarding the need for treatment after radionuclide contamination given the recent occurrence of nuclear disasters, such as that seen at the Fukushima Daiichi Nuclear Power Plant in March 2011, and the vulnerability of nuclear facilities and materials as the result of terrorism, sabotage or military actions.[1,2] Orally effective chelating agents are the most practical therapy to reduce radiological and chemical

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toxicities resulting from internal contamination with radioisotopes of the actinide series,[3,4] and the lack of existing treatment solutions has prompted significant research and development efforts in the area of medical countermeasures against nuclear threats over the past decade.[5] As a result of these efforts, the octadentate hydroxypyridinonate ligand 3,4,3-LI(1,2-HOPO) received an Investigational New Drug (IND) designation from the U.S. Food and Drug Administration (FDA) in 2014 and is currently undergoing development as an orally effective actinide decorporation agent for the treatment of internal contamination with plutonium (Pu), americium (Am), curium (Cm), uranium (U), or neptunium (Np) (Figure 1). The active pharmaceutical ingredient 3,4,3-LI(1,2-HOPO) has been shown to be more potent and sequester a wider spectrum of radionuclides than diethylenetriaminepentaacetic acid (DTPA), the only drug currently approved by the FDA for treatment of internal contamination by Pu, Am, and Cm.[6] While DTPA-based products must be delivered intravenously or by nebulizer, 3,4,3-LI(1,2-HOPO) has the advantage of being efficacious in the oral delivery format, which is highly desirable from a logistical standpoint in a mass casualty setting.

It is unethical to conduct radionuclide decorporation efficacy studies in humans, and advanced development of 3,4,3-LI(1,2-HOPO) as a drug product must therefore rely heavily on data acquired from animal models as per the Animal Efficacy rule established by the FDA.[7] While studies in animals are essential for drug approval, detailed pharmacological and metabolic profiling of the drug candidates using *in vitro* systems is desirable to minimize the number of animals used. In addition, different species have significant differences in metabolic pathways, which should be recognized early so that the best predictive animal model can be selected for efficacy studies.[8,9] Standard *in vitro* metabolism assays were used to characterize the microsomal, plasma, and gastrointestinal fluid stability of 3,4,3-LI(1,2-HOPO), as these parameters are important considerations when choosing animal models in lieu of human studies. We also report the inhibition potential of 3,4,3-LI(1,2-HOPO) for six cytochrome P450 (CYP) isoforms as well as prediction of intestinal absorption using the Caco-2 cell line.[10]

MATERIALS AND METHODS

The ligand 3,4,3-LI(1,2-HOPO) was synthesized and characterized by Ash Stevens, Inc. (Detroit, MI) as previously described.[4] Purity was determined to be 97.3% by high performance liquid chromatography (HPLC) analysis (data not shown). All other chemicals were obtained from commercial suppliers and used as received. Purified deionized water, using Millipore Milli-Q reverse osmosis, was used to prepare aqueous solutions. Pooled plasma samples and male and female liver microsomes from Sprague Dawley rat, beagle dog, and human were purchased from Bioreclamation, Inc. (Hicksville, NY). All samples were stored at −80°C until use. Either [$^{13}C_4$]-3,4,3-LI(1,2-HOPO), with a chemical purity of 99.3% (Moravek Biochemicals, Inc., Brea, CA), or ethyl nicotinate (Sigma Chemicals, St. Louis, MO) was used as the internal standard during the liquid chromatography coupled with mass spectrometric (LC-MS or LC-MS/MS) analyses.

In Vitro Metabolic Stability using Pooled Human Liver Microsomes

The metabolic stability of 3,4,3-LI(1,2-HOPO) was measured by incubation with human microsomes and assayed by LC-MS/MS. The test ligand 3,4,3-LI(1,2-HOPO) (10 and 50 μM final) was incubated with pooled mixed gender human liver microsomes (active and heat-inactivated, 0.5 mg/mL) and appropriate cofactors (2.5 mM NADPH and 3.3 mM MgCl$_2$) in 0.1 M phosphate buffer, pH 7.4, at 37°C. Reactions were started with the addition of the NADPH/MgCl$_2$ mixture and stopped by removing 100 μL aliquots at selected time points (0, 15, 30 and 60 min) and mixing with 200 μL aliquots of acetonitrile containing ethyl nicotinate as the internal standard (200 ng/mL for 10 μM samples and 1,000 ng/mL for 50 μM samples). Midazolam (10 μM final), a known substrate of CYP3A4, was included as a control. Following brief vortexing and centrifugation, the supernatants were diluted 20- and 100-fold (for 10 and 50 μM samples, respectively) in a 96-well plate using a solution consisting of 10 mM di-sodium ethylenediaminetetraacetic acid (Na$_2$-EDTA) in water; midazolam samples were diluted 20-fold. All samples were assayed in duplicate on a Shimadzu LC-20AD HPLC pump coupled with an API SCIEX 4000 Q TRAP system. Chromatographic separation was achieved on a Synergi Fusion column (Phenomenex, Torrance, CA, USA; 4 μm, 2 $\times$ 50 mm) maintained at 35°C with two mobile phases [(A) 5 mM ammonium acetate, pH 3.5, in methanol-water (5:95, v/v) and (B) 0.5% formic acid in acetonitrile]. Samples (20 μL) were eluted using a gradient from 2% B to 90% B over 1.7 min. The flow rate was maintained at 0.4 mL/min. Analytes and internal standards were detected by multiple reaction monitoring (MRM) after electrospray ionization (ESI) in the positive ion mode, using the following transitions: $775 \to 195$ (3,4,3-LI(1,2-HOPO)) and $152 \to 124$ (ethyl nicotinate).

