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Determination of Optimal Rhodamine Fluorophore for *In Vivo* Optical Imaging

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Abstract

Optical imaging has the potential to improve the efficacy of surgical and endoscopic approaches to cancer treatment; however, the optimal type of fluorescent probe has not yet been established. It is well known that rhodamine-core-derived fluorophores offer a combination of desirable properties such as good photostability, high extinction coefficient, and high fluorescence quantum yield. However, despite the ubiquitous use of rhodamine fluorophores for *in vivo* optical imaging, it remains to be determined, however, if unique chemical properties among individual rhodamine core family members affect fluorophore parameters critical to *in vivo* optical imaging applications. These parameters include: preserved fluorescence intensity in low pH environments, similar to that of the endolysosome; efficient fluorescence signal despite conformational changes to targeting proteins as may occur in harsh subcellular environments; persistence of fluorescence after cellular internalization; and sufficient signal-to-background ratios to permit the identification of fluorophore-targeted tumors. In the present study, we conjugated 4 common rhodamine-core based fluorescent dyes to a clinically feasible and quickly internalizing D-galactose receptor targeting reagent, galactosamine serum albumin (GmSA), and conducted a series of *in vitro* and *in vivo* experiments using a metastatic ovarian cancer mouse model to determine if differences exist among rhodamine fluorophores and if so, which rhodamine core possesses optimal characteristics for *in vivo* imaging applications. Herein, we demonstrate that the rhodamine-fluorophore, TAMRA, is the most robust of the 4 common rhodamine fluorophores for *in vivo* optical imaging of ovarian cancer metastases to the peritoneum.

INTRODUCTION

The application of targeted optical imaging probes as an aid to surgical or endoscopic procedures offers the potential for the identification and treatment of malignancies, such as ovarian and colon cancer, at sub-visible volumes when the opportunity for cure is the greatest. However, maximal clinical benefit requires probes with high target-to-background ratios which are sufficient to permit identification and detection of cancer tissues in the human body. In addition, optical imaging probe selection must take into consideration imaging application-specific parameters. For example, in applications permitting access to superficial surfaces of targeted tissues, green dyes such as rhodamine core fluorophores are well-suited because of their high emission efficiency and high target-to-background ratios. In comparison, applications where greater depth of penetration is required, as occurs in submucosal lesions, near infrared (NIR) probes are generally preferred (1–5). Another important consideration in

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the selection of an optimal fluorophore is the effect that internalization and subsequent entry into the endolysosome, with its harsh acidic microenvironment, has on the fluorescence of molecular probes (6).

Rhodamine core optical fluorophores are widely utilized fluorophores in research and among the green fluorescent dyes, Rhodamine Green has been shown to possess the highest emission efficiency and the longest sustained fluorescence even after cellular internalization (6). It remains to be determined, however, if unique chemical properties among individual rhodamine core family members affect fluorophore parameters critical to *in vivo* optical imaging. These parameters include: preserved fluorescence intensity in low pH environments, similar to that of the endolysosome; efficient fluorescence signal despite possible conformational changes of targeting proteins, as may occur in harsh subcellular environments; persistence of fluorescence after cellular internalization; and sufficient target-to-background ratios of fluorophore-targeted tumors to permit identification of subvisible disease.

In the present study we conjugated 4 common rhodamine-core based fluorescent dyes to a clinically feasible and quickly internalizing D-galactose receptor targeting reagent, galactosamine serum albumin (GmSA) (7–9) and conducted a series of *in vitro* and *in vivo* experiments using a mouse model of metastatic ovarian cancer to determine if differences exist among rhodamine fluorophores and if so, which rhodamine core derivative possesses optimal characteristics for *in vivo* imaging.

