Published in final edited form as:

Mol Imaging Biol. 2012 August ; 14(4): 443–451. doi:10.1007/s11307-011-0520-4.

Glucosamine-Linked Near-Infrared Fluorescent Probes for Imaging of Solid Tumor Xenografts

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Abstract

Purpose—Near-infrared fluorescence (NIRF) imaging is an attractive technique for studying diseases at the molecular level *in vivo*. Glucose transporters are often used as targets for *in vivo* imaging of tumors. The efficiency of a tumor-seeking fluorescent probe can be enhanced by attaching one or more glucosamine (GlcN) moieties. This study was designed to evaluate the use of previously developed GlcN-linked NIRF probes for *in vitro* and *in vivo* optical imaging of cancer.

Procedures—Cellular uptake of the probes (1 μ M) was investigated in monolayer cultures of luciferase-expressing PC3 (PC3-luc) cells. The prostate tumors were established as subcutaneous xenografts using PC3-luc cells in nude mice. The biodistributions and tumor-targeting specificities of cypate (cyp), cypate- α -(+)-glucosamine (cyp-GlcN), and α -(+)-glucosamine-cypate- α -(+)-glucosamine (cyp-2GlcN) were studied. The tumor, muscle, and major organs were collected for *ex vivo* optical imaging.

Results—The tumor cell uptake of the probe containing two glucosamine residues, cyp-2GlcN, was significantly higher than the uptake of both the probe with one glucosamine residue, cyp-GlcN, and the probe without glucosamine, cyp only. Similarly, in *in vivo* experiments, cyp-2GlcN demonstrated higher maximum fluorescence intensity and longer residence lifetime in tumors than cyp-GlcN or cyp. The *ex vivo* biodistribution analysis revealed that tumor uptake of cyp-2GlcN and cyp-GlcN was four- and twofold higher than that of cyp at 24 h post-injection, respectively.

Conclusion—Both cyp-GlcN and cyp-2GlcN NIRF probes exhibited good tumor-targeting properties in prostate cancer cell cultures and live mice. The cyp-2GlcN probe showed the highest uptake with good retention characteristics *in vivo*. The uptake of cyp-2GlcN and cyp-GlcN is likely mediated by glucosamine-recognizing transporters. The uptake mechanism is being explored further for developing cypate-glucosamine-based probes for *in vivo* imaging.

Keywords

Optical imaging; Near-infrared fluorescence; Tumor; Prostate cancer; Glucosamine; Cypate

Introduction

Near-infrared fluorescence (NIRF) imaging is an inexpensive technique for studying diseases at the molecular level, detecting small tumors, monitoring targeted drug delivery, and evaluating therapeutic response [1-3]. The low absorption and increased photon penetration depth in the near-infrared (NIR) tissue transparency window (650–900 nm) are some of the major benefits of using NIR light for *in vivo* imaging [4-6]. NIRF rapid imaging technique provides high-throughput imaging with relatively high sensitivity. Moreover, this technique poses no risks associated with techniques involving ionizing radiation. Image contrast and diagnostic accuracy can be improved significantly by using tumor-targeted NIRF imaging probes, which would serve as an active agent to enhance the optical signal from disease-specific molecular markers. Targeted probes usually link a signaling unit (organic or inorganic dye, quantum dots, *etc.*) to a targeting arm provided by a specific ligand, peptide, antibody, sugar, or other small molecule [7-9]. A targeted diagnostic approach with a high signal to noise ratio can provide increased sensitivity and additional tumor specificity with potential extension to a targeted theragnostics (or theranostics), which is a treatment strategy that combines therapeutics with diagnostics [10].

Malignant cells have altered metabolism and increased requirements for glucose and glutamine. Enhanced tumor uptake of glucose is facilitated by the overexpression of glucose transporter proteins (GLUTs), which is widely observed in tumor tissue [11-13]. The most commonly used positron-emitting radiopharmaceutical, 2-deoxy-2-[¹⁸F]fluoro-D-glucose (FDG), is a glucose analog used extensively in cancer detection and therapeutic monitoring utilizing positron emission tomography (PET) [14]. Increased uptake of FDG and increased activity of hexokinase in tumors result in the accumulation of FDG in cancer cells independent of glucose uptake [15, 16].

