Design and Evaluation of a Novel Trifluorinated Imaging Agent for Assessment of Bile Acid Transport Using Fluorine Magnetic Resonance Imaging

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Received 7 May 2014; revised 17 July 2014; accepted 23 July 2014

Published online 5 September 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24131

ABSTRACT: Previously, we developed a trifluorinated bile acid, CA-lys-TFA, with the objective of noninvasively assessing bile acid transport *in vivo* using ^{19}F magnetic resonance imaging (MRI). CA-lys-TFA was successfully imaged in the mouse gallbladder, but was susceptible to deconjugation *in vitro* by choloylglycine hydrolase (CGH), a bacterial bile acid deconjugating enzyme found in the terminal ileum and colon. The objective of the present study was to develop a novel trifluorinated bile acid resistant to deconjugation by CGH. CA-sar-TFMA was designed, synthesized, and tested for *in vitro* transport properties, stability, imaging properties, and its ability to differentially accumulate in the gallbladders of normal mice, compared with mice with known impaired bile acid transport (deficient in the apical sodium-dependent bile acid transporter, ASBT). CA-sar-TFMA was a potent inhibitor and substrate of ASBT and the Na^+/taurocholate cotransporting polypeptide. Stability was favorable in all conditions tested, including the presence of CGH. CA-sar-TFMA was successfully imaged and accumulated at 16.1-fold higher concentrations in gallbladders from wild-type mice compared with those from Asbt-deficient mice. Our results support the potential of using MRI with CA-sar-TFMA as a noninvasive method to assess bile acid transport *in vivo*.
 J Pharm Sci 103:3782–3792, 2014
Keywords: fluorine MRI; bile acid malabsorption; enterohepatic circulation; imaging methods; intestinal absorption; site-specific delivery; transporters; targeted drug delivery; bile acid transporters; biliary excretion

INTRODUCTION

Bile acid homeostasis is maintained by balancing hepatic synthesis with an efficient enterohepatic recirculation, and the major biosynthetic enzymes and transporters have been identified. After their synthesis from cholesterol in the liver, bile acids are conjugated to glycine or taurine, and secreted into bile by the bile salt export pump (BSEP; *ABCB11*) and to a lesser extent by the multidrug resistance-associated protein 2 (MRP2; *ABCC2*). Between meals, bile is stored in the gallbladder. In response to a meal, the gallbladder contracts and bile acids are emptied into the duodenum where they increase lipid solubility through micelle formation. Bile acids are passively absorbed throughout the small intestine, as well as actively absorbed by enterocytes in the terminal ileum via the apical sodium-dependent bile acid transporter (ASBT, *SLC10A2*). After uptake by enterocytes, bile acids are effluxed into the portal circulation by the organic solute transporters (OSTα-OSTβ; *SLC51A*, *SLC51B*) and to a lesser extent by multidrug resistance protein 3 (MRP3; *ABCC3*). At the liver, the bile acids are taken up by hepatocytes via the Na^+/taurocholate cotransporting polypeptide (NTCP, *SLC10A1*) and the organic anion transporting polypeptides (OATPs) for resecretion into bile. This enterohepatic circulation of bile acids maintains the human bile acid pool between 2 and 4 g. Bile acids circulate several times daily with less than 10% lost in feces.[1,2]

Bile acid malabsorption (BAM), characterized by excess fecal bile acids and chronic watery diarrhea, is often misdiagnosed as diarrhea-predominant irritable bowel syndrome (IBS-D).[3] BAM is considered responsible for 30%–50% of unexplained chronic diarrhea.[4–7] Although BAM can be attributed to ileal