EXPERIMENTAL PROCEDURES

Selection of Rhodamine Fluorophores

Four fluorophores within the rhodamine fluorophore family, which differed with regard to structure of the amine side chains were selected for this study—Rhodamine Green (RG; 9-[2-Carboxy-4(or 5)-[[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]phenyl]-3,6-bisamino-xanthylium); Carboxyrhodamine 6G (CaRG; 9-[2-carboxy-4-[[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]phenyl]-3,6-bis(ethylamino)-2,7-dimethyl-xanthylium); Carboxytetramethylrhodamine (TAMRA; 5-[[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]phenyl]-3,6-bis(dimethylamino)-xanthylium); and Carboxy-X-Rhodamine (ROX; 1-[[[2',3',6',7',12',13',16',17'-octahydro-3-oxospiro[isobenzofuran-1(3H),9'-[1H,5H,9H,11H,15H]xantheno[2,3,4-ij:5,6,7-i'j']diquinolizin]-5(or 6)-yl]carbonyl]oxy]-2,5-Pyrrolidinedione). Each of these 4 dyes—RG, CaRG, TAMRA, and ROX—possessed distinct side chains with primary, secondary, tertiary, or cyclic amines, respectively (Figure 1).

Synthesis of Galactosamine Serum Albumin–Rhodamine Fluorophore Conjugates

Galactosamine Serum Albumin (GmSA), which has approximately 23 galactosamine residues per Bovine Serum Albumin (BSA), was purchased from Sigma Chemical (St Louis, MO, USA) and amino-reactive RG, CaRG, TAMRA, and ROX were purchased from Molecular Probes (Eugene, OR, USA). At room temperature, 400 µg of GmSA in 196 µL Na₂HPO₄ was incubated with 4 µL of a 6 mM solution (24 nmol) of each one of 4 amino-reactive rhodamine dyes in dimethyl sulfoxide for 15 min. The mixture was then purified with a Sephadex G-50 Gel Filtration System (PD-10; GE Healthcare, Milwaukee, WI, USA). GmSA–rhodamine conjugate samples were then kept at 4°C as stock solutions.

Comparison of Fluorescence Intensity at Various pH Values

To compare the fluorescence capability of the 4 fluorophore-conjugates, fluorescence intensity and emission spectra were measured in arbitrary units using the Maestro™ In-Vivo Imaging System (CRi Inc., Woburn, MA, USA). 5 µg of GmSA-RG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX were pipetted into a nonfluorescent 96-well plate and diluted in 390 µL of a

buffer solution containing appropriate ratios of sodium dihydrogen phosphate and phosphate to obtain pH values of 2.3, 3.3, 5.2, 6.4, or 7.4 and spectral fluorescence imaging was performed. The ratio of RG, CaRG, TAMRA, and ROX was ~3 molecules per albumin molecule. Appropriate band pass and long pass filter sets (445 to 490 nm/515 nm for RG; 480 to 520 nm/550 nm for CaRG; 503 to 555 nm/580 nm for both TAMRA and ROX) were used for emission and excitation light, respectively. The tunable filter was automatically stepped in 10 nm increments from 500 to 800 nm while the camera captured images at each wavelength interval with constant exposure times. Spectral unmixing algorithms that subtracted any background autofluorescence were applied to create an unmixed image for each of the 4 dye conjugates, permitting accurate evaluation of the fluorescence intensity of each conjugate. A region of interest (ROI) was drawn over the entirety of each well to determine the fluorescence intensity and emission spectra for each of the 4 GmSA-dye conjugates.

Effect of Target Protein Unfolding on Fluorescence Intensity of GmSA-Rhodamine Fluorophore Conjugates

To evaluate the effect of target protein unfolding on the fluorescence characteristics of GmSA-fluorophore conjugates, each of the 4 conjugates were incubated with 5% SDS in PBS for 10 min at room temperature. Experimental controls were established by incubating each conjugate in normal PBS without SDS. The fluorescence signal intensity of each sample and control was measured using both a fluorescence spectrometer (Perkin-Elmer LS55, Perkin-Elmer, Shelton, CT, USA) and the Maestro™ In-Vivo Imaging System (CRi Inc., Woburn, MA, USA). The excited wave lengths were 505, 524, 555 and 576 nm for GmSA-RG, GmSA-CaRG, GmSA-TAMRA and GmSA-ROX, respectively. To investigate the effect of target protein unfolding on the energy absorbance of each protein-fluorophore conjugate, the number of fluorophore molecules per GmSA was calculated. The GmSA concentration was determined by Coomassie Plus protein assay kit (Pierce Biotechnology) by measuring the absorption at 595 nm using a UV-Vis system (8453 Value UV-Vis system, Agilent Technologies, Santa Clara, CA, USA). The fluorophore concentration was then determined by measuring the absorbance at the maximum absorption wavelength of each fluorophore to confirm the number of rhodamine fluorophore molecules conjugated to each GmSA molecule.