In Vitro CYP Inhibition

The inhibitory effect of 3,4,3-LI(1,2-HOPO) on *in vitro* CYP activity in human liver microsomes was determined using a high-throughput multiple CYP assay with LC-MS/MS analysis. Pooled human liver microsomes (0.5 mg/mL) and cofactors (2.5 mM NADPH and 3.3 mM MgCl$_2$) were incubated with the test article (1 and 10 μM final) and a cocktail of seven different CYP probe substrates in 0.1 M phosphate buffer, pH 7.4 (final volume of 200 μL). The probe substrate concentrations approximated the K_m for each reaction and consisted of the following final concentrations: 25 μM phenacetin (CYP1A2), 25 μM bupropion (CYP2B6), 10 μM diclofenac (CYP2C9), 20 μM mephenytoin (CYP2C19), 10 μM bufuralol (CYP2D6), 50 μM testosterone (CYP3A4) and 4 μM midazolam (CYP3A4). The incubations contained a final organic solvent concentration of 0.3% DMSO. Specific inhibitor control samples were incubated and analyzed in the same manner as the test article incubation samples but contained the following final concentrations of inhibitors in place of the test article: 10 μM furafylline (CYP1A2), 10 μM thioTEPA (CYP2B6), 3 μM sulfaphenazole (CYP2C9), 10 μM nootkatone (CYP2C19), 2 μM quinidine (CYP2D6), and 5 μM ketoconazole (CYP3A4). Control samples that did not contain the test article or specific inhibitors were prepared in the same manner. Reactions were started with the addition of pre-warmed NADPH/MgCl$_2$ mixture and terminated after 20 min of incubation at 37°C by the addition of 200 μL of ice-cold acetonitrile containing 2 μM dextrorphan (as internal standard). Samples were chilled on ice for 20 min and centrifuged at 1,500 rpm for

20 min at 10°C. The supernatants (~300 μL each) were transferred to a 96-well plate, evaporated under nitrogen for about 15 min until approximately 150 μL remained, then analyzed by LC-MS/MS on a Shimadzu LC-20AD HPLC pump coupled with an API SCIEX 4000 Q TRAP system. Chromatographic separation was achieved on a Phenomenex Synergi Fusion column (4 μm, 2×50 mm) maintained at 35°C with two 0.1% formic acid mobile phases [(A) in water and (B) in acetonitrile]. Samples (10 μL) were eluted using a gradient from 2% B to 95% B over 4.5 min. The flow rate was maintained at 0.4 mL/min. Analytes and internal standards were detected by MRM after positive-ion ESI, using the following transitions: $775 \rightarrow 195$ (3,4,3-LI(1,2-HOPO)), $152 \rightarrow 124$ (ethyl nicotinate), $258 \rightarrow 201$ (dextrorphan), $152 \rightarrow 110$ (acetominophen), $256 \rightarrow 130$ (hydroxybupropion), $312 \rightarrow 230$ (4′-hydroxydiclofenac), $235 \rightarrow 150$ (4′-hydroxymephenytoin), $278 \rightarrow 186$ (1′-hydroxybufuralol), $305 \rightarrow 269$ (6′-hydroxytestosterone), and $342 \rightarrow 168$ (1′-hydroxymidazolam). The percent CYP activity in test article or specific-inhibitor samples relative to the control samples was calculated as follows:

[Substrate metabolite response (peak area ratio, PAR) in presence of inhibitor or test article/Substrate metaboli

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Protein Binding Assay

The plasma protein binding of 3,4,3-LI(1,2-HOPO) was determined using gel filtration and LC-MS/MS analysis. The test ligand 3,4,3-LI(1,2-HOPO) (2.65, 13.3, and 40.0 μM final) was incubated in rat, dog and human plasma for 2 h at 37°C. The plasma proteins in these mixtures were separated by gel filtration through Sephadex G-25 beads (5 min centrifugation at $200 \times g$). The Sephadex G-25 eluates were then processed in duplicate for LC-MS/MS analysis, in parallel with a set of 3,4,3-LI(1,2-HOPO) calibration standards in plasma at 15 different concentrations between 0 and 66.7 μM. Sample preparation entailed the addition of 1 mM EDTA solutions (50 μL) to 50 μL of the test sample or calibration standard, followed by extraction with 1 mL of 3:1 chloroform:methanol (v:v). The organic extract was removed and discarded, and the aqueous phase received 400 μL of acetonitrile to precipitate any remaining plasma proteins. The samples were then centrifuged for 10 min at $18,000 \times g$, after which the supernatants were transferred to new tubes and evaporated under vacuum. The dry residues were reconstituted with 60 μL of 1 mM EDTA in water, centrifuged for 3 min at $18,000 \times g$, and transferred to individual wells of a plastic 96-well plate (deep well; 2 mL volume) for analysis by LC-MS/MS. Chromatographic separation was achieved on a Phenomenex Luna C_{18} column (5 μm, 2×50 mm) maintained at 20°C with two mobile phases [(A) 5 mM ammonium acetate, pH 3.5, in methanol-water (5:95, v/v) and (B) 0.5% formic acid in acetonitrile]. Samples (20 μL injection volume) were eluted isocratically at 11% B with a flow rate of 0.4 ml/min, and the column was then washed with a gradient taking the mobile phase to a composition of 98% and then re-equilibrated to 11% B. The analyte 3,4,3-LI(1,2-HOPO) was detected by MRM after positive-ion ESI using the $751 \rightarrow 195$ transition. Protein concentrations were measured in the spiked plasma samples (not gel-filtered) and the Sephadex G-25 column eluates by the microbiuret method of Itzahki and Gill.[11] After appropriate dilution with phosphate buffer saline (PBS), protein concentrations were determined with the use of calibration standards prepared using bovine serum albumin in PBS at 10 concentrations between 0 and 0.9 mg/mL. To determine the percent of 3,4,3-LI(1,2-HOPO) that was bound to plasma proteins,

