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Real-Time Intraoperative Assessment of the Extrahepatic Bile Ducts in Rats and Pigs Using Invisible Near-Infrared Fluorescent Light

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Abstract

Background—Currently, only x-ray fluoroscopy is available for visualization of the extrahepatic bile ducts intraoperatively. We hypothesized that with an appropriate fluorophore and imaging system, invisible near-infrared (NIR) light could be used for image-guided procedures on the extrahepatic bile ducts.

Methods—We quantified the performance of three 800 nm NIR fluorophores, differing primarily in their degree of hydrophilicity, for real-time imaging of the extrahepatic bile ducts in rats and pigs: IR-786, indocyanine green (ICG), and the carboxylic form of IRDyeTM 800CW (CW800-CA). The signal-to-background ratio (SBR) of the CBD relative to liver and pancreas was measured as a function of the dose of contrast agent, injection site, and kinetics using a previously described intraoperative NIR fluorescence imaging system. Bile samples were examined by high performance liquid chromatography tandem mass spectrometry (HPLC/MS) to determine the chemical form of fluorophores in bile.

Results—Non-sulfonated (IR-786) and di-sulfonated (ICG) NIR fluorophores had poor efficiency and kinetics of excretion into bile. Tetra-sulfonated CW800-CA, however, provided sensitive, specific, and real-time visualization of the extrahepatic bile ducts after a single low-dose given either intraportally or intravenously via systemic vein. A SBR ≥ 2 provided sensitive assessment of extrahepatic bile duct anatomy and function, including the detection of millimeter-sized, radiolucent inclusions in pigs, for over 30 min post-injection. CW800-CA remained chemically intact after secretion into bile.

Conclusion—The combination of invisible NIR light and an IV injection of CW800-CA provides prolonged, real-time visualization of the extrahepatic bile ducts, without ionizing radiation, and without changing the look of the surgical field.

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Keywords

Common Bile Duct; Extrahepatic Ducts; Intraoperative Imaging; Near-Infrared Fluorophores; Near-Infrared Fluorescence Imaging

INTRODUCTION

Intraoperative x-ray cholangiography (IOC), performed by direct injection of iodine contrast medium into the CBD, is used for its anatomic visualization, as well as intraoperative diagnosis of stones and injuries. The use of IOC is still controversial, and opinions are split between routine^{1,2,3} and selective use.⁴ Nevertheless, the incidence of CBD stones is approximately 3.3%,⁵ and the detection rate of CBD abnormalities or injuries intraoperatively is reported in the range of 18 to 37%,^{6,7,8} suggesting that biliary complications might be detected earlier and more frequently with its routine use.⁶ In addition, early diagnosis of stones and injuries is reported to improve treatment outcome.^{5,9,10} Thus, if there were a more convenient method for routine intraoperative assessment of the extrahepatic bile ducts, outcomes might be improved. Intraoperative assessment might also potentially prevent or reduce severe bile duct injuries.

There are various cholegraphic techniques available, including excellent preoperative imaging of stones using ERCP, three-dimensional CT, and MRCP.^{11,12} However, none of these techniques are ideal for intraoperative imaging. First, it takes a relatively long time (≈ 30 –60 min) to detect the bile duct following administration of contrast agents (CT) or just to perform the study (ERCP and MRCP).¹³ Second, it is difficult to merge pre-operative scans with what the surgeon sees on the operative field. Third, and most important, iodine-based agents have a low, but clinically relevant, rate of adverse reactions.^{14,15} Presently, there are no efficient intraoperative imaging modalities for real-time visualization of extrahepatic biliary anatomy and function in the operative field, other than direct injection of iodine contrast agent in conjunction with x-ray fluoroscopy or ultrasonography.