Cell Culture

An established ovarian cancer cell line, SHIN3, was used for *in vitro* fluorescence microscopy, flow cytometry, and intraperitoneal ovarian cancer mouse models (10). The cell lines were grown in RPMI 1640 medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), 0.03% L-glutamine at 37°C, 100 units/mL penicillin, and 100 µg/mL streptomycin in 5% CO₂.

Fluorescence Microscopy

SHIN3 cells (1×10^4) were plated on a cover glass bottom culture well and incubated for 16 h, after which GmSA-RG, GmSA-CaRG, and GmSA-TAMRA, or GmSA-ROX was added to the medium (3 µg/mL), and the cells were incubated with conjugates for either 0h, 6h, or 24h. After the respective incubation period, cells were washed five times with PBS and fluorescence microscopy was performed using an Olympus BX51 microscope (Olympus America Inc., Melville, NY) equipped with excitation filters (470–490 nm) and emission filters (515 nm long pass). Fluorescence microscopy images of cells treated with each conjugate for each incubation period were obtained.

Flow Cytometry

One-color flow cytometry was performed to evaluate the fluorescing capability of GmSA-fluorophore conjugates in SHIN3 cancer cells. SHIN3 cells (1×10^4) were plated on a 12-

chamber culture well and incubated for 16 h, after which, GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX was added to the medium (30 $\mu\text{g/mL}$), and the cells were incubated with fluorophore conjugates for 3 hours. Cells were washed twice with PBS and incubated in RPMI 1640 medium without fluorophore conjugates for 0h, 6h, or 24h. Cells were then washed once with PBS, trypsinized, and flow cytometry was performed at each incubation time point 0h, 6h, and 24h, after transfer to conjugate-free media. The argon ion 488 nm laser was employed for excitation. Signals from cells were collected using a 530/30 nm band-pass filter. Cells were analyzed in a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ) and all data were analyzed using *CellQuest* software (Becton Dickinson, Franklin Lakes, NJ). The fluorescing capability of each fluorophore conjugate was referred to as the mean fluorescence index (MFI).

Tumor Model

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the local Animal Care and Use Committee. The intraperitoneal tumor implants were established by intraperitoneal (i.p.) injection of 2×10^6 cells suspended in 200 μL of PBS in female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD). Experiments with tumor-bearing mice were performed at 21 days after injection of the cells.

In Vivo Spectral Fluorescence Imaging

12.5 μg each of GmSA-RhodG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX were diluted in 300 μL PBS and injected into the peritoneal cavities of mice bearing disseminated peritoneal SHIN3 ovarian cancer implants. Three hours after injection of the fluorophore conjugate, each mouse was sacrificed individually with carbon dioxide. Immediately after sacrifice, the abdominal cavity was exposed. Mice were placed on nonfluorescent black plates and whole abdominal multispectral fluorescence images were obtained using the Maestro In-Vivo Imaging System (CRi, Inc., Woburn, MA). A band-pass filter from 445 to 490 nm and a long-pass filter over 515 nm were used for emission and excitation light, respectively. The tunable filter was automatically stepped in 10 nm increments from 500 to 800 nm, while the camera captured images at each wavelength interval with constant exposure time. The spectral fluorescence images consisting of autofluorescence spectra and the spectra for the specific GmSA-fluorophore conjugate were obtained and then unmixed on the basis of their spectral patterns using commercial software (*Maestro* software, CRi, Inc., Woburn, MA). The experiment was repeated three times ($n = 3$ mice per fluorophore). Spectral fluorescence images were obtained for the purpose of determining the target-to-background ratios of tumor implants versus normal anatomy (see discussion below).

