Published in final edited form as:

Mol Imaging Biol. 2016 February ; 18(1): 52–61. doi:10.1007/s11307-015-0870-4.

700 nm Zwitterionic Near-Infrared Fluorophores for Dual-Channel Image-Guided Surgery

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Abstract

Purpose—To develop a family of 700 nm zwitterionic pentamethine indocyanine near-infrared fluorophores that would permit dual-channel image-guided surgery.

Procedures—Three complementary synthetic schemes were used to produce novel zwitterionic chemical structures. Physicochemical, optical, biodistribution, and clearance properties were compared to Cy5.5, a conventional pentamethine indocyanine now used for biomedical imaging.

Results—ZW700-1a, ZW700-1b, and ZW700-1c were synthesized, purified, and analyzed extensively *in vitro* and *in vivo*. All molecules had extinction coefficients $199,000 \text{ M}^{-1}\text{cm}^{-1}$, emission 660 nm, and stability 99% after 24 h in warm serum. In mice, rats, and pigs, 80% of the injected dose was completely eliminated from the body via renal clearance within 4 h. Either alone or conjugated to a tumor targeting ligand, ZW700-1a permitted dual-channel, high SBR, and simultaneous imaging with 800 nm NIR fluorophores using the FLARE[®] imaging system.

Conclusions—Novel 700 nm zwitterionic NIR fluorophores enable dual-NIR image-guided surgery.

Keywords

Near-infrared fluorescence; optical imaging; image-guided surgery; signal-to-background ratio

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CONFLICT OF INTEREST STATEMENT

Dr. Frangioni is CEO of Curadel, LLC, a for-profit company that has licensed FLARE[™] technology from the Beth Israel Deaconess Medical Center.

INTRODUCTION

Because of four key physical principles, near-infrared (NIR) fluorescence has the potential to revolutionize image-guided surgery [1, 2]. First, photon absorption in living tissue reaches local minima between 650–900 nm. Second, photon scatter is much lower in the NIR than it is in the visible spectrum. Third, tissue autofluorescence is low in the NIR. And fourth, NIR light is invisible to the human eye and therefore doesn't change the look of the surgical field. The first two properties permit the interrogation of living tissue up to 5 mm below its surface, the third minimizes background, and the fourth ensures that normal clinical workflow is undisturbed.

Although image-guided surgery systems that “see” NIR fluorescence have matured rapidly over the last decade, clinical contrast agent chemistry lags far behind (reviewed in [3]). In 2011, we reported a new class of heptamethine indocyanines, fluorescing ≈ 800 nm, that were geometrically balanced, polyionic, net neutral compounds termed “zwitterionic” for convenience [4]. Because of their unique chemical structure, these NIR fluorophores displayed extremely low non-specific binding and uptake *in vitro* and *in vivo*, one-step conjugation to targeting ligands, and rapid elimination of unbound dose from the body via renal clearance into urine [4–6]. While promising, this family of contrast agents only permits single-channel NIR fluorescence imaging. However, most clinical applications require 2 independent targets to be visualized simultaneously. For example, during tumor resection, the malignant cells need to be highlighted to ensure complete resection, but so do normal structures, such as nerves, blood vessels, or internal lumens, so that they can be avoided. Indeed, the major morbidity of most human surgery comes from damage to normal structures that are not otherwise visible through blood and tissue [7, 8].

To solve this longstanding problem, and to exploit the dual-NIR channel capability of the FLARE[®] image-guided surgery system [9, 10], we developed a family of novel zwitterionic NIR fluorophores, which fluoresce ≈ 700 nm, and studied their physicochemical, optical, and physiological properties.

MATERIALS AND METHODS

Synthesis of the ZW700-1 series of NIR fluorophores

As shown in Fig. 1a, starting materials obtained from Sigma-Aldrich (Saint Louis, MO) and Alfa Aesar (Ward Hill, MA) were used to prepare the 700 nm emitting zwitterionic pentamethine indocyanine fluorophores ZW700-1a-c. All compounds were obtained in high purity as indicated by TLC analyses using C18 adsorbents and high-resolution ¹H and ¹³C nuclear magnetic resonance (NMR) spectra. Chemical purity was also confirmed using ultra-performance liquid chromatography (UPLC, Waters, Milford, MA) combined with simultaneous evaporative light scatter detection (ELSD), absorbance (photodiode array), fluorescence, and electrospray time-of-flight (ES-TOF) mass spectrometry (MS). See Supplementary Materials for detailed chemical syntheses and analyses. Cy5.5 (GE Healthcare, Piscataway, NJ) was used as a control in all experiments.