the measured concentration of 3,4,3-LI(1,2-HOPO) in the Sephadex G-25 eluates was corrected for any dilution of the plasma proteins that attended passage through the Sephadex G-25 column as follows:

$$[3,4,3-\mathrm{LI}(1,2-\mathrm{HOPO})]_{\mathrm{bound, corrected}}=([\mathrm{Protein}]_{\mathrm{unfiltered}}/[\mathrm{Protein}]_{\mathrm{G-25\ eluate}})\times[3,4,3-\mathrm{LI}(1,2-\mathrm{HOPO})]_{\mathrm{G-25\ eluate}}.$$

. The percent of 3,4,3-LI(1,2-HOPO) that was protein bound is thus expressed as:

$$\%\mathrm{Bound}=100\times\{[3,4,3-\mathrm{LI}(1,2-\mathrm{HOPO})]_{\mathrm{bound, corrected}}/[3,4,3-\mathrm{LI}(1,2-\mathrm{HOPO})]_{\mathrm{total}}\}.$$

Stability in Plasma

The stability of 3,4,3-LI(1,2-HOPO) in mouse, rat, dog, and human plasma was investigated by UPLC-MS analysis. Plasma samples were diluted 1:1 with PBS (pH 7.4, 100 mM) to help maintain the pH. Samples were prepared and assayed in duplicate. To 679 μL of plasma-buffer mixture, 21 μL of 3,4,3-LI(1,2-HOPO) was added (15 μM final). At 0, 5, 15, 30, and 60 min, 100 μL aliquots of test samples were added to 200 μL aliquots of chilled acetonitrile containing [^{13}C$_4$]-3,4,3-LI(1,2-HOPO), as the internal standard (5 μM). The solution mixtures were transferred to 0.2 μm membrane centrifugal filter and then centrifuged for 10 min at 10,000 rpm to remove proteins. Supernatants (10 μL) were assayed in duplicate on a UPLC Waters system interfaced to a QTOF mass spectrometer (Waters Corporation, Milford, MA) in Micromass Z-spray geometry. Chromatographic separation was achieved on an analytical Zorbax Eclipse column (Agilent, XDB-C$_{18}$, 1.8 μm, 4.6×50 mm) maintained at ambient temperature with two 0.1% formic acid mobile phases [(A) in water and (B) in acetonitrile]. Samples (10 μL) were eluted using a gradient initially held constant at 10% B for 1.4 min, then progressed to 95% B in the next 0.9 min. Mobile phase B was held constant at 95% for 1 min and was rapidly switched to 10% B and held until 5 min for equilibration. The flow rate was maintained at 0.5 mL/min. The mass spectrometer equipped with an ESI source was operated in positive ion mode and mass spectra were acquired in the continuum mode across the *m/z* range of 100 – 1000, at 5 s per scan. Calibration was performed by directly infusing a mixture of NaOH 0.1 M – formic acid 10% (50:50) diluted (1:50) with acetonitrile-water (80:20) at a flow rate of 10 μL/min. Selected-ion monitoring mode was used to target ions [M+H]$^+$ at *m/z* 751 and 755 for 3,4,3-LI(1,2-HOPO) and internal standard, respectively. Diltiazem (Sigma Aldrich, St. Louis, MO) and water served as the positive and negative controls, respectively.

Stability in Acidic Solutions and Simulated Gastric Fluid

The stability of 3,4,3-LI(1,2-HOPO) in acidic aqueous buffered solutions and simulated gastric fluid (SGF) was probed and analyzed by LC-MS. Three standard acidic buffer solutions from the U.S. Pharmacopeial Convention (USP) were prepared separately to probe a wide acidic pH range: (i) hydrochloric acid buffer USP (pH 1.40), (ii) acetate buffer USP (pH 4.50), and (iii) phosphate buffer USP (pH 6.80). Test solutions of 3,4,3-LI(1,2-HOPO) at 10 mg/mL were incubated at 25°C/60% relative humidity (RH) throughout the study in sealed scintillation glass vials that were protected from light. Analysis was performed on aliquots sampled at 1, 3, 6, 24, and 48 h and diluted 10-fold. SGF at a final pH of 1.2