Invisible NIR light in the range of 700 nm to 900 nm has the potential to provide sensitive, specific, and real-time imaging of the extrahepatic bile ducts (reviewed in¹⁶). Photon absorption, scatter, and autofluorescence in this wavelength range are relatively low, which provides a low “background” to which a “signal” from exogenous NIR fluorophores can be added. This setting is in sharp contrast to UV and visible wavelength fluorescence,¹⁷ which suffer from high background and relatively low signal. We have described previously an intraoperative NIR fluorescence imaging system that simultaneously acquires color video (i.e., surgical anatomy) along with NIR fluorescence of the surgical field.^{18–20} By introducing an exogenous NIR fluorophore that highlights the target of interest, such as extrahepatic bile ducts, the NIR fluorescence channel can be used to pinpoint the location of any desired structure in the operative field and/or to assess its function. To date, this system has been used for high sensitivity sentinel lymph node mapping,^{20–32} assessment of tissue and vascular calcification,^{33–36} assessment of ischemic reperfusion-related cell injury and death,³⁷ detection of obscure gastrointestinal bleeding,³⁸ detection of acute thrombosis,³⁹ and delivery of gene therapy.⁴⁰

Heptamethine indocyanines are a class of NIR fluorophores for which vast human data are available. The prototype molecule, indocyanine green (ICG), has been FDA-approved for other indications since 1958 and has a remarkable safety record.^{41,42} In this study, we compare three different 800 nm heptamethine indocyanines differing primarily in their degree of sulfonation, i.e., hydrophilicity. For each compound, we quantify their efficiency of transport from the bloodstream to extrahepatic bile ducts, their SBR in the bile duct over time, and their performance in assessing extrahepatic biliary anatomy and function.

MATERIALS AND METHODS

Preparation of NIR Fluorophores

IR-786 perchlorate (IR-786) and sodium ICG were purchased from Sigma (St. Louis, MO). CW800-CA, the carboxylic acid form of IRDyeTM 800CW was obtained from LI-COR (Lincoln, NE). IR-786 was diluted to 100 μ M in phosphate-buffered saline (PBS) supplemented with 10% Cremophor EL (Sigma) and 10% absolute ethanol immediately before use. Stock solutions of 10 mM ICG and 18 mM CW800-CA were stored in DMSO at -80°C in the dark. ICG and CW800-CA were diluted in PBS as indicated below before use. Quantum yields of contrast agents in 100% fetal bovine serum (FBS) were calculated using ICG in dimethyl sulfoxide (quantum yield 13%⁴³) as the calibration standard, under conditions of matched fluorophore absorbance.

Animal Model Systems

Animals were used in accordance with an approved institutional protocol. Adult, male, 330 g, Sprague-Dawley rats from Charles River Laboratories (Wilmington, MA) were anesthetized with 65 mg/kg intraperitoneal pentobarbital. Adult, female, 30 kg Yorkshire pigs (E.M. Parsons and Sons, Hadley, MA) were induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Labs, Fort Dodge, IA), then intubated and maintained with 1.5–2.0% isoflurane/balance O₂.

Bile Duct Imaging

Portal vein injection in rats (N = 3 rats per NIR fluorophore) was performed with a 30-gauge, 1/2" needle using the following volumes and concentrations of stock solutions: IR-786 (50 μ L of a 100 μ M stock; 0.015 μ mol/kg final; 9 μ g/kg final), ICG (50 μ L of a 1 mM stock; 0.15 μ mol/kg final; 120 μ g/kg final), and CW800-CA (50 μ L of a 100 μ M stock; 0.015 μ mol/kg final; 15 μ g/kg final). Fluorescence intensities of the CBD, pancreas, and right lobe of the liver were measured with the same regions of interests at 3, 5, 10, 20, and 30 min following injection of each NIR fluorophore.

For quantifying the dose-response of CW800-CA, the CBD was visualized after systemic (tail vein) IV injection (N = 4 rats per dose) or portal vein injection (N = 4 rats per dose) of 50 μ L of CW800-CA having a stock concentration of 10, 20, 50, and 100 μ M, corresponding to final doses of 0.0015 μ mol/kg (1.5 μ g/kg), 0.003 μ mol/kg (3 μ g/kg), 0.0075 μ mol/kg (7.5 μ g/kg), and 0.015 μ mol/kg (15 μ g/kg), respectively. Fluorescence intensities of the CBD, pancreas, and right lobe of the liver were measured with the same regions of interests at 3, 5, 10, 20, and 30 min following injection of each NIR fluorophore.