Live cell imaging and cell viability assay

The C2C12 mouse myoblast cell line was obtained from ATCC (Manassas, VA, USA). Cultured myoblasts were seeded onto 24-well plates (5×10^4 cells per well), and incubated at 37°C in a humidified 5% CO₂ incubator in DMEM/F12, supplemented with 10% FBS and 1% Pen/Strep. When the cells reached approximately 50% confluence, the seeded cells were rinsed twice with PBS and the NIR fluorophore was added to each well at a concentration of 2 µM or 10 µM and incubated for 30 min at 37°C in a humidified 5% CO₂ incubator. The cells were observed on a 4-channel NIR fluorescence microscope as described previously [11]. The excitation and emission filter used for microscopy was 650 ± 22 nm and 710 ± 25 nm, respectively.

Cytotoxicity of ZW700-1 fluorophores was assessed by a modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide (MTT, Sigma-Aldrich) assay. The cultured myoblasts were seeded onto 24-well plates (3×10^4 cells per well). Cells were treated with 2 or 10 µM of each NIR fluorophore (n = 6) for 30 min and cultured for 24 h post-treatment. For each assay time point, cell media was replaced with 1 mL of fresh media. 100 µL of MTT solution (5 mg/mL stock in PBS) was added to each well and incubated for 4 h at 37°C in a humidified 5% CO₂ incubator. Cell media was carefully removed and the formed crystals were re-dissolved in 1 mL of DMSO and plated into 96-well microtiter plates for measuring the absorption intensity at 590 nm using a microplate reader (E-max, Molecular Device, USA). The data was normalized to the dye-untreated control group. Statistical significance was determined by one-way analysis of variance (ANOVA).

Biodistribution and animal models

Animals were housed in an AAALAC-certified facility and all animal studies were performed under the supervision of BIDMC's IACUC in accordance with approved institutional protocol #155-2008. Male CD-1 mice weighing ≈ 20 g and Sprague-Dawley rats weighing ≈ 250 g were purchased from Charles River Laboratories (Wilmington, MA), and female NCr nu/nu mice averaged 5 to 6 weeks of age and weighed 22 ± 3 g were purchased from Taconic Farms (Germantown, NY). Animals were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine intraperitoneally (Webster Veterinary, Fort Devens, MA). Female Yorkshire pigs averaging 35 kg were purchased from E.M. Parsons and Sons (Hadley, MA), induced with 4.4 mg/kg intramuscular Telazol (Fort Dodge Labs, Fort Dodge, IA), intubated, and maintained with 2% isoflurane (Baxter Healthcare Corp., Deerfield, IL). For biodistribution and clearance studies, ZW700-1 fluorophores in saline were administered intravenously, and animals were imaged with the FLARE[®] real-time intraoperative imaging system as described in detail previously [9, 12]. To quantify the blood clearance rate and urinary excretion, intermittent sampling from tail vein was performed over 4 h. Approximately 20 µL of blood and urine was collected at the following time points using glass capillary tubes: 0, 1, 2, 5, 10, 30, 60, 90, 120, 180, and 240 min. To measure total body excretion in animals, urine was collected at 30, 60, 90, 120, 180, and 240 min, and the amount of each fluorophore in urine was quantified by measuring absorbance intensity, which was converted to concentration (%ID/g) or amount (%ID) based on the

extinction coefficient of NIR fluorophore, respectively. Major organs and tissues were harvested 4 h post-injection immediately after intraoperative imaging.

To develop integrin- α v β 3-positive tumor-bearing mice, LNCaP human prostate cancer cells (ATCC) and MG-63 human osteosarcoma cells MG63 (ATCC) were cultured in DMEM, supplemented with 10% FBS and 1% P/S under a 5% CO₂ atmosphere. After anesthesia, a mixture of LNCaP cells (2.5×10^6) and MG63 cells (2.5×10^6) resuspended in 50% Matrigel (BD Bioscience, Bedford, MA) were injected subcutaneously in the left flank. Within 15 days post-inoculation, animals developed angiogenic tumors of approximately 5 to 8 mm in diameter. At least 3 animals were used for each study, and data are representative of N = 3 independent experiments per condition.