Abbreviations used: ACN, acetonitrile; ASBT, the apical sodium-dependent bile acid transporter; BAM, bile acid malabsorption; BSEP, the bile salt export pump; CGH, choloylglycine hydrolase; DCM, dichloromethane; DMEM, Dulbecco's modified Eagle medium; DMF, dimethyl formamide; DPBS, Dulbecco's phosphate buffered saline; DMSO, dimethyl sulfoxide; EtOH, ethanol; FBS, fetal bovine serum; FGF, fibroblast growth factor; FLASH, fast low angle shot; HBSS, Hank's balanced salt solution; HBTU, hexaflourophosphate; HCAT, homocholic acid-taurine; HEK, human embryonic kidney; HOBt, hydroxybenzotriazole; HSQC, heteronuclear single quantum coherence; IBS, irritable bowel syndrome; i.p., intraperitoneal; LC/MS/MS, liquid chromatography/tandem-mass spectrometry; MDCK, Madin–Darby canine kidney; MRI, magnetic resonance imaging; MRP2, the multidrug resistance-associated protein 2; MRP3, the multidrug resistance-associated protein 3; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate; NaOH, sodium hydroxide; NMR, nuclear magnetic resonance; NTCP, the Na^+/taurocholate cotransporting polypeptide; OATPs, the organic anion transporting peptides; OSTα-OSTβ, the organic solute transporters; PEG, polyethylene glycol; RARE, rapid acquisition with relaxation enhancement; ROI, region of interest; SFB, sodium-free buffer; SIF, simulated intestinal fluid with pancreatic enzymes; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; WT, wild type.

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This article contains supplementary material available from the authors upon request or via the Internet at http://onlinelibrary.wiley.com/.

Journal of Pharmaceutical Sciences, Vol. 103, 3782–3792 (2014)

resection/damage or rare ASBT mutations, the cause of most cases of idiopathic BAM is unknown. Recent advances in understanding this disease suggest that overproduction of bile acids resulting from deficient fibroblast growth factor (FGF)-19 may be a common feature of BAM.[8] FGF19 is part of the mechanism for feedback inhibition of hepatic bile acid synthesis from cholesterol, and regulation of bile acid synthesis is impaired in its absence. This dysregulation leads to hepatic overproduction of bile acids, exceeding the ileal absorptive capacity, thereby increasing colonic exposure and diarrhea.

In the United States, diagnosis of BAM is limited by the lack of sensitive, specific, and cost-effective tests. ^{75}Se-homocholic acid-taurine (HCAT), a ^{75}Se-labeled gamma-emitting synthetic bile acid used to measure intestinal uptake of bile acids, is available for use in selected European countries, but has not been approved by the United States Food and Drug Administration (US FDA).[9] Diagnostic criteria for this test are based on the percentage of ^{75}Se-HCAT retained in the body 1 week after oral administration. Other methods to diagnose BAM include ^{14}C-taurocholate stool measurement,[10] 7α-hydroxy-4-cholesten-3-one serum measurement as a biomarker of bile acid formation,[11] and blood FGF19 measurement[12] (inversely related to 7α-hydroxy-4-cholesten-3-one levels). However, these methods are time consuming, difficult, not readily available, or not validated clinically. Hence, BAM is often diagnosed by administering bile acid sequestrants, such as colesevelam, in a therapeutic trial,[13,14] an approach that is not US FDA approved for this indication, lacks specificity, and has a high rate of false-negative diagnosis.[7]

To address these limitations, we previously proposed to develop a novel approach to diagnosing BAM by using *in vivo* imaging of a fluorinated bile acid analogue with ^{19}F magnetic resonance imaging (MRI).[15] We hypothesized that tracking a tagged bile acid molecule by imaging its accumulation in the gallbladder would allow differentiation between normal and impaired bile acid transport. ^{19}F MRI was chosen because it is noninvasive and involves no ionizing radiation. ^{19}F, the naturally occurring, stable (i.e., nonradioactive) isotope of fluorine, is second to only ^{1}H MRI in terms of MRI sensitivity. Unlike ^{1}H MRI, with ^{19}F MRI there is no endogenous background signal,[16] thereby providing the potential for improved signal-to-noise-ratio. ^{19}F MRI signal intensity increases proportionally to fluorine concentration, so tracer amounts can be compared and quantified.[17]