In anesthetized pigs, 5 mL of 100 μ M of CW800-CA (0.015 μ mol/kg; 15 μ g/kg final) were injected into the portal vein (N = 3 pigs) and 5 mL of either 50 μ M (N = 3 pigs; 0.0075 μ mol/kg; 7.5 μ g/kg) or 100 μ M (N = 3 pigs; 0.015 μ mol/kg; 15 μ g/kg) of CW800-CA were injected systemically (ear vein). Radiolucent globular beads (2.5 mm in diameter) or cylindrical beads (3.5 mm in long axis) were inserted into the CBD via the papilla of Vater after duodenotomy (N = 2 pigs). These were used to simulate different sizes and shapes of CBD stones seen in patients. An additional N = 2 pigs received direct injection of 10 mL of 10 μ M ICG into the cystic duct.

A previously described²⁰ simultaneous color video/NIR fluorescence intraoperative imaging system was employed for quantification of all NIR fluorescence images. White light (400–700 nm) and NIR fluorescence excitation light (725–775 nm) was used at 1 mW/cm² and 5 mW/cm², respectively. Spatial resolution at a field-of-view of 20 $\times$ 15 cm is 625 μ m, and at a field-of-view of 4 $\times$ 3 cm is 125 μ m. After computer-controlled camera acquisition via custom LabVIEW (National Instruments, Austin, TX) software, anatomic (white light) and functional

(NIR fluorescence light) images were displayed separately and merged. To create a single image that displays both anatomy (color video) and function (NIR fluorescence), the NIR fluorescence image was pseudo-colored in lime green and overlaid with 100% transparency on top of the color video image of the same surgical field. All images were refreshed up to 15 times per second. The entire apparatus was suspended on an articulated arm over the surgical field, thus permitting non-invasive and non-intrusive imaging. Hands-free operation utilizing motorized zoom and focus lenses, a four-pedal footswitch, and a multifunction data acquisition board were described in detail previously.⁴⁴

Gel-Filtration Chromatography

Bile was collected from the CBD using a needle and syringe, flash frozen in LN₂, and stored at -80°C until analyzed. After thawing, samples were diluted immediately 1:1 in phosphate-buffered saline, pH 7.4 (PBS) and separated using an AKTA Prime chromatography system (Amersham Bioscience, Piscataway, NJ) equipped with an Econo-Pac P6 (6,000 MW cutoff; Bio-Rad Laboratories, Hercules, CA) gel-filtration cartridge. The mobile phase was PBS and the flow rate was 0.5 ml/min. Online, full-spectrum absorbance (200–1100 nm) and fluorescence (350–1000 nm) were measured as described in detail previously.⁴⁵ Fractions having the highest 783 nm absorbance and 803 nm fluorescence were collected for analysis by mass spectrometry.

High-Performance Liquid Chromatography/Tandem Mass Spectrometry (LC/MS)

Twenty-five microliters of each gel-filtration sample was analyzed on a Waters (Milford, MA) LCT electrospray (ES) time-of-flight (TOF) LC/MS equipped with dual-absorbance detectors, fluorescence detector, Sedex model 75 evaporative light scatter detector (ELSD; Richards Scientific, Novato, CA), and lockspray, as described in detail previously.⁴⁶ Briefly, the absorbance detectors were set to 280 nm and 700 nm (the maximum permitted wavelength). The fluorescence detector was set to 803 nm using 770 nm excitation light. Bovine heart myoglobin (5 µM) in water was used as a mass reference for the lockspray. Buffer A was 10 mM triethylammonium acetate (TEAA; Glen Research, Sterling, VA) and Buffer B was absolute methanol (Aaper Alcohol and Chemical, Shelbyville, KY). Using a gradient of 0 to 70% B over 20 min, bile samples were resolved on a 4.6 × 150 mm Symmetry (Waters) C₁₈ column at a flow rate of 1 ml/min, with the eluate split equally to the ELSD and MS detectors. The ion mode was set to ES⁻, and cone and capillary voltages were 30 V and 3000 V, respectively. Desolvation and source temperature were 350°C and 140°C, respectively. Data were analyzed with MassLynx (Waters) software.