Determination of Target-to-Background Ratios of Tumors Compared to Normal Tissue

Spectral fluorescence images of the whole abdominal cavity were obtained as previously described. Monochromatic images corresponding to the peak emission wavelength for each fluorophore conjugate were then generated from multispectral whole abdominal images using the MaestroTM In-Vivo Imaging System (CRi Inc., Woburn, MA, USA) software single wavelength channel extraction function. The target-to-background ratios of tumor implants compared to surrounding normal tissue were determined by placing an ROI over tumors and ROIs over the normal tissue of the liver, intestines, and skin. Intestinal tissue without substantial fluorescence due to food was chosen for intestinal ROI placement to establish target-to-background ratios indicative of normal intestinal autofluorescence with minimal fluorescence contribution due to feed, which often varies between research centers and investigative endpoints. ROI average signal intensity for tumors was then compared to the versus ROI average signal intensity of the liver, intestines, and skin, respectively, were determined for each

animal (Table 2). In addition, to approximate tumor-to-background ratios of tumor implants to normal tissue in general, ROI average signal intensity of tumors was compared a calculated average ROI signal intensity that combined the signal intensity measurements of all normal anatomic features (liver, intestines, and skin combined) for each animal.

RESULTS AND DISCUSSION

Comparison of Fluorescence Intensity at Various pH Values

After cellular internalization, the GmSA-fluorophore probe enters the highly acidic lysosomal vesicle. This isolated low pH environment may affect fluorophore chemical composition and therefore has the potential to alter fluorescence. To evaluate the effect of pH on GmSA-fluorophore probe fluorescence, fluorescence intensity and emission spectra were measured under the same conditions: total dose, concentration of solution, number of fluorophores per GmSA, and exposure time. Excitation/emission filters varied according to which filter set was optimal for each dye. The effect of pH on fluorescence intensity was determined by measuring the fluorescence intensity for each dye at pH values 2.3, 3.3, 5.2, 6.4, and 7.4 (Figure 2). Measurements of the fluorescence intensities at neutral pH (7.4) for the four conjugates, GmSA-RG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX, demonstrated that GmSA-CaRG had the highest signal intensity while GmSA-RG had the lowest. All of the conjugates with the exception of GmSA-TAMRA, demonstrated a decrease in fluorescence intensity value when comparing values obtained at pH 7.4 to pH 2.3 (Figure 2). At pH 2.3, fluorescence intensity was reduced for all dyes with the exception of GmSA-TAMRA, which demonstrated an increase in fluorescence intensity with decreasing pH (Figure 2). Both GmSA-RG and GmSA-CaRG retained near optimal fluorescence at pH values as low as 3.3, after which lowering pH to 2.3 resulted in an approximately 20% reduction in fluorescence intensity (Figure 2). GmSA-ROX retained near optimal fluorescence at pH 7.4 and 6.4 but reduction in pH to 5.2 resulted in decrease in fluorescence intensity. At every pH value tested, GmSA-CaRG demonstrated the highest fluorescence intensity (Figure 2).

These results demonstrate that GmSA-RG, GmSA-CaRG, and GmSA-TAMRA retain fluorescence in low pH environments. Interestingly, only GmSA-TAMRA displayed increasing fluorescence signal with decreasing pH; although none of the conjugates demonstrated substantial changes. The ability of GmSA-RG, GmSA-CaRG, and GmSA-TAMRA to retain fluorescence at low pH, suggests that these agents may be preferred for *in vivo* imaging if the molecular probe is internalized within the lysosome.

Effect of Target Protein Unfolding on Fluorescence Intensity of GmSA-Rhodamine Fluorophore Conjugates

The harsh chemical environment of the endolysosomal system may potentially alter the tertiary structure of the targeting protein. To evaluate the effect of structural changes of targeting proteins on the fluorescence properties of GmSA-fluorophore conjugates, fluorescence intensity values were measured for each of the 4 conjugates in a control solution of PBS and as well as after treatment with a detergent solution containing SDS, and ratios comparing fluorescence with and without SDS treatment were generated. Treatment with SDS demonstrated higher fluorescence-intensity in all four GmSA-rhodamine conjugates compared to controls (Table 1). When measured using a fluorescence spectrometer (Perkin-Elmer LS55, Perkin-Elmer, Shelton, CT, USA), ratios for GmSA-RG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX with and without SDS treatment were 6.9, 11.3, 34.3, and 3.3 respectively (Table 1). When measured with a multispectral camera (Maestro™ In-Vivo Imaging System CRi Inc., Woburn, MA, USA), fluorescence intensity ratios for GmSA-RG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX with and without SDS treatment were 6.6, 5.0, 18.1, and 4.1 (Table 1). Unfortunately, due to limitations in excitation filters available for the multispectral camera,

excitation wavelengths were closer to optimal for some dyes than for others and therefore comparison of ratios obtained with the two measurement techniques is not valid.

