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 min, 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 coadministered 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 bidirectional 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. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:1832–1838, 2015

Keywords: chelation therapy; stability; microsomes; ADME ; protein binding; cytochrome P450; intestinal absorption

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 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 last decade.[5] As a result of these efforts, the octadentate hydroxypyridinonate ligand 3,4,3-LI(1,2-HOPO) received an investigational new drug designation from the US 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, or neptunium (Fig. 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] Although 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] Although 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, Michigan) as

Abbreviations used: Am, americium; Cm, curium; CYP, cytochrome P450; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FDA, US 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; PBS, phosphate buffer saline; Pu, plutonium; RH, relative humidity; SGF, simulated gastric fluid; UPLC, ultra-performance liquid chromatography; USP, US Pharmacopeial Convention.

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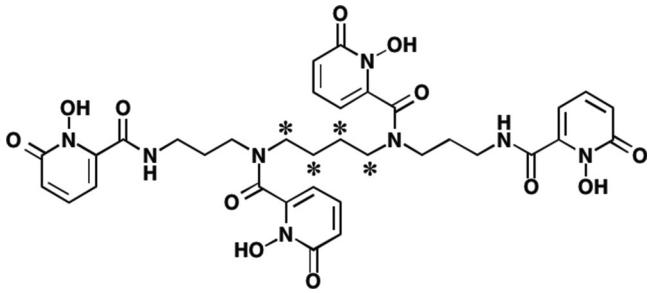

Figure 1. Structure of 3,4,3-LI(1,2-HOPO); * indicates ^{13}C labels in the labeled internal standard [$^{13}C_4$]-3,4,3-LI(1,2-HOPO).

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, New York). 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, California), or ethyl nicotinate (Sigma Chemicals, St. Louis, Missouri) was used as the internal standard during the liquid chromatography coupled with mass spectrometry (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 nicotinamide adenine dinucleotide phosphate (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 1000 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 disodium 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, California; 4 μm, 2 × 50 mm^2) 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 → 195 [3,4,3-LI(1,2-HOPO)] and 152 → 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% dimethyl sulfoxide. 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 prewarmed 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*g* 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^2) 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 → 195 [3,4,3-LI(1,2-HOPO)], 152 → 124 (ethyl nicotinate), 258 → 201 (dextrorphan), 152 → 110 (acetominophen), 256 → 130 (hydroxybupropion), 312 → 230 (4′-hydroxydiclofenac), 235 → 150 (4′-hydroxymephenytoin), 278 → 186 (1′-hydroxybufuralol), 305 → 269 (6′-hydroxytestosterone), and 342 → 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 the presence of inhibitor or test article/substrate metabolite mean PAR in control] × 100.

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.