GLUT1 was the first facilitative sugar transporter discovered; it primarily transports glucose, although it can also recognize and transport galactose, mannose, and glucosamine (GlcN). GLUT1 is responsible for basal glucose uptake required to maintain glycolysis and respiration in cells. GLUT2 is a glucose transporter with high affinity for glucosamine. GLUT4 is an insulin-regulated glucose transporter able to move glucosamine and dehydroascorbic acid [13]. GLUT1 and GLUT4 are transcriptionally repressed by p53, a tumor suppressor protein that is important in cell cycle control and in promoting apoptosis [17]. Since PC3 human prostate cancer cells do not express p53 protein [18], repression of GLUT1 and GLUT4 is not expected. The common feature of these GLUTs is their ability to transport glucosamine. Whereas GLUT1 and GLUT4 have similar apparent affinities for glucose and glucosamine, GLUT2 has a ~20-fold higher affinity for GlcN than for glucose, but is mostly expressed in the liver, basolateral surface of kidney absorptive cells, and in pancreatic β cells [13, 19]. Elevated GLUT1 expression has been reported in many cancers including PC3 cell line [20, 21]. Therefore, increased accumulation of glucosamine-based probes can be expected in tumors which overexpress GLUT1. It is well known that FDG-PET has high sensitivity and demonstrated clinical applicability for tumor detection and imaging [22]. However, PET imaging has several limitations, such as exposure to ionizing radiation, difficulty of synthesizing compounds labeled with positron-emitting isotopes, short half-life of positron-emitting nuclide, and relatively low spatial resolution. To overcome some limitations of PET, researchers have been developing optical probes for metabolic imaging [23-25]. Cyanine fluorophores are suitable for *in vitro* and *in vivo* imaging because of their high molar extinction coefficient, biocompatibility, desirable NIR spectral properties between 650 and 900 nm, and relatively high fluorescence efficiency. The main advantages of choosing cypate (cyp) for this study were minimal tissue autofluorescence, decreased absorption and decreased scattering in the excitation and

emission regions of cyp, all of which contributed to a photon penetration of several centimeters through tissue [4].

The use of GlcN-linked targeted NIRF probes has potential for enhanced tumor specificity and increased sensitivity. The goal of this study was to investigate some previously designed GlcN-linked targeted NIRF probes [25] for optical imaging of human prostate cancer tumor cells and solid tumor xenografts in mice. A preliminary screen suggested the superiority of multivalent probes over monovalent probes for efficient targeted imaging of tumors [25].

In this investigation, the *in vitro* and *in vivo* uptake of intravenously (i.v.) injected cypate (cyp), cypate conjugated to a single GlcN molecule, cypate-D-(+)-glucosamine (cyp-GlcN), and cypate conjugated to two GlcN molecules, D-(+)-glucosamine-cypate-D-(+)-glucosamine (cyp-2GlcN) was monitored in a mouse xenograft model of human prostate cancer. The biodistribution and tumor-targeting specificity of cyp, cyp-GlcN, and cyp-2GlcN in luciferase-expressing PC3 (PC3-luc) tumors were studied. In addition, competitive uptake of the optical probes in the presence of different sugar derivatives was also evaluated. Overall, GlcN-linked probes appear to label tumors *in vivo* with fairly high specificity and long retention times. The cyp-2GlcN probe showed the highest uptake with good retention characteristics *in vivo*. Slow degradation/clearance provides a longer fluorescence-enhanced window, which can be attractive for the future application in areas such as tumor detection, fluorescence-guided surgical procedures, and theranostics. The uptake mechanism is being explored further for developing cypate-glucosamine-based probes for *in vivo* applications.

Materials and Methods

Cell Culture and Animal Model

The human prostate cancer cell line transfected with luciferase gene, PC-3M-luc (Caliper Life Sciences, Inc., Hopkinton, MA), was used to test the efficiency of the probe *in vitro* and *in vivo*. Cells were maintained as exponentially growing monolayer cultures in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% heat-inactivated fetal bovine serum and 50 µg/ml each of penicillin, streptomycin, and neomycin (Invitrogen Corporation, Carlsbad, CA). Subcutaneous xenograft tumors of PC-3M-luc were induced subcutaneously into the flank of 6–8-week-old athymic nude mice (Harlan, Indianapolis, IN) by injecting 1×10^7 subconfluent cells in 100 µl Dulbecco's phosphate-buffered saline. All studies were performed in accordance with institutional guidelines for animal use after obtaining approval for the experimental protocols from Howard University IACUC.