Although an important characteristic of xanthene ring fluorophores is their ability to undergo a synthesis reaction through the amino-reactive form by adding a succinimidyl ester, ultimately permitting conjugation with virtually any protein or peptide via the branched amine-residues on the lysine or the N-terminal amine, when the amino-reactive forms of fluorescence dyes are conjugated with proteins or peptides, it is well known that the emission efficacy (extinction coefficient) of fluorophores is generally decreased depending on the distinct couplings of fluorescence dyes and proteins. However, our data suggest that fluorescence intensity losses due to conjugation may be minimized by exposure of conjugates to lysosomal microenvironments (6).

After cellular internalization, probes encounter isolated chemical environments that may alter targeting protein conformation and possibly protein-fluorophore interactions. We attempted to simulate these changes with SDS treatment. Interestingly, we have found that fluorophore-conjugated proteins can emit more light than the original conjugates after either treatment with SDS, or after lysosomal catabolism, as previously demonstrated (6). Among the 4 conjugates tested here, GmSA-TAMRA showed the largest increase in fluorescence after SDS-induced protein unfolding (Table 1). This feature is important for imaging applications as it enables target cells to have “activatable” potential, allowing successfully targeted probes to emit more light than the unbound agent. We have previously reported several conjugates, NuAv-BODIPY-FL and Avidin/GSA-ROX, which demonstrate such activatable characteristics and are capable of highly specific tumor targeted imaging (9,11). Although we have observed this to be a general property of the rhodamine family of dyes, the data presented here suggest that the rhodamine family of dyes possess an inherent activatable characteristic. This “rhodamine effect” is most notably demonstrated by TAMRA.

Activation strategies are often independent and may have an additive effect when combined. Therefore, utilization of the rhodamine fluorescence characteristic in conjunction with other activation approaches, such as photon electron transfer, may aid in the development of fully activatable probes that exhibit fluorescence only after cellular internalization (12). Such probes represent an important goal in optical probe development as fluorescence due to non-specific binding would be substantially reduced, providing maximum contrast of targeted tumors.

Intracellular Fluorescence Evaluation

To confirm fluorescence retention after cellular internalization, SHIN3 cells were incubated with each of the 4 fluorophore conjugates for either 0h, 6h, or 24h, and then imaged using fluorescence microscopy (Figure 3). Fluorescence microscopy revealed that all four GmSA-rhodamine conjugates demonstrated fluorescence after cellular internalization and that longer incubation resulted in increased brightness likely due to increased intracellular accumulation of the respective fluorophore conjugate (Figure 3). However, the excitation filters were not optimized equally for all four conjugates. For instance, the excitation of ROX was not optimized in comparison to the other three conjugates. **Flow Cytometry.** Not only do targeting probes need to remain fluorescent immediately after cellular internalization, but probes must also retain fluorescence for prolonged periods to be used clinically. To evaluate fluorescence longevity, one-color flow cytometry was performed using fluorophore conjugates in SHIN3 cancer cells. SHIN3 cells (1×10^4) were plated on a 12-chamber culture well and incubated for 16 h. GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX was added to the medium (3 μ g/ml), and the cells were incubated for 3 hours to ensure dye internalization and then rinsed and placed in conjugate-free media. Flow cytometry was performed at 0h, 6h, and 24 h after transfer to conjugate-free media. Flow cytometry data for GmSA-RG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX confirmed the presence of the 4 fluorophore conjugate

treated cell populations (Figure 4, upper) and revealed that over time, the mean fluorescence index (MFI) decreased for each dye by 41.7%, 40.3%, 23.1%, and 5.0%, respectively, at 24h. The rate of reduction in MFI was comparable for GmSA-RG and GmSA-CaRG (Figure 4, lower). Although the green excitation laser excited ROX less efficiently than the other 3 fluorophores, which may result in a slight underestimation of relative decrease of fluorescence intensity, GmSA-ROX demonstrated the lowest reduction in MFI over the 24 hour time period and GmSA-TAMRA demonstrated the second lowest decrease in MFI over 24 hours (Figure 4).