Real-time intraoperative imaging using dual-channel NIR fluorescence

For simultaneous dual-channel imaging, a mixture (500 μ L) of ZW700-1a and ICG was injected intravenously into a 250 g Sprague-Dawley rat 1 h prior to imaging (N = 3). The abdominal cavity was opened to reveal renal and hepatobiliary clearances, and color, 700 nm, and 800 nm NIR fluorescence images were taken by the FLARE[®] imaging system simultaneously. To perform image-guided tumor surgery, 25 nmol (50 μ L of 500 μ M) of cRGDyK (cyclic Arg-Gly-Asp peptide) conjugated ZW800-1 (cRGD-ZW800-1; [11]) was administered intravenously into a xenograft tumor-bearing mouse 4 h prior to imaging, and 10 nmol (50 μ L of 200 μ M) of ZW700-1a labeled bovine serum albumin (BSA-ZW700-1a) was injected intravenously into the same mouse 1 h prior to imaging (N = 3). White light (400 to 650 nm) was at 40,000 lx. Color video and 2 independent channels (700 nm and 800 nm) of NIR fluorescence images were acquired simultaneously with custom FLARE[®] software at rates up to 15 Hz over a 15 cm diameter field of view. 670 nm and 760 nm excitation fluence rates were 4.0 and 11.0 mW/cm² respectively. In the color-NIR merged image, 700 nm fluorescence (ZW700-1a) and 800 nm fluorescence (ICG or ZW800-1) were pseudo-colored red and lime green, respectively, and overlaid onto the color image in real time.

Quantitative analysis

At each time point, the fluorescence (FL) and background (BG) intensity of a region of interest (ROI) over each organ/tissue was quantified using custom FLARE[®] software. The contrast-to-background ratio (CBR) was calculated as $CBR = (\text{fluorescence} - \text{background}) / \text{background}$, where background is the system background (the sum of camera noise and filter light leakage), using ImageJ version 1.45q. All NIR fluorescence images for a particular fluorophore were normalized identically for all conditions of an experiment. At least 3 animals were analyzed at each time point. Statistical analysis was carried out using a one-way ANOVA followed by Tukey's multiple comparisons test. Results were presented as mean $\pm$ S.D. and curve fitting was performed using Prism version 4.0a software (GraphPad, San Diego, CA). *P* values less than 0.05 were considered significant: **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

RESULTS

Synthesis of the ZW700-1 series of NIR fluorophores

Three types of ZW700-1 fluorophores were synthesized through different reaction pathways (Fig. 1a). The synthesis of Vilsmeier-Haack type reagent **2** functionalized at the *meso*-position with Sodium 3-(4-oxidophenyl)propanoate proceeded using substitution of the bromine in compound **1** in DMSO at 65°C. ZW700-1a was prepared from compound **2** and the heterocyclic salt **3** in anhydrous ethanol. Synthesis of ZW700-1b began with the preparation of **4** from methyl 5,5-dimethoxyvalerate using oxalyl chloride in anhydrous dimethylformamide and dichloromethane. Further reaction with sodium hydroxide formed the dialdehyde intermediate, which was converted to the modified malonaldehyde dianil hydrochloride salt after the addition of benzenammonium chloride. Compound **4** was reacted with the heterocyclic salt **3** in anhydrous ethanol using acetic anhydride and sodium acetate to yield ZW700-1b. ZW700-1c was synthesized by Suzuki-Miyaura coupling using Tetrakis(triphenylphosphine)palladium(0) catalyst and cesium carbonate in a mixture of ethanol and water resulting in a firm *meso*-aryl propionic acid linker [13]. The purified ZW700-1 fluorophores were analyzed by ultra-performance liquid chromatography (UPLC, Waters) equipped with simultaneous PDA, fluorescence, ELSD, and ES-TOF MS. All ZW700-1 fluorophores showed clear single peaks and high purities prior to *in vitro* cell and *in vivo* animal studies (Figs. S1 and S2 in Supplementary Materials).