RESULTS

NIR Fluorescent Contrast Agents for Imaging the Extrahepatic Bile Ducts

The heptamethine indocyanines IR-786, ICG, and CW800-CA possess 0, 2, and 4 sulfonate groups, respectively, and hence increasing hydrophilicity (Figure 1A). Although all three have similar chemical structures and peak emission wavelengths ≈800 nm, their quantum yield in serum increases with hydrophilicity. So, too, does the efficiency of excretion into bile (this study) and urine⁴⁷ after intravenous injection, with the tetra-sulfonated compound CW800-CA showing superior performance.

Portal Vein Injection of NIR Fluorescent Contrast Agents

To optimize visualization of the extrahepatic bile ducts, NIR fluorescent contrast agents were injected initially into the portal vein. As shown in Figure 1B, all three agents provided image-guided identification of the extrahepatic bile ducts but differed greatly in their performance. IR-786 and ICG were excreted poorly into bile with an extremely low signal relative to liver

background. Also, ICG required ten-fold higher doses to achieve reasonable bile duct labeling. On the contrary, CW800-CA was transferred from the liver into bile with extremely low liver retention (Figure 1B) and background (Figure 1C). Indeed, a single portal vein injection of CW800-CA resulted in an SBR ≥ 2.5 starting 3 min after injection, peaking at 5–10 min and continuing for at least 30 min. The dose-response curve of CW800-CA (Figure 2, top) suggested that doses greater than 0.0015 $\mu\text{mol/kg}$ provided the highest SBR, with a dose of 0.0075–0.015 $\mu\text{mol/kg}$ being optimal for prolonged visualization.

Systemic Injection of NIR Fluorescent Contrast Agents

Because injection into the portal vein injection is not ideal, we next determined whether a simple, peripheral IV injection of CW800-CA would permit sensitive imaging of the extrahepatic bile ducts. As shown in Figure 2 (bottom), a single IV injection of CW800-CA into rats at doses greater than 0.0015 $\mu\text{mol/kg}$ resulted in a high SBR of the CBD for at least 30 min post-injection, with a dose of 0.0075 $\mu\text{mol/kg}$ being optimal.

Visualization of Normal and Abnormal Bile Ducts in Large Animal Model Systems

Based on the results from rats, CW800-CA was chosen for large animal studies. Initial dose-response curves (data not shown) suggested that a dose of 0.015 $\mu\text{mol/kg}$ injected into the portal vein ($N = 3$ pigs), and either 0.0075 $\mu\text{mol/kg}$ ($N = 3$ pigs) or 0.015 $\mu\text{mol/kg}$ ($N = 3$ pigs) injected systemically, resulted in SBR ≥ 2.5 starting from 5 min and continuing for at least 30 min post-injection. Figure 3A shows representative imaging of the normal extrahepatic bile duct system after a single systemic IV injection of 0.0075 $\mu\text{mol/kg}$ CW800-CA. Visualization lasted at least 30 min, and the systemic injection could be repeated as many times as necessary (data not shown) to complete the operation. Importantly, contrast in the CBD is great enough to detect the presence of small (2.5–3.5 mm) radiolucent inclusions (Figure 3B). Because ICG is already FDA-approved for other indications and is available in the clinic, and since neither portal vein nor systemic injection results in a high and predictable biliary concentration (Figure 1B), we tested the direct injection of ICG into the extrahepatic bile duct via the cystic duct. A dose of 10 μM was chosen, because it provides the highest possible fluorescence emission without dye quenching.²⁴ As shown in Figure 3C, direct injection provided immediate imaging of both distal and proximal extrahepatic bile ducts with an SBR ≥ 3 for over 30 min.