consisting of 3.2 g/L of purified pepsin, derived from porcine stomach mucosa, with activities of 800 to 2,500 units per mg of protein (JT Baker, Center Valley, PA); 2 g/L NaCl (Mallinckrodt Inc., Paris, KY); and 8 mL/L 6 N HCl (JT Baker, Center Valley, PA) in water was prepared and stored at 2–8°C. To 990 μL of SGF pre-warmed to 37°C, 10 μL of 3,4,3-LI(1,2-HOPO) was added (1 and 10 μM final) and incubated at 37°C for 0 and 60 min. Samples were prepared and assayed in triplicate with 1 μM final concentrations of erythromycin (Acros, Geel, Belgium) and propanolol (EMD Chemicals, San Diego, CA) as positive and negative controls, respectively. Aliquots (150 μL) of the reactions were stopped at the appropriate time by addition of sodium carbonate (0.1 M), and 150 μL of chilled acetonitrile containing the ^{13}C labeled internal standard. Particulates were removed by centrifugation for 5 min at 10,000 rpm over a 0.2-μm membrane centrifugal filter (VRW International, Radnor, PA). LC-MS analysis was performed on 10 μL supernatants. For both acidic solution and SGF stability samples LC-MS analysis followed the same procedure as that described above for the metabolic stability studies.

Bi-Directional Permeability Study Using Caco-2 Cells

The bi-directional permeability of 3,4,3-LI(1,2-HOPO) was assessed using a standard Caco-2 cell-based assay with LC-MS/MS analysis. A CacoReady™ HTS Transwell®-24 plate consisting of differentiated Caco-2 cells plated on polycarbonate microporous filters (6.5 mm diameter, 0.33 cm^2 growth area, and 0.4 μm pore size) was purchased from ADMEcell, Inc. (Emeryville, CA). Cells were maintained according to the manufacturer's instructions. On the day of the experiment, the integrity of the cell monolayer was assessed by measuring the transepithelial electrical resistance value in each well, using an epithelial volt-ohm meter (EVOM instrument, World Precision Instruments, Sarasota, FL). For apical-to-basal permeability (A-B), test and control compound solutions were prepared in Hanks' Balanced Salt Solution (HBSS) at pH 6.0 and added to the apical side of the cell monolayer. For basal-to apical permeability (B-A), test and control compound solutions were prepared in HBSS at pH 7.4 and added to the basal side of the cell monolayer. Permeability through the cell barrier was measured by taking aliquots from the receiving compartment—from the basal side (lower compartment) for A–B permeability, and from the apical side (upper compartment) for B-A permeability. Incubations with test article (10 and 50 μM final) were performed in duplicate wells in each direction (A–B and B-A). Control incubations were performed in single wells (due to the limited number of wells on the 24-well plate) in both the A–B and B-A directions. Aliquots (100 μl) were removed from the appropriate compartment at different time points (30, 60, 90, and 120 min) and transferred to tubes containing 50 μl of 10 mM EDTA for test article samples or directly into scintillation vials (controls), then replaced with an equal volume of fresh medium. Aliquots were removed from the donor wells at the beginning and end of the experiment to determine recovery of the test article. Controls consisted of: 1) 1 μM digoxin (mix of unlabeled and ^{3}H-labeled, 1μCi/mL) as a substrate of P-glycoprotein (P-gp), and b) 1 μM digoxin (mix of unlabeled and ^{3}H-labeled, 1 μCi/mL) with 100 μM ketoconazole, a known inhibitor of P-gp transport. Radiolabelled control samples containing ^{3}H-digoxin were analyzed by liquid scintillation counting. Test article samples were further diluted with 10 mM EDTA:methanol (97:3, v:v) containing internal standard into a 96-well plate then analyzed by LC-MS/MS in MRM mode using positive-ion ESI. Donor samples (0 and 120 min) were diluted 12-fold with

ethyl nicotinate (1000 ng/mL) followed by 10-fold dilution with 10 mM EDTA:methanol (97:3, v:v); and receiving well samples (30, 60, 90, and 120 min) were diluted 9-fold with ethyl nicotinate (100 ng/mL). LC-MS analysis was performed on 10 μL samples following the same procedure as that described above for human liver microsomal metabolic stability studies. Apparent permeability (P_{app}) was calculated using the following equation:

$P_{app} = (dQ/dt) \times v \times A \times C_0$, where dQ/dt is the rate of drug transport, v is the volume of the receiving compartment, A is the surface area of the membrane, and C_0 is the initial concentration. Efflux ratio was calculated using the following equation:

$ER = P_{app,B-A}/P_{app,A-B}$, where $P_{app, B-A}$ is the apparent permeability of basal-to-apical transport, and $P_{app, A-B}$ is the apparent permeability of apical-to-basal transport. An efflux ratio greater than 2 indicates that a test article is likely to be a substrate for P-gp transport. An efflux ratio that is less than 2, or significantly reduced, when test article is incubated in the presence of a P-gp inhibitor further confirms that the test article is a substrate of P-gp.[10,12]

Statistical Analysis

One-way analysis of variance was used to compare values between groups, where applicable, using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA). Differences were considered significant when p values were <0.05.

RESULTS

Metabolic Stability using Pooled Human Liver Microsomes

The results for the metabolic stability of 3,4,3-LI(1,2-HOPO) using pooled human liver microsomes are presented in Figure 2. When incubated with active microsomes for up to 60 min at both 10 and 50 μM, there was no loss of parent test article, indicating that 3,4,3-LI(1,2-HOPO) was metabolically stable in the chosen conditions. Results for the positive control midazolam were consistent with historical laboratory results, demonstrating the integrity of the assay.