Synthesis of Glucosamine-Cypate Probes

Cyp, cyp-GlcN, and cyp-2GlcN were synthesized as previously described by Ye *et al.* [25]. In short, the carboxylate-terminating multivalent near-infrared carbocyanine probes from a dicarboxylic acid precursor dye (cypate) were prepared via its iminodiacetic acid derivatives. Conjugation of the probes with D-(+)-glucosamine afforded dendritic arrays of the carbohydrates on an inner NIR chromophore core (Fig. 1). The cypate labeling efficiencies have been determined previously [25]. Absorption spectra were used to determine probe concentration. The fluorescence of the diluted probes was measured before all experiments. All the multicarboxylate probes and their glucosamine conjugates had similar UV–vis–NIR absorption and fluorescence characteristics. The absorption and emission spectra maxima were centered on 780 and 810 nm, respectively.

In Vitro Cell Fluorescence Study

The fluorescence microscopy was performed using a Zeiss Axio Observer (Carl Zeiss MicroImaging, LLC, Thornwood, NY) fluorescence microscope equipped with 769/832 nm

band-pass (40 nm) excitation/emission filters. A Xenogen IVIS Spectrum imaging system (Caliper Life Sciences, Inc., Hopkinton, MA) was used for quantitative analysis of fluorescent probe uptake (excitation/emission=710/820 nm, 30/20 nm band-pass filters). PC-3M-luc cells were plated at 5,000 cells per well in an 8-well chamber slide (for fluorescence microscopy) and 96-well plates (for quantitative optical imaging). After 24 h, competitive uptake of the probes was imaged in the presence of competing substrates for cellular uptake. Briefly described, cells were incubated for 1 h with 1 μ M of cyp, cyp-GlcN, or cyp-2GlcN and one of the following competitive substrates: 1 mM D-glucosamine (D-GlcN), 5 mM D-glucosamine, 5 mM L-glucose (L-Glc), 25 mM L-Glc, 5 mM D-glucose (D-Glc), 25 mM D-Glc, 25 mM N-acetyl-D-glucosamine (GlcNAc), or medium (control). The cells were washed three times with PBS and fixed for fluorescence microscopy or subjected directly to optical imaging. All experiments were carried out at 37°C. Each experiment was repeated at least three times, and the results were averaged for further analysis. MTT assays were used to normalize fluorescence intensities in 96-well plate experiments. No cytotoxic effects were observed in cells treated with probes for 3 h in order to determine cellular uptake of the probes.

In Vivo and Ex Vivo Optical Imaging

NIR-based fluorescent imaging was performed with a Xenogen IVIS Spectrum imaging system. Imaging and quantification of signals were controlled by the acquisition and analysis software Living Image (Caliper Life Sciences, Inc., Hopkinton, MA). Mice were subjected to imaging when the tumor size reached 6–7 mm in diameter. Mice were placed onto the warmed stage inside the light-tight camera box and anesthetized by continuous exposure to 1.5% isoflurane. Animals were given 100 μ l (10 nmol) of the cypate-based NIRF probe for each tail vein injection. The whole animal was imaged dynamically every 10–30 min during the first 6 h, and then twice a day for 20–30 days. Imaging parameters were: excitation/emission=710/820 nm (30 and 20 nm band-pass filters, respectively); field of view=6.6 $\times$ 6.6 cm²; f/stop=4; bin=8; and exposure time, 5 s. The signal intensity was expressed as the average radiance (photons per second per centimeter squared per steradian). The contralateral leg muscle served as normal control in these experiments. At the end of the *in vivo* imaging experiments, the mouse was sacrificed by cervical dislocation under isoflurane anesthesia. The tumor, muscle, and the major organs were collected for *ex vivo* optical imaging studies. The normalized signal was calculated by comparing the average fluorescence intensities in the tumor and the thigh muscle from the same animal.

Competitive *in vivo* uptake experiments were done in the manner described above with one more additional step(s) before intravenous injection of the probes. First, the mice were given an intraperitoneal injection of D-Glc (2 g/kg), or D-GlcN (0.5 g/kg), or GlcNAc (2 g/kg) in a total volume of 0.1 ml. After 30 min, cyp-2GlcN (10 nmol) was injected via i.v. Mice were imaged continuously for 24 h and sacrificed, and major organs were collected for *ex vivo* imaging of the biodistribution of the fluorescent probe. Mice were divided into six groups (six mice per group) for the 24-h study of probe uptake as well as biodistribution (Cyp, cyp-GlcN, cyp-2GlcN, cyp-2GlcN+D-Glc, cyp-2GlcN+D-GlcN, and cyp-2GlcN+GlcNAc). Three more groups (three mice per group) were used for a 30-day study of probe clearance and biodistribution of the probes, Cyp, cyp-GlcN, and cyp-2GlcN.