In vitro characterization of ZW700-1 fluorophores

The physicochemical and optical properties of ZW700-1 fluorophores are detailed in Table 1. Of particular importance is the geometrical balance of charge (net zero) over each molecule's surface (Fig. 1b), which stands in stark contrast to the most widely used 700 nm NIR fluorophore Cy5.5, of which the carboxylated form has a net charge of -4 . Indeed, the energy-minimized 3D structures of ZW700-1 molecules and Cy5.5 showed significantly different distributions of charge and hydrophobicity over the molecular surface. LogD values at pH 7.4 for the ZW700-1 NIR fluorophores ranged from -3.52 to -5.65 , which is highly hydrophilic compared to conventional NIR fluorophores, such as ICG (8.05) and IRDye 800CW (2.51) [4], but is similar to Cy5.5 (-4.57). The total polar surface area (TPSA) values of ZW700-1 molecules are greater than $140 \text{ \AA}^2$, which suggests poor permeation of cell membranes in accordance with Lipinski's rule. These parameters help predict the bioavailability and retention of fluorophores in the human body [14], with optimal agents for imaging extracellular epitopes having high hydrophilicity and low cell permeation. ZW700-1 NIR fluorophores also exhibit relatively higher molar extinction coefficients (ϵ) and quantum yields in serum compared to clinically available methylene blue (Table 1) and ICG ($\epsilon = 121,000 \text{ M}^{-1}\text{cm}^{-1}$, QY = 9.3%) [4]. Serum protein binding was measured by gel filtration chromatography (GFC) after incubating each fluorophore in bovine serum albumin solution at 37°C for 4 h. ICG and Cy5.5 showed a significant serum protein binding due to the hydrophobicity of ICG and net negative charge (-4) of Cy5.5. However, ZW700-1 fluorophores with high hydrophilicity and balanced net charge distributed evenly over their surface did not absorb to serum proteins (Fig. S3 in Supplementary Materials).

Cellular properties of ZW700-1 fluorophores

To confirm cell membrane impermeability of the charge-balanced fluorophores, C2C12 mouse myoblast cells, A549 human alveolar basal epithelial cells, and PC-3 human prostate cancer cells were incubated with the ZW700-1 fluorophores, respectively, for 1 h at 37°C and gently washed with DMEM. No fluorescence signals were detected at 700 nm under any condition (Fig. 2a). Also, no cell death was observed even with the highest concentrations of ZW700-1 fluorophores ($P > 0.05$), while Cy5.5 showed ($*P < 0.05$, Fig. 2b). These results confirm that zwitterionic molecules do not interact with cell membranes, consistent with TPSA theory, and do not show obvious cytotoxic effects.

In vivo biodistribution and clearance of ZW700-1 fluorophores

In order to study *in vivo* behavior, ZW700-1 fluorophores were administered intravenously into mice and rats, and their biodistribution and clearance were measured in real time over 4 h. As shown in Fig. 3, all ZW700-1 fluorophores were eliminated from the body by renal clearance into urine without measurable uptake in other tissues and organs. The excreted amount of injected fluorophores as a function of injected dose for small animals is summarized in Table 2. To explore the effect of increasing dose on *in vivo* performance, initial tests in mice spanned a range of doses (30, 60, and 120 nmol per 20 g mouse) that included the equivalent maximum dose anticipated in human studies [15, 16]; that is, 25 mg in a 60 kg adult, corresponding to 0.11 mg (120 nmol) in a 20 g mouse. At a dose of 10 nmol per mouse, $\approx 90\%$ of the injected dose was eliminated into urine after only 4 h. Elimination was consistent among the three ZW700-1 fluorophores up to 30 nmol per mouse. Even at the highest dose, ZW700-1a and ZW700-1c fluorophores showed renal elimination of over 55–65 %ID within 4 h post-injection, although ZW700-1b exhibited lower relative excretion at higher doses, likely due to its physicochemical properties (e.g., extreme LogD). At the highest injected dose, some background signal in the abdominal wall was seen due to the circulating fluorophores in the blood stream, with this signal disappearing by 8 h post-injection as fluorophores were continually filtered from blood. Eventually, all ZW700-1 fluorophores detectable by imaging were eliminated from the body by 24 h post-injection (Fig. S4 in Supplementary Materials). Based on mass spectrometric analysis, the ZW700 series of NIR fluorophores were apparently unchanged after excretion into urine, which further confirms *in vivo* stability (Fig. 3b).