CYP Inhibition

The test ligand 3,4,3-LI(1,2-HOPO) was incubated at two concentrations (1 and 10 μM final) with human liver microsomes, cofactors, and specific CYP probe substrates. Samples were extracted and assayed for metabolites of the CYP probe substrates using a high throughput LC-MS/MS analytical method. The results from the CYP inhibition experiment are displayed in Figure 3. At both concentrations, 3,4,3-LI(1,2-HOPO) did not significantly influence the activities of any of the CYP isoforms that were screened. Yet, the positive control compounds yielded inhibition of CYP enzyme activities consistent with expected values, demonstrating the integrity of the assay. Thus, 3,4,3-LI(1,2-HOPO) is unlikely to cause drug-drug interactions by inhibiting the metabolic clearance of co-administered drugs metabolized by these CYP enzymes.

Protein Binding

Figure 4 shows the results of the protein binding experiments of 3,4,3-LI(1,2-HOPO) in rat, dog, and human plasma using gel filtration and LC-MS/MS analysis. Evaluation of 3,4,3-LI(1,2-HOPO) at concentrations of 2, 10, and 30 μg/mL (2.7, 13.3, and 40.0 μM, respectively) revealed species and concentration dependent differences in the extent of plasma protein binding. Protein binding was greatest in dog, intermediate in human, and lowest in rat and generally increased with increasing amounts of 3,4,3-LI(1,2-HOPO).

Stability of 3,4,3-LI(1,2-HOPO) in plasma from different species

The stability of 3,4,3-LI(1,2-HOPO) was also compared in mouse, rat, dog, and human plasma (Figure 5). No degradation of 3,4,3-LI(1,2-HOPO) was observed for any of the species when 3,4,3-LI(1,2-HOPO) was tested at 15 μM for up to 1 h at 37°C in plasma diluted 1:1 with PBS. This result suggests that the compound is not sensitive to enzymatic hydrolysis. Diltiazem, used as a positive control to ensure the integrity of the experiment, was degraded in mouse, rat, dog, and human plasma at rates consistent with published values.[13]

Stability of 3,4,3-LI(1,2-HOPO) in simulated gastric fluid

Before reaching the systemic circulation, orally administered compounds must first endure the harsh conditions of the gastrointestinal tract. Thus, the stability of an orally administered compound such as 3,4,3-LI(1,2-HOPO) becomes an important consideration during the drug development process. The ligand was stable in three acidic buffer conditions (pH 1.40, 4.50, and 6.80) over 48 h at 25°C/60%RH, indicating that despite the incorporation of several hydrolytically labile amide groups, the structure of 3,4,3-LI(1,2-HOPO) is not readily hydrolyzed in acidic solutions. However, after incubation in simulated gastric fluid (pH 1.2) at 37°C for 60 min, 76 and 72% of the initial 3,4,3-LI(1,2-HOPO) content at concentrations of 1 and 10 μM, respectively, remained. The moderate extent of degradation observed is likely from enzymatic processes as the stability of 3,4,3-LI(1,2-HOPO) in hydrochloric acid buffer (pH 1.4) without pepsin was stable for 3 h. Erythromycin, known to be degraded in gastric fluid,[14] and propranolol, known to be stable in gastric fluid,[15] were included in the assay and served as positive and negative controls.

Bi-Directional Permeability Study Using Caco-2 Cells

For both 10 and 50 μM solutions, permeability could not be calculated since the concentration of 3,4,3-LI(1,2-HOPO) in the receiving wells was below the lower limit of quantitation (0.5 μM) and could not be detected. The mean % recovery of 3,4,3-LI(1,2-HOPO) ranged from 81–121% (Table 1), with the majority being recovered in the donor well at the end of the experiment, thus confirming the lack of permeability of 3,4,3-LI(1,2-HOPO) across the Caco-2 monolayer. The results for the controls were within the expected values. In the absence of ketoconazole, the efflux ratio for digoxin was determined to be 2.75, a value greater than 2, which confirms that it is a substrate of P-gp. In the presence of ketoconazole, an acceptable P-gp inhibitor,[10,12] the efflux ratio was reduced to 0.48. This indicates that in the presence of ketoconazole, P-gp transport of digoxin was inhibited, demonstrating the suitability of this system as a model for P-gp transport.

DISCUSSION

Understanding the *in vitro* behavior of 3,4,3-LI(1,2-HOPO) in biological matrices is an important step in developing this compound as a pharmaceutical ingredient. Solution stability is decisive for good oral bioavailability and should be assessed in the early stage of drug development. When developing an oral drug, it is also essential that the compound does not undergo significant first-pass metabolism, unless the compound is a pro-drug. The metabolic stability of 3,4,3-LI(1,2-HOPO) using pooled human liver microsomes demonstrated no loss of parent test article, indicating that 3,4,3-LI(1,2-HOPO) is metabolically stable.