Statistical Analysis

All the data are presented as the mean of at least three experiments (three runs for *in vitro* or three to six animals from the same group for *in vivo* studies). The results were evaluated using statistical software OriginPro V.7 (OriginLab Corporation, Northampton, MA, <http://www.originlab.com>). Student's *t* test (two tailed) was applied for analysis of statistical

differences. A P value <0.05 was considered as a significant difference between any two sets of data.

Results and Discussion

In Vitro Uptake Studies

Cellular uptake of cyp, cyp-GlcN, and cyp-2GlcN was investigated in human prostate cancer cell cultures by fluorescence microscopy. Figure 2 shows fluorescence images of PC3-luc cells after incubation with 1 μ M of cyp, or cyp-GlcN, or cyp-2GlcN for 1 h at 37°C. NIRF probes were spread over the entire cytoplasm and exhibited a granular pattern of distribution. The probes did not accumulate in the nucleus or periphery of PC3-luc cells. cyp-GlcN and cyp-2GlcN showed a higher degree of cellular uptake compared to cyp alone, and the highest uptake was observed in cells treated with cyp-2GlcN. The results indicate that the uptake of GlcN-linked probes is an active process involving a transporter that recognizes GlcN, and the cyp-2GlcN probe which has two GlcN residues is taken up more actively than the other two probes examined in this study. Similar granular patterns in micrographs were observed by Li *et al.* [24] in various cancer cell lines for mono GlcN NIRF probes. The authors showed that the probes have good tumor-targeting specificity with a tendency to localize in lysosomes.

To explain whether cellular uptake of the probes is related to GLUTs and to quantify the cellular uptake, PC3-luc cells were incubated in 96-well plates with 1 μ M of each NIRF probe alone, and in combination with a competing sugar derivative such as D-GlcN (1 and 5 mM), L-Glc (5 and 25 mM), D-Glc (5 and 25 mM), or GlcNAc (25 mM) for 1 h at 37°C. The GlcN-linked probes incubated without sugars showed the ability to accumulate in PC3-luc prostate cancer cells (Fig. 3, control). The uptake of cyp-2GlcN was significantly higher than the uptake of the cyp-GlcN or cyp. The uptake of cyp-GlcN was about 40% more than that of cyp alone. It is interesting to note that the cellular uptake of cyp-2GlcN was nearly double that of cyp-GlcN.

The uptake of cyp, which lacks a targeting carbohydrate moiety, was not inhibited by any of the tested sugars ($p>0.05$). Moreover, the uptake of cyp-2GlcN and cyp-GlcN was not significantly inhibited ($p>0.05$) by D-Glc, L-Glc, and GlcNAc (data not shown). These results may suggest that actively transported dyes such as cyp-2GlcN and cyp-GlcN are not taken up into prostate cells through glucose transporters, or that the GLUT-mediated transport was not completely blocked by co-incubation with the sugar derivatives listed above. It is worth noting that similar results were reported by Cheng *et al.* [23] and Li *et al.* [24] experimenting with GlcN and NIRF dyes with molecular weight (MW) above 500. In contrast, the uptake of 2-deoxy-D-Glc with MW=342.26 can be inhibited by D-Glc but not L-Glc, thus suggesting the probe delivery and trapping in cells by GLUT/hexokinase pathway [26-28].

The co-incubation with D-GlcN exhibits an enhancement effect on the cellular uptake of the cyp-2GlcN and cyp-GlcN probes compared to uptake of free cyp ($p<0.05$; see Fig. 3). This indicates that the GlcN transport system was activated by the excess amount of D-GlcN, thus facilitating the uptake of the GlcN-linked probes by PC3-luc cells. Different GLUTs might be involved in the uptake process. From this competitive study, we can pinpoint that transport of cyp-GlcN and cyp-2GlcN NIRF probes were active processes that were enhanced by D-GlcN, and could not be blocked by D-Glc. The results indicate that the uptake of cyp-2GlcN and cyp-GlcN is likely mediated by increased GlcN affinity of GLUTs or other transporters in the presence of GlcN. Due to complexity of sugar derivative metabolism in cancer cell, we cannot draw a final conclusion about the uptake mechanism

and pathways involved in this process. More studies are needed in order to clarify the exact uptake and retention mechanism of these probes *in vitro*.