To determine whether these results were unique to rodents, we repeated the biodistribution and clearance study by injecting 4.3 mg (5 μ mol; human equivalent dose of 9.4 mg) of ZW700-1a into 35 kg Yorkshire pigs. ZW700-1a was chosen for large animal studies because of its red-shifted emission and high solubility in DMSO (which facilitates the synthesis of reactive derivatives) compared to ZW700-1b and ZW700-1c (Table S1 in Supplementary Materials). As shown in Fig. 4, ZW700-1a showed significant renal excretion 4 h post-intravenous injection, and no nonspecific uptake was observed in any tissue or organ. Over 90 %ID of injected ZW700-1a was eliminated into urine 4 h post-injection, which is consistent with the rodent data. The blood half-life ($t_{1/2\beta}$) of ZW700-1a in pigs was 45.7 min (*cf.* ICG: 3.6 min) [6], which is ideal for ligand-targeted applications as discussed below.

Real-time intraoperative imaging using dual-channel NIR fluorescence

We studied the utility of ZW700-1 fluorophores for simultaneous, dual-channel NIR imaging using ZW700-1a (to visualize the urinary system) and ICG (to visualize the hepatobiliary system) by injecting both agents together and intravenously into a Sprague-Dawley rat. As shown in Fig. 5a for the 1 h time point, as ZW700-1a was filtered by the kidney and progressed down the ureters to the bladder, highlighting the entire urinary track using the 700 nm fluorescence channel of FLARE[®]. ICG, which is extracted from blood by the liver and excreted into bile for elimination into feces, permitted imaging of the entire hepatobiliary system using the 800 nm fluorescence channel of FLARE[®]. Both physiological systems could be imaged simultaneously and in real time.

Next, we studied dual-channel, image-guided cancer surgery by targeting tumor tissue using ZW800-1 conjugated cRGDyK (cRGD-ZW800-1; 800 nm fluorescence; Fig. S5 in Supplementary Materials) while avoiding vital vasculature using ZW700-1a labeled BSA conjugates (BSA-ZW700-1a; 700 nm fluorescence). As shown in Fig. 5b, cRGD-ZW800-1 accumulated in the tumor site over the course of 4 h [5] while BSA-ZW700-1a provided continuous highlighting of the vasculature over the same time period.

DISCUSSION

The ZW700-1 series of NIR fluorophores described in this study have several key features engineered into their chemical structures. First, excitation and emission of these pentamethine indocyanines were tuned to the 700 nm channel (685-735 nm) of the FLARE[®] imaging system to permit dual-channel NIR fluorescence imaging when used with the 800 nm (> 785 nm) channel and previously developed heptamethine indocyanines. Thus two independent targets can now be imaged simultaneously and in real time. Although, 800 nm NIR fluorophores have an approximately 2- to 5-fold theoretical advantage over 700 nm NIR fluorophores when considering the combined effects of extinction coefficient, tissue autofluorescence, and tissue attenuation, much of this advantage is neutralized by the high QY of 700 nm NIR fluorophores and the fact that virtually all silicon-based CCD cameras are 2-fold less sensitive at 800 nm than 700 nm. Second, extinction coefficients and quantum yields in warm serum of the ZW700-1 family were maximized, resulting in the highest possible fluorescence signal *in vivo*. Third, ZW700-1 molecules were engineered for high stability in warm serum. And, finally, strong surface charges (sulfonates and quats) have been distributed evenly and alternating over the molecular volume, resulting in a geometrically balanced molecule with zero net charge (zwitterionic for convenience). For targeting ligands spanning the spectrum from 1 nm small molecules [4–6] to 5 nm quantum dots [11], zwitterionicity appears to greatly reduce non-specific background binding, thus proportionally raising the signal-to-background ratio (SBR). To the best of our knowledge, these are the first 700 nm zwitterionic NIR fluorophores described to date.

Another key feature engineered into these molecules is efficient elimination into urine within a few hours after intravenous injection. The importance of this should not be underestimated. Contrast agents injected intravenously but not bound to their target should be efficiently eliminated from the body. Otherwise, long circulation times and non-specific

uptake result in high background, as seen previously with conventional tetrasulphonated NIR fluorophores [17, 18], and therefore potential toxicity.

ZW700-1a exhibits a blood half-life in swine of ≈ 45 min, compared to 3.6 min for ICG. This is due to the fact that ICG is actively translocated by the liver from the systemic circulation to the biliary canicular system, whereas zwitterionic NIR fluorophores distribute throughout the blood volume and are cleared passively by the kidney through filtration.