Many *in vivo* applications of optical imaging agents involve administration of the targeted fluorophores before surgical or endoscopic procedures. It is therefore necessary for targeted imaging probes to have prolonged fluorescence half lives to permit the completion of endoscopic or surgical procedures. Fluorescence-longevity, which would derive from chemical, biological and photo-physical properties of a dye, is an important parameter to consider in probe selection. Unlike radionuclide counterparts, optical probes are prone to chemical degradation that cannot be “decay adjusted.” Among the four conjugates GmSA-ROX and GmSA-TAMRA demonstrated the lowest decrease in MFI 24 hours after probe administration and therefore the most persistent fluorescence. However, when considering multi-modality and/or multiple color imaging (13), optical probes may not be the time-determining agent in the cocktail and therefore fluorescence-longevity becomes an increasingly important parameter.

Target-to-Background Ratio of Fluorophore Targeted Tumors versus Normal Tissue

Ultimately, the goal of *in vivo* optical imaging, as applied to the detection of cancer, is to enable effective identification and removal of targeted tissues. This requires targeting probes that are easily recognized among a complex anatomic background that often possess autofluorescent properties. To evaluate which GmSA-rhodamine probe offered the highest fluorescence signal over normal surrounding anatomic features, each of the 4 GmSA-fluorophore conjugates was injected into the peritoneal cavities of mice with disseminated peritoneal SHIN3 ovarian cancer implants (n=3 for each fluorophore conjugate) and multispectral fluorescence images of the whole abdominal cavity were obtained 3 hours later. Monochromatic images corresponding to the wavelength of the emission spectra peak for each fluorophore conjugate (Figure 5) were created from whole abdominal multispectral images and signal-to-background ratios of targeted tumors were determined by placing an ROI over tumors and ROIs over the surrounding normal tissues of the skin, intestines, and liver, and a ratio of ROI average signal intensity value of targeted tumors versus selected anatomic features was generated. The average signal-to-background ratios of tumors targeted with GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX to normal tissue was 7.5, 7.2, 11.0, and 5.9, respectively (Table 2). Thus, GmSA-TAMRA demonstrated the highest target-to-background ratio, while GmSA-ROX, demonstrated the lowest ratio. In addition, when comparing target to-background values, GmSA-TAMRA demonstrated the highest signal-to-background ratio in each case with the exception of the intestines, in which GmSA-RG was slightly better (Table 2).

The clinical application of optical imaging to surgical and endoscopic procedures requires optical probes that permit *in vivo* identification of targeted tissues. Optimal visualization of optical probes *in vivo* relies upon both probe fluorescence intensity and the level of contrast of tumor targeted fluorophores compared to the naturally occurring autofluorescence of skin and visceral organs. GmSA-TAMRA and GmSA-RG demonstrated the highest signal-to-background ratios, implying that these probes may permit the best visualization of targeted tissues *in vivo*.

Optical imaging represents a powerful technology for cancer research. Currently, *in vivo* imaging applications include: 1) the study of cancer biology *in vivo* through expression and

imaging of endogenously expressed fluorescent proteins and 2) the development of clinical applications for the treatment of cancer with disease-specific fluorescent probes. Early studies involving imaging of fluorescent proteins revealed the power of optical imaging in cancer biology, such as for the repeated imaging of cancer invasion and metastasis in longitudinal studies (14–16). One limitation to the use of fluorescence proteins for cell labeling in humans is that this process necessitates gene transfection, which is typically achieved in animal models with use of viral infection. Small fluorescent molecules such as organic fluorophores, however, may be used similarly to conventional contrast agents and are therefore more well-suited for development of optical probes for clinical applications as they do not require manipulation of the host genome. Additionally, fluorophores are smaller and easily conjugated to targeting proteins such as antibodies.