Analysis of NIR Fluorophore Chemical Form in Bile

Although there was an NIR fluorescent substance in bile after intravenous injection of CW800-CA, it is possible that the fluorophore was metabolized during excretion. To determine the chemical form of the NIR fluorophore in bile, a combination of gel-filtration and LC/MS analyses were performed. As shown in Figure 4A, separation of control bile on a 6,000 Da cutoff column confirmed that there are no endogenous NIR fluorophores present. Separation of bile aspirated from the CBD 15 min after injection of 0.015 $\mu\text{mol/kg}$ CW800-CA demonstrated that 94% of the NIR fluorescence was non-protein bound, running at $\approx 1,000$ Da in size. The peak fraction was collected and subjected to LC/MS analysis using a C_{18} column for separation. As shown in Figure 4B, a single NIR absorbing and fluorescing peak was present, with a retention time (21 min) and mass spectrometric pattern identical to pure CW800-CA. These data suggest that $\approx 94\%$ of CW800-CA is excreted unchanged into bile and is the major contributor to the 800 nm NIR fluorescence signal in the extrahepatic bile ducts.

DISCUSSION

NIR fluorescence bile duct imaging offers several advantages over IOC by x-ray fluoroscopy including prolonged (at least 30 min), high sensitivity visualization after a single, low-dose, systemic injection, no exposure of patients and caregivers to ionizing radiation, real-time imaging with no moving parts, patient contact, or image reconstruction, and simultaneous

acquisition of surgical anatomy (via color video) and bile duct function (via NIR fluorescence). Contrast agents can be stored as dry powders in sterile vials, and resuspended in sterile saline immediately prior to use. Contrast agent injection can also be repeated as many times as needed for long cases. And, bile duct injury can be detected in real-time using this technology (data not shown), as we described previously for ureteral injury.⁴⁷ Moreover, because both NIR fluorescence excitation and emission light is invisible to the surgeon, and the NIR fluorophores are used at extremely low doses, there is no alteration to the look of the surgical field.

However, several limitations deserve mention. First, peak contrast doesn't occur for approximately 10 min after IV injection. Second, NIR light has finite depth penetration into scattering tissue, which limits detection to 3–5 mm from the surface. However, at least in rats and pigs, there was never much difficulty in finding quickly and assessing the CBD, because autofluorescence from surrounding structures is low, and the SBR is relatively high. And, third, IV injection of a contrast agent always carries the risk of toxicity. CW800-CA, however, belongs to a class of heptamethine indocyanines with low inherent toxicity.⁴⁸ In fact, the chemically-related molecule ICG has one of the lowest reported toxicities for any pharmaceutical administered to patients,⁴¹ with toxicity seen only at doses 10-fold higher than those used in the present study.

The chemical form and injection dose of the NIR fluorescent contrast agent are of critical importance for successful visualization of the extrahepatic bile ducts using this technology. First, there appears to be an important effect of sulfonation, and therefore hydrophilicity, on the efficiency of transfer of NIR fluorescent contrast agents from the bloodstream to the bile. Indeed, the excretion of the tetra-sulfonated compound CW800-CA occurred so quickly that the background signal in the liver was barely detectable (Figures 1B and 3). On the contrary, non-sulfonated IR-786 and di-sulfonated ICG had tremendously high liver uptake and poor efficiency of excretion into bile.

An optimal dose of CW800-CA would result in a final CBD concentration of $\approx 10 \mu\text{M}$, which is the concentration at which fluorescence emission reaches its peak, and dye stacking and quenching are minimal.²⁴ As demonstrated in Figure 2, increasing the dose beyond its optimum results in a lower SBR in the CBD. It also results in the tinting of bile slightly green (the color of CW800-CA in solution), indicating that the concentration in the CBD is too high and fluorescence quenching is occurring. Nevertheless, at least 94% of CW800-CA remains non-protein bound in bile and is chemically unchanged. This observation, coupled with the low dose required ($0.0075 \mu\text{mol/kg}$ or $250 \mu\text{g}$ in a 33 kg pig), and the chemical similarity to ICG, suggests that translation to the clinic in the near-term should be possible.

Although open surgery for gallbladder disease is still being performed, laparoscopic surgery plays an ever more important role. For this reason, we have adapted our NIR fluorescence imaging system for laparoscopic surgery (unpublished data). The importance of the present study, though, is that the NIR fluorescent contrast agent chemistry for imaging of the extrahepatic biliary system is now defined, and can be applied equally to open surgery and laparoscopic surgery to permit real-time, image-guided assessment of the anatomy and function of the extrahepatic bile ducts.