The inhibitory effect of 3,4,3-LI(1,2-HOPO) on *in vitro* CYP activity in human liver microsomes was determined using a high-throughput multiple CYP assay for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. In the presence of 1 or 10 μM 3,4,3-LI(1,2-HOPO), none of the CYP isoforms were significantly influenced, suggesting that the compound is not a substrate for any of the CYP enzymes tested. These data suggest that co-administration of 3,4,3-LI(1,2-HOPO) with another drug is not likely to inhibit the metabolism of the latter. This is important as co-administration of multiple drugs can potentially influence drug metabolism, effecting plasma levels, thereby leading to adverse drug reactions and/or toxicity.

The plasma protein binding of 3,4,3-LI(1,2-HOPO) was determined using gel filtration. The extent of protein binding of 3,4,3-LI(1,2-HOPO) in rat, dog, and human plasma was species- and concentration-dependent. In general, protein binding was greatest in dog, intermediate in human, and lowest in rat and generally increased with increasing amounts of 3,4,3-LI(1,2-HOPO). The amount of 3,4,3-LI(1,2-HOPO) bound to human protein was below the lower limit of quantification at the tested concentration of 2 μg/mL. When orally administered, the plasma concentration of 3,4,3-LI(1,2-HOPO) (225 mg/kg) is typically less than 2 μg/mL.[8] Since only free drug in whole blood or plasma can elicit therapeutic responses, minimal protein binding of 3,4,3-LI(1,2-HOPO) is expected *in vivo* at physiologically relevant oral dose. Future studies will also have to address the question whether the metal complexes formed between the radionuclides and 3,4,3-LI(1,2-HOPO) exhibit different protein-binding properties.

Stability of 3,4,3-LI(1,2-HOPO) in plasma was investigated as microsomal enzymes are different than plasma enzymes.[16] Assessing plasma stability early on in drug discovery and development is crucial as compounds that are rapidly degraded in plasma may not have sufficient concentration to produce pharmacological activity at the intended target(s).[17] Plasma contains a large number of hydrolytic enzymes such as esterases and deamidases and the level and types of enzymes present in plasma varies from species to species;[17,18] it is therefore important to evaluate the compound stability in plasma of different species and compare with human plasma. Despite hydrolytically labile functional groups present in the structure of 3,4,3-LI(1,2-HOPO) that may result in rapid clearance in plasma, the compound exhibited stability under the test conditions. One explanation for the observed stability of 3,4,3-LI(1,2-HOPO) in plasma may be that it is hydrolyzed by enzymes with low catalytic capacities.

The bi-directional permeability study confirmed the low permeability of 3,4,3-LI(1,2-HOPO). To determine whether 3,4,3-LI(1,2-HOPO), due to its large size, could permeate through the membrane of the Transwell plate itself, a second identical experiment was performed in a 24-well Transwell plate not containing Caco-2 cells. The results indicated that while detectable levels of 3,4,3-LI(1,2-HOPO) were able to permeate the membrane in the absence of Caco-2 cells, when the experiment was performed in the presence of cells, the test article either was not able to permeate through the cells or if a small amount was able to pass through the cells, it was below the limit of quantitation and could not be detected. Future studies will explore the reasons for the low permeability of 3,4,3-LI(1,2-HOPO), including for example its potential interactions with the plasma membrane P-glycoprotein.

In conclusion, this work highlights the stability of 3,4,3-LI(1,2-HOPO) in various biological matrices. To improve permeability of 3,4,3-LI(1,2-HOPO), various compounds with known permeability enhancement qualities are currently being evaluated. Based on a series of *in vivo* actinide decorporation efficacy studies,[4,8,9] and despite its poor permeability, 3,4,3-LI(1,2-HOPO) is, as of today, the most promising actinide chelating agent for administration as an oral therapeutic; continued development is therefore warranted.

Acknowledgments

List of Abbreviations

Am	Americium
Cm	Curium
CYP	Cytochrome P450
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionization
FDA	U.S. Food and Drug Administration
HBSS	Hanks' Balanced Salt Solution
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography coupled with Mass Spectrometry
MRM	Multiple Reaction Monitoring
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Np	Neptunium
PBS	Phosphate Buffer Saline

Pu	Plutonium
QTOF	Quadrupole Time-of-Flight
RH	Relative Humidity
SGF	Simulated Gastric Fluid
U	Uranium
UPLC	Ultra performance liquid chromatography
USP	U.S. Pharmacopeial Convention