Rhodamine core-based fluorophores are characterized by a xanthene ring core with flanking amine-residues (Figure 1). However, the structure of the amine side chains varies between core constructs (Figure 1), which results in distinct energy differences between basic and excited states and the characteristic fluorescence emission wavelengths of each dye. It is possible that variation in amine residues affects fluorescence properties of rhodamine-targeting protein conjugates *in vitro* and *in vivo*. In this study, we chose 4 well-known rhodamine fluorescence dyes with xanthene ring cores and distinct flanking amine residues; RG with primary amines, CaRG with secondary amines, and TAMRA and ROX with tertiary amines (Figure 1). In addition, variation exists within the chemical structure of the tertiary amine-containing rhodamine dyes as ROX possesses cyclic tertiary amines while TAMRA possesses dimethyl amines (Figure 1). These structural differences likely affect fluorescence behavior *in vitro* and *in vivo*. For example, the bis-primary amine of RG permits effective hydrogen bonding and the stereochemistry enables increased accessibility of the electron pair, likely enhancing basicity compared to secondary or tertiary amines. Differences in emission wavelength due to distinct side chains also affect the signal-to-background ratios of targeted tissue for *in vivo* imaging.

It is well known that rhodamine core derived fluorophores offer a combination of desirable properties for optical imaging, including good photostability, high extinction coefficient, and high fluorescence quantum yield. Herein, we demonstrated that the rhodamine-fluorophores, CaRG and TAMRA, may represent superior probes for *in vivo* optical imaging applications (Table 3). Advantages to the use of CaRG include high fluorescence intensity at low pH and increased fluorescence intensity of fluorophore-targeting protein constructs in low pH environments. Advantages of TAMRA include superior fluorescence in low pH, persistent fluorescence after internalization, and importantly, superior *in vivo* signal-to-background ratios compared to other rhodamine core fluorophores. In addition, the data presented here suggests that rhodamine dyes possess an inherent activatable characteristic that is most markedly demonstrated by the TAMRA fluorophore. This is an important finding as creation of activatable probes would be of significant development for *in vivo* optical imaging.

Optical imaging represents an important technological advance that has the potential to improve the efficacy of surgical and endoscopic approaches to cancer treatment (17,18). The physical accessibility of tumor implants has been shown to be an important criterion for optical probe selection. However, an equally important consideration is the effect of *in vivo*-specific conditions on optical probe function. It was previously shown that RG possesses the highest emission efficiency after cellular internalization (6). With this in mind, we evaluated rhodamine core fluorophores with the following parameters critical to the *in vivo* application of optical imaging probes: probe function at low pH and protein-denaturing chemical environments, fluorescence persistence after internalization, and ultimately target-to-background ratios of fluorophore-targeted tissues. Using *in vivo* assays directly and *in vitro* assays aimed to simulate

living cell conditions we demonstrated that members of the rhodamine core fluorophore family differ with regard to *in vivo* imaging functionality.

CONCLUSION

The rhodamine-core fluorophores represent a widely used group of fluorescent dyes for *in vivo* optical imaging. However, despite their ubiquitous use, a direct comparison of the performance of the rhodamine probes was previously lacking. Herein, we presented a comparison of 4 rhodamine-core fluorophores and determined that the TAMRA fluorophore represents a superior probe for *in vivo* optical imaging applications. Advantages to use of TAMRA include superior fluorescence in low pH and protein-denaturing environments, preserved fluorescence longevity post-cellular internalization, and importantly, superior *in vivo* signal-to-background ratios compared to other rhodamine core fluorophores.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The chemical structures of the four rhodamine core fluorophores compared in this study. Note that all dyes possess a xanthene ring but each is unique in the structure of flanking amine side chains.

Figure 2.

Fluorescence intensity of GmSA-rhodamine conjugates at neutral and acidic pH values. 5 μ g of GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX was placed in 390 μ L PBS (pH 7.4) or in a mixture of sodium dihydrogen phosphate (pH 6.4, 5.2, 3.3, or 2.3) and then placed in a nonfluorescent 96-well plate and emission spectra and fluorescence intensity was measured. The ratio of RG, CaRG, TAMRA, and ROX molecules to GSA was approximately 3 in all cases. All four dyes possessed distinct emission peak spectra. At neutral pH (7.4), GmSA-CaRG had the highest signal intensity whereas GmSA-RG had the lowest. With the exception of GmSA-TAMRA, all other conjugates demonstrated a decrease in fluorescence intensity value when comparing values obtained at pH 7.4 to the lowest pH value tested, pH 2.3. At pH 2.3, fluorescence intensity was substantially reduced for all GmSA-rhodamine conjugates with the exception of GmSA-TAMRA, which demonstrated an increase in fluorescence intensity with decreasing pH at all values tested.