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ABBREVIATIONS

CBD	common bile duct
CT	computed tomography
ERCP	endoscopic retrograde cholangiopancreatography
ICG	indocyanine green
IOC	intraoperative cholangiography
IV	intravenous
MRCP	magnetic resonance cholangiopancreatography
NIR	near-infrared
PBS	phosphate-buffered saline, pH 7.4, SBR, signal-to-background ratio

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A.

IR-786 ICG CW800-CA

Molecular Weight	584 Da	776 Da	999 Da
Sulfonation	0	2	4
Peak Absorbance in FBS	768 nm	800 nm	783 nm
Peak Fluorescence in FBS	803 nm	811 nm	801 nm
Quantum Yield in FBS	3.3%	9.0%	12.0%
Excretion into Bile	-/+	+	+++
Excretion in to Urine	-	-	+++

B.

Color VideoNIR FluorescenceColor-NIR MergeIR-786 ICG CW800-CA

C.

Figure 1. NIR fluorescent contrast agents for real-time assessment of the extrahepatic bile ducts

A. Chemical structures (top) of IR-786, ICG, and CW800-CA and their optical properties (bottom) in fetal bovine serum (FBS).

B. CBD imaging in rat 10 min after portal vein injection of 0.015 $\mu\text{mol/kg}$ IR-786 (top row), 0.15 $\mu\text{mol/kg}$ ICG (middle row), and 0.015 $\mu\text{mol/kg}$ CW800-CA (bottom row). Shown are the color video (left), NIR fluorescence (middle), and pseudo-colored (lime green) merged images of the two (right). The CBD is marked with a white arrow. NIR fluorescence exposure time is 100 msec. Images are representative of $N = 3$ independent animals studied per contrast agent.

C. Quantification of the SBR in the CBD over time. The SBR (mean $\pm$ SEM) of the CBD relative to liver (left) and pancreas (right) was quantified over time after portal vein injection of IR-786, ICG, and CW800-CA as described in Figure 1B.

Figure 2.

The SBR dose-response of CW800-CA for CBD imaging. The SBR (mean $\pm$ SEM) of the CBD relative to liver (left) and pancreas (right) was quantified over time, following portal vein injection (top) or systemic IV injection (bottom) of CW800-CA, at the doses shown, in $N = 3$ animals per dose.

Figure 3. CBD visualization and assessment in large animals approaching the size of humans

A. Typical visualization of the CBD 15 min after systemic IV injection of 0.0075 $\mu\text{mol/kg}$ CW800-CA, showing the left (L) hepatic duct, right (R) hepatic duct, and common (C) bile ducts. Shown are the color video (left), NIR fluorescence (middle), and merged images of the two (right). NIR fluorescence exposure time was 100 msec. Image is representative of N = 9 animals total.

B. Detection of three spherical radiolucent inclusions of 2.5 mm diameter (arrowhead) and one cylindrical radiolucent inclusion of 3.5 mm long axis (arrow) using CW800-CA as described in Figure 3A. Image is representative of N = 2 animals total.

C. NIR fluorescence IOC was performed by direct injection of 10 ml of 10 μM ICG into the cystic duct (Cy). Visualization of the CBD (arrowhead) and the proximal second order branch of the left hepatic duct (L) is shown.

Figure 4. LC/MS analysis of bile

A. Initial separation of low molecular weight (<6,000 Da) components of bile by gel-filtration. Shown are chromatograms for control bile (left) and bile collected 15 min after intravenous injection of 0.015 $\mu\text{mol/kg}$ CW800-CA (right). The fraction collected from the post-contrast specimen for LC/MS analysis is marked with an arrow.

B. LC/MS analysis of pure CW800-CA (left) and the bile fraction described in Figure 4A (right). Shown are the absorbance at 700 nm (top tracings), fluorescence at 803 nm (middle tracings), and mass spectrographs of the 21 min peak (bottom tracings).