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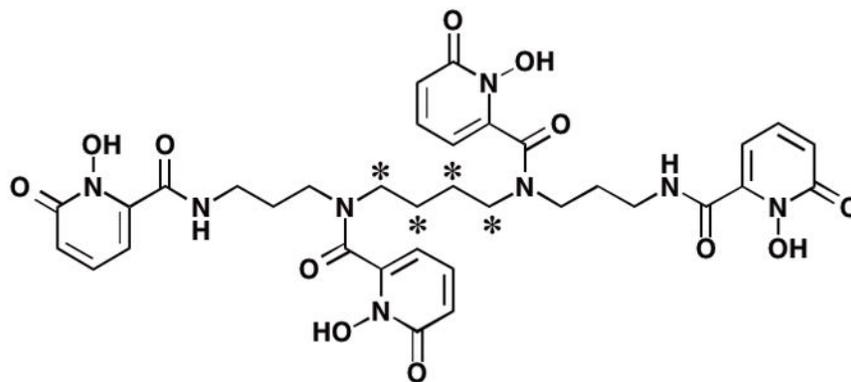
Figure 1.
Structure of 3,4,3-LI(1,2-HOPO); the * indicate ^{13}C labels in the labeled internal standard [$^{13}C_4$]-3,4,3-LI(1,2-HOPO).
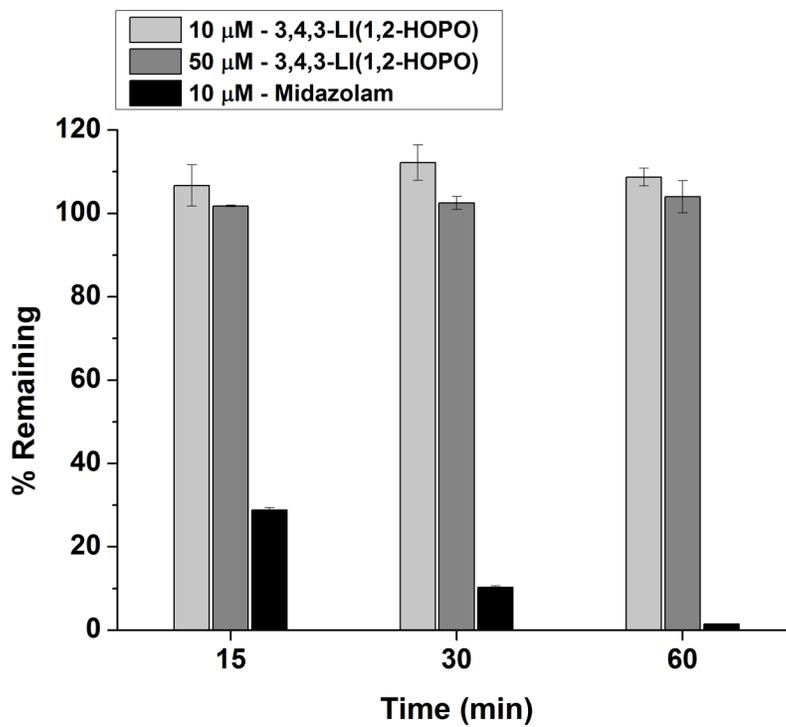
Figure 2.
Metabolic stability of 3,4,3-LI(1,2-HOPO) (10 and 50 μM) and midazolam (10 μM) using pooled, mixed gender human liver microsomes. Data expressed as % of test article remaining (mean ± SD, n = 3) relative to the control samples (t = 0 min, 100%).
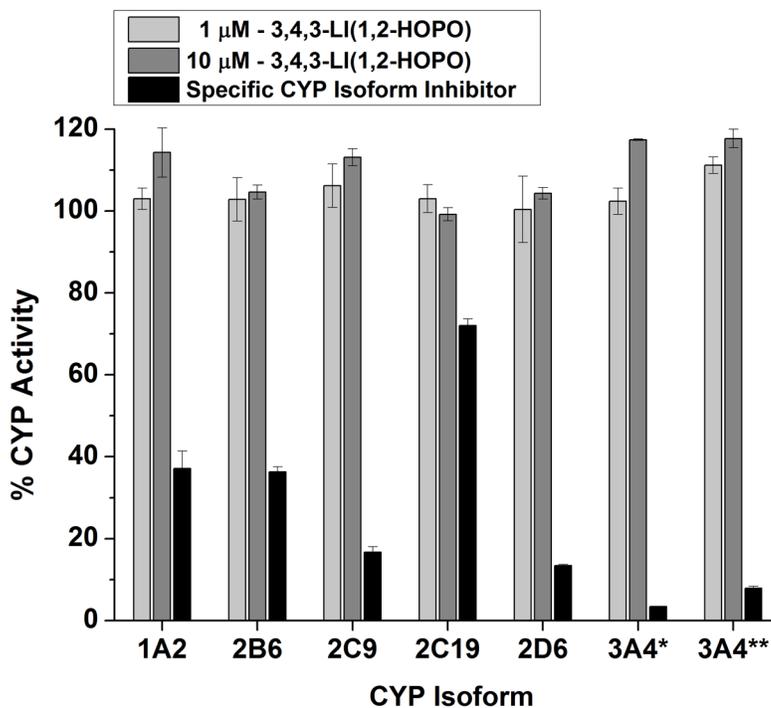
Figure 3.
Effect of 3,4,3-LI(1,2-HOPO) (1 and 10 µM) on CYP activity in human liver microsomes. Seven specific CYP probe substrates [phenacetin (CYP1A2), bupropion (CYP2B6), diclofenac (CYP2C9), mephenytoin (CYP2C19), bufuralol (CYP2D6), testosterone (CYP3A4*) and midazolam (CYP3A4**)] and six specific inhibitors [furafylline (CYP1A2), thioTEPA (CYP2B6), sulfaphenazole (CYP2C9), nootkatone (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A4)] were included in the assay. Data expressed as % CYP activity in test article or specific inhibitor samples (mean ± SD, n = 3) relative to the control samples (no test article/inhibitor).
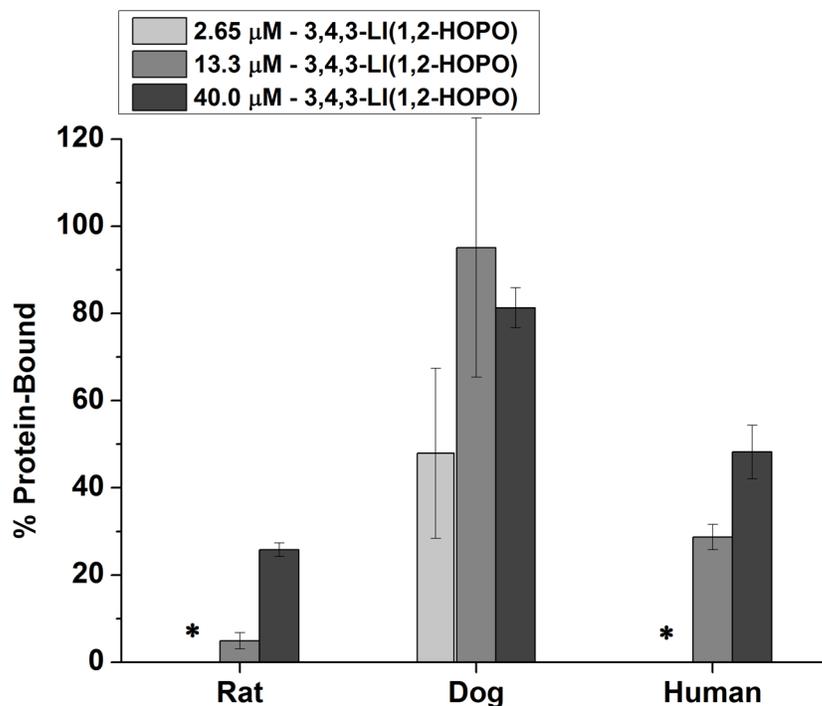
Figure 4.