CA-lys-TFA, a conjugate of trifluoroacetyl-lysine and cholic acid, was previously synthesized and tested *in vitro* for stability and ASBT and NTCP transport affinity.[15] After a preliminary pharmacokinetic profile was obtained in mice, CA-lys-TFA was orally dosed and imaged *in vivo* in the mouse gallbladder by ^{19}F MRI.[18] CA-lys-TFA accumulated in significantly larger amounts in wild type (WT) mouse gallbladders compared with gallbladders of Asbt-deficient (*Slc10a2*$^{-/-}$) mice, which have severely impaired bile acid absorption, a reduced bile acid pool, and higher levels of fecal bile acids.[19] The gallbladders of Asbt-deficient mice showed no fluorine signal when imaged using ^{19}F MRI, whereas those of WT mice showed robust ^{19}F signals. Overall, these studies showed that ^{19}F MRI of a fluorinated bile acid probe is a feasible means to identify impaired bile acid transport *in vivo*.

An important limitation of CA-lys-TFA as an imaging agent is its susceptibility to bacterial metabolism. Like native bile acids, Ca-lys-TFA is susceptible to removal of its amino acid side chain by choloylglycine hydrolase (CGH), a bacterial bile acid deconjugating enzyme located predominantly in the colon and in smaller amounts in the terminal ileum.[15] Here, our objective was to synthesize and test a fluorinated bile acid resistant to bacterial deconjugation. We believe this attribute will diminish the potential for differences in intestinal microbiota to alter probe concentrations in the enterohepatic circulation and also increase the half-life of the fluorinated bile acid probe. We report the synthesis of the novel trifluorinated bile acid CA-sar-TFMA, and its *in vitro* stability and affinity for both ASBT and NTCP. A pilot *in vivo* disposition study in mice confirmed that CA-sar-TFMA can be imaged in the gallbladder using ^{19}F MRI. Additionally, using Asbt-deficient mice as a test model, we show that oral administration of CA-sar-TFMA has potential as a novel method to diagnose impaired intestinal bile acid uptake. Collectively, our results support the suitability of CA-sar-TFMA as a ^{19}F MRI tracer to diagnose BAM.

EXPERIMENTAL

Materials

Taurocholate, cholic acid, trifluoroacetic anhydride, rat liver S9 fraction, trifluoroacetic acid (TFA), rat plasma, and CGH from *Clostridium perfringens* were obtained from Sigma–Aldrich (St. Louis, Missouri). N-boc-ethylene diamine was purchased from Oakwood Chemical (West Columbia, South Carolina). [^{3}H]-taurocholate (10 μCi/mM) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri). Trypsin, geneticin, fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were purchased from Invitrogen (Rockville, Maryland). All other reagents and chemicals were of the highest purity available commercially.

Methods

Synthesis of CA-sar-TFMA

CA-sar-TFMA was synthesized as in Figure 1. Two milliliters (12.6 mmol) of N-boc-ethylene diamine was stirred for 15 min with 2 eq. (25.2 mmol) sodium hydroxide (NaOH) in dimethyl formamide (DMF). To this mixture, 0.6 eq. (7.6 mmol) benzyl bromoacetate was added and stirred overnight at room temperature. DMF was diluted with ethyl acetate and washed three times with 30 mL water. The product was dried with sodium sulfate and ethyl acetate was removed by vacuum. The resulting clear oil was separated by silica gel column chromatography, using an eluent of 1:1 ethyl acetate:hexane. The resulting product (Fig. 1, compound 1) showed an appropriate mass spectrometry (MS) peak of [M+1] 309.1.

Compound 1 was stirred with 1:1 dichloromethane (DCM):TFA for 15 min to remove the N-boc protecting group. Excess solvent was evaporated, yielding compound 2. Next, the compound was stirred in DCM at 0°C and 0.6 eq. (3.5 mmol) triflouroacetic anhydride was added. The mixture was allowed to return to room temperature and was stirred overnight. DCM was then evaporated under vacuum and the product was dissolved in ethyl acetate and washed with saturated sodium bicarbonate. The organic layer was dried with sodium sulfate and ethyl acetate was evaporated under vacuum. The resulting product was purified using flash column chromatography with a solvent of 30% hexane in ethyl acetate. MS showed [M+1] of 305.1 (Fig. 1, compound 3) and thin-layer chromatography