Figure 3.

Serial fluorescence microscopy images of SHIN3 ovarian cancer cells treated with 3 µg/mL of GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX. SHIN3 cells were incubated with each of the four GmSA-rhodamine fluorophore conjugates for 0h, 6h, or 24h and fluorescence microscopy was then performed after washing with PBS. No fluorescence was detected in cells imaged immediately after treatment with GmSA-rhodamine fluorophore conjugates (at the incubation time point of 0h) suggesting that optical probes had not yet undergone cellular internalization. Intracellular fluorescence was detected for all conjugates at the 6h and 24h incubation time points.

Figure 4.

Change in fluorescence intensity over time. SHIN3 cancer cells treated with 30 $\mu\text{g/mL}$ of either GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX for 3 hours then rinsed and placed in dye-free media. One-color flow cytometry was performed after 0h, 6h, or 24h incubation in dye-free media. (Upper) One-color flow data confirmed the presence of the 4 GmSA-rhodamine conjugate treated cell populations. (Lower) Graphical depiction of flow data for GmSA-RG, GmSA-CaRG, GmSA-TAMRA, and GmSA-ROX. The rate of reduction in mean fluorescence index (MFI) was comparable for GmSA-RG and GmSA-CaRG, while GmSA-ROX demonstrated the lowest reduction in MFI over the 24 hour time period and GmSA-TAMRA demonstrated the second lowest decrease in MFI over 24 hours.

Figure 5.

Representative monochromatic whole abdominal images of four mice with intraperitoneally disseminated SHIN3 ovarian cancer obtained 3 hours after intraperitoneal injection of 12.5 μ g of GmSA-RG, GmSA-CaRG, GmSA-TAMRA, or GmSA-ROX. Image wavelength corresponded to the peak emission-spectra wavelength for each GmSA-rhodamine conjugate. These images demonstrate that rhodamine optical probes effectively target SHIN3 cell tumors and result in tumor enhancement over background normal tissue. Images are depicted in the monochromatic color that corresponds to the fluorescence of each probe for presentation value only. Gray-scale monochromatic images similar to those shown here were used to calculate signal-to-background ratios of rhodamine fluorophore-targeted tumors.

Table 1

Fluorescence intensity ratios of GmSA-rhodamine with and without SDS treatment.

	Fluorescence Intensity Ratio (spectrometer)	Fluorescence Intensity Ratio (multispectral camera)
GmSA-RG	6.9	6.6
GmSA-CaRG	11.3	5.0
GmSA-TAMRA	34.3	18.1
GmSA-ROX	3.3	4.1

Table 2

Signal-to-background ratios of GmSA-rhodamine conjugate targeted tumors versus surrounding normal anatomy. Fluorophore-conjugate rank in each category is shown in parenthesis.

Conjugate	Tumor vs. Normal Tissue	Tumor vs. Intestines	Tumor vs. Liver	Tumor vs. Skin
GmSA-RG	7.5 (2)	6.4 (1)	10.5 (2)	4.5 (4)
GmSA-CaRG	7.2 (3)	4.9 (3)	10.4 (3)	7.3 (2)
Gm-SA- TAMRA	11.0 (1)	6.0 (2)	15.1 (1)	9.1 (1)
GmSA-ROX	5.9 (4)	3.8 (4)	7.6 (4)	6.1 (3)

Table 3Summary of fluorophore performance in parameters critical to *in vivo* optical imaging.

<i>In Vivo</i> Imaging Criteria	Fluorophore Performance	Impact on images
Fluorescence intensity at low pH	CarRhodG > TAMRA > RhodG > ROX	Minor
Fluorescence intensity after targeting protein unfolding	TAMRA > CarRhodG or RhodG > ROX	Major
Fluorescence-persistence after cellular internalization	ROX > TAMRA > CarRhodG > RhodG	Minor
Target-to-background ratio	TAMRA > RhodG > CarRhodG > ROX	Moderate