Note that the ZW700-1 series was also engineered with a single carboxylic acid group for one-step conjugation to targeting ligands, resulting in a stable amide bond. Because these fluorophores also exhibit the lowest possible nonspecific binding to various normal and cancer cells, tissues and organs, have a blood half-life permitting adequate contact time, yet are efficiently eliminated from the body, they should be particularly valuable for researchers developing high-performance 700 nm targeted NIR fluorescent contrast agents.

It should be emphasized that the ZW700-1 series differs significantly from currently available 700 nm NIR fluorophores, all of which have either strong surface charges (negative or positive), moderate extinction coefficient or quantum yield, and/or relatively high nonspecific tissue uptake after intravenous injection [4–6]. Methylene blue, for example, has an extinction coefficient of only 71,200, a QY of 3.8%, cannot be conjugated to other molecules, and crosses the plasma membrane of cells. Although Cy5.5 can be conjugated to targeting ligands and has an excellent extinction coefficient and QY, it exhibits high non-specific binding and uptake due to its highly anionic chemical structure. Conventional 700 nm NIR fluorophores also exhibit significant hepatobiliary clearance (i.e., by the liver into bile), thus contaminating the gastrointestinal tract and precluding image-guidance during many common surgical procedures.

Because of the relative ease of synthesis, achievable purity, and stability in warm serum, we believe that the ZW700-1 family of zwitterionic NIR fluorophores has a relatively high probability of clinical translation. Although the selection of which one to translate will require more detailed analysis of cGMP-compatible synthetic pathways, ZW700-1 fluorophores could someday be used clinically for highlighting the ureters to help avoid damage during abdominopelvic procedures, for performing NIR fluorescence angiography of virtually any tissue or organ, or for conjugation to targeting ligands such as antibodies and small molecules.

CONCLUSIONS

The novel 700 nm zwitterionic NIR fluorophores described and characterized in this study lay the foundation for dual-NIR channel image-guided surgery. This, in turn, should enable, for the first time, complex surgeries to be performed under real-time optical guidance, which will hopefully improve patient outcomes while minimizing morbidity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This study was supported by the following grants from the National Institutes of Health: NCI BRP grant R01-CA-115296, NIBIB grants R01-EB-010022 and R01-EB-011523, as well as a grant from the Dana Foundation Program in Brain and Immuno-Imaging. This paper's contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. We thank David J. Burrington, Jr. for editing and Eugenia Trabucchi for administrative assistance.

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Figure 1. Synthetic schemes and 3D structures of ZW700-1 NIR fluorophores
 a) Synthetic scheme for the three molecules studied. b) 3D structures and net charges of ZW700-1 and Cy5.5. Red: negative charge; blue: positive charge; gray: hydrophobic.

Figure 2. Live cell imaging and cytotoxicity of NIR fluorophores in C2C12 mouse myoblast cells
a) Shown are phase contrast and NIR images of each cell line tested at a concentration of 2 μ M (exposure time = 200 msec). Scale bars = 100 μ m. **b)** Cell viability was plotted 1 day post-treatment of each NIR fluorophore at a concentration of 2 μ M (left) or 10 μ M (right). Data are representative of N = 6 independent experiments per condition (mean $\pm$ S.D.). * P < 0.05.

Figure 3. *In vivo* clearance and elimination of ZW700-1 fluorophores in mice

a) 10 nmol of ZW700-1 NIR fluorophores were injected intravenously into 25 g CD-1 mice 4 h prior to imaging. Exposure time = 200 msec. Abbreviations used are: Bl, bladder; Du, duodenum; In, intestine; Ki, kidneys; Li, liver; Lu, lungs; Pa, pancreas; Sp, spleen. Scale bars = 1 cm. **b)** UPLC chromatography and ESI-TOF mass spectrometry of ZW700-1 fluorophores in mouse urine 4 h post-injection. Desalting microcolumns, packed with Poros R2 resin (Applied Biosystems, Framingham, MA), were used to remove excess salts and

contaminants from urine samples. Data are representative of $N = 3$ independent experiments per condition.

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Figure 4. *In vivo* clearance and elimination of ZW700-1 fluorophores in pigs
 5 μ mol of ZW700-1 NIR fluorophores were injected intravenously into 35 kg Yorkshire pigs 4 h prior to imaging. Exposure time = 200 msec. Abbreviations used are: Bl, bladder; Ki, kidneys; Li, liver; Sp, spleen, St, stomach, Ur, ureter. Scale bars = 1 cm. For blood clearance (%ID/g), blood half-life (mean $\pm$ 95% confidence intervals), and urine elimination of ZW700-1a and ICG in pig. Data are representative of N = 3 independent experiments per condition (mean $\pm$ S.D.). *** P < 0.001.