Determination of 3,4,3-LI(1,2-HOPO) (2.65, 13.3, and 40.0 μM) binding to rat, dog, and human plasma proteins by gel filtration (2 h incubation, 37°C). Data expressed as % protein-bound (mean ± SD, n = 3), with values for 2.65 μM samples in rat and human plasma (*) below the lower limit of quantitation.
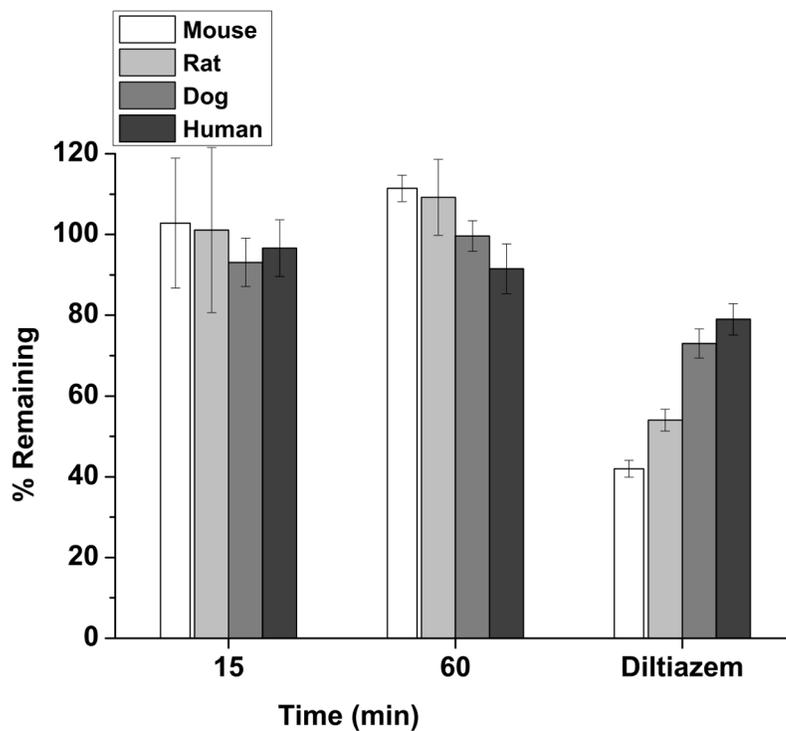
Figure 5.
tability of 3,4,3-LI(1,2-HOPO) (15 μM) in mouse, rat, dog, and human plasma. Data expressed as % of test article remaining (mean ± SD, n = 2) relative to the initial samples (t = 0 min, 100%). Diltiazem was used as a positive control for the assay.

Table 1

In vitro bi-directional permeability of 3,4,3-LI(1,2-HOPO) using Caco-2 cells.

	$P_{app} \times 10^{-6}$ cm/sec		Efflux Ratio	% Recovery of Test Article[b]	
	A–B (mean)	B-A (mean)		A–B (mean)	B-A (mean)
10 µM 3,4,3-LI(1,2-HOPO)	NC[a]	NC	NC	112.2	87.2
10 µM 3,4,3-LI(1,2-HOPO) + 100 µM ketoconazole	NC	NC	NC	120.6	90.3
50 µM 3,4,3-LI(1,2-HOPO)	NC	NC	NC	105.5	97.6
50 µM 3,4,3-LI(1,2-HOPO) + 100 µM ketoconazole	NC	NC	NC	114.9	85.7
1 µM digoxin	3.1	8.6	2.8	90.8	80.9
1 µM digoxin + 100 µM ketoconazole	3.7	1.8	0.5	93.0	85.3

[a] NC = not calculated. P_{app} could not be calculated, since test article could not be detected in receiving wells.

[b] % recovery of test article at the end of experiment.