Figure 5. Simultaneous *in vivo* dual-channel NIR fluorescence imaging in the same animal
a) Renal vs. hepatobiliary clearance: 50 nmol of ZW700-1a and ICG were intravenously injected into a 250 g Sprague-Dawley rat 1 h prior to imaging (N =3). Kidneys, bladder, and ureters (arrows) were imaged using the 700 nm NIR channel (exposure time = 200 msec), while liver, duodenum, and bile duct (arrowhead) were imaged using the 800 nm (exposure time = 100 msec). Abbreviations used are: Bl, bladder; Du, duodenum; In, intestine; Ki, kidneys; Li, liver. **b)** Vasculature and tumor imaging: 10 nmol of BSA-ZW700-1a and 25 nmol of cRGD-ZW800-1 were injected into a 25 g xenograft tumor mouse 1 h and 4 h prior

to imaging, respectively (N =3). Vasculature was imaged using the 700 nm NIR channel (exposure time = 200 msec), while integrin- α v β 3 overexpressing tumor (Tu; arrowheads) was imaged using the 800 nm channel (exposure time = 100 msec). The 700 nm and 800 nm images were pseudo-colored in red and green, respectively to generate the merged image. Scale bars = 1 cm.

Physicochemical and optical properties of NIR fluorophores in 100% serum, pH 7.4. *In silico* calculations of the 3D charge, Log D at pH 7.4, and total polar surface area were calculated using Marvin and JChem calculator plugins (ChemAxon, Budapest, Hungary).

Table 1

Property in 100% Serum, pH 7.4	ZW700-1a	ZW700-1b	ZW700-1c	Cy5.5	Methylene Blue
Molecular Weight (Da)	877.12	785.03	861.12	1031.32	319.85
LogD at pH 7.4	-4.34	-5.65	-3.52	-4.57	-0.62
Total Polar Surface Area (Å ²)	170.01	160.78	160.78	272.35	18.61
Extinction Coefficient (M ⁻¹ cm ⁻¹)	205,500	271,500	199,000	250,000	71,200
Absorbance Maximum (nm)	653	642	646	682	665
Emission Maximum (nm)	672	660	663	695	685
Stokes Shift (nm)	19	18	17	13	20
Quantum Yield (%)	19.4	22.8	19.5	21.3	3.8
Stability (%) at 24 h, 37°C	99	99	99	99	99

Table 2

In vivo excretion amount (% ID) of ZW700-1 fluorophores 4 h post-injection into mice (N = 3) and rats (N = 3).

Fluorophore	Species	Body Weight (g)	%ID of elimination into urine 4 h post-injection ^[a]			
			10 nmol ID	30 nmol ID	60 nmol ID	120 nmol ID
Cy5.5	Mouse	20	17.5 ± 1.0	18.1 ± 2.4	13.0 ± 3.7	14.7 ± 4.5
	Rat	250	N.D.	11.4 ± 3.3	N.D.	N.D.
ZW700-1a	Mouse	20	89.4 ± 2.1	71.2 ± 1.8	71.9 ± 9.9	55.0 ± 8.8
	Rat	250	N.D.	77.1 ± 7.1	N.D.	N.D.
ZW700-1b	Mouse	20	82.1 ± 0.4	80.3 ± 0.7	49.7 ± 6.5	33.6 ± 3.1
	Rat	250	N.D.	74.3 ± 2.6	N.D.	N.D.
ZW700-1c	Mouse	20	91.3 ± 0.2	76.0 ± 1.0	72.2 ± 5.0	65.3 ± 10.1
	Rat	250	N.D.	87.9 ± 2.7	N.D.	N.D.

^[a]The %ID of renal excretion was calculated based on the following equation: %ID = [excreted amount into bladder (nmol)/amount of injected dose (nmol)] × 100%, where the amount of fluorophore in bladder was calculated by measuring the fluorescence intensity of urine using a spectrophotometer in the context of a calibration curve created using known concentrations of fluorophores diluted in urine from the same animal. Shown for each measurement is mean ± S.D. N.D. = not done.