In Vivo and Ex Vivo Optical Imaging

Whole-body dynamic optical imaging of mice bearing PC3-luc human prostate tumor xenografts was performed after systemic administration of cyp and GlcN-linked NIRF probes, cyp-GlcN and cyp-2GlcN. cyp-2GlcN demonstrated the highest tumor-specific uptake and overall retention time in tumor as compared to the uptake and accumulation in surrounding tissues. The typical *in vivo* uptake of cyp-2GlcN is shown in Fig. 4. For cyp-2GlcN, the fluorescence in the tumor area was clearly observed up to 30 days after injection, while the maximum fluorescence intensity was evident 1 h after i.v. injection of the probe. However, remarkable fluorescence was observed for up to 6 h in the regions of the major excretion organs, such as the liver and kidneys. At 24 h after injection, a significant contrast in the tumor region was seen.

A quantitative analysis of whole-body dynamic optical imaging has been performed. Fluorescence intensities (average radiance, photons per second per centimeter squared per steradian) in PC3-luc prostate tumor xenografts and surrounding normal tissues as a function of time are shown in Fig. 5. It was found that cyp-GlcN and cyp-2GlcN exhibit specific uptake by tumors. The cyp-2GlcN demonstrated higher maximum fluorescence intensity and longer residence lifetime than cyp-GlcN and cypate molecule. The fluorescence of cyp-2GlcN, cyp-GlcN, and cyp reached its maximum intensity in the tumor region at 60, 30, and 1 min after i.v. injection, respectively. Maximum average radiance of cyp-2GlcN, cyp-GlcN, and cyp in the tumors was 9.6×10^9 , 1.35×10^8 , and 4.7×10^7 p/s/cm²/sr, respectively, thus showing nearly one order higher uptake of probes with each addition of GlcN to the cyp core. The half-life of the NIRF probe was defined at the 50% of maximum of average radiance. The cyp-2GlcN possesses a higher half-life of 15 h compared to cyp-GlcN (1.7 h) and cyp (15 min). These results suggest that the addition of the second GlcN to a cyp imaging core can significantly improve tumor-targeting ability and retention time of the GlcN-linked NIRF probes *in vivo*.

The *ex vivo* biodistribution of cyp, cyp-GlcN, and cyp-2GlcN at 24 h after injection was investigated in tumor-bearing mice, and the results are shown in Fig. 6. Tumor uptakes of cyp-2GlcN and cyp-GlcN were four and twofold higher than that of cyp at 24 h after probe injection, respectively. The tumor muscle ratios at 24 h after i.v. administration of cyp-2GlcN, cyp-GlcN, and cyp were 7.8:1, 3.8:1, and 2.0:1, respectively. The fluorescence signal of cyp-2GlcN after 20 days of i.v. injection was nearly one order less but still quite high (2.8×10^7 p/s/cm²/sr), and the tumor to muscle signal ratio was around 3.7:1. The *ex vivo* biodistribution results are consistent with results of *in vivo* study except that the tumor to muscle signal ratios are higher in *ex vivo* studies. This may reflect the lack of skin emission and absence of light penetration depth effects on the fluorescence intensities from different organs and tumors in *ex vivo* experiments. It is noticeable that cyp-2GlcN was also retained in the liver and kidneys for prolonged duration with relatively high uptake in the liver. There could be two reasons for probe accumulation in these organs. This may be explained partly on the basis of probe entrapment in the rich reticuloendothelial system characteristic of the liver and kidneys and escape from engulfment by macrophages. The high levels of GLUT2, which is a high-affinity glucosamine transporter [19], in the liver and kidneys [13] could also account for the high levels of the probe in these organs. The washout of the probe from other organs and tissues could be mediated through renal and biliary routes and cause additional amounts of the probe to be in transit through the liver and kidneys. *Ex vivo* biodistribution studies show that the probe with two GlcN moieties (cyp-2GlcN) exhibits the highest uptake in the liver and kidneys compared to probe linked to one GlcN unit (cyp-GlcN) and the unconjugated dye (cyp). On the other hand, the *in vivo* biodistribution study

clearly showed high levels of accumulation and retention of cyp-2GlcN in PC3-luc prostate tumor xenografts in mice.

The *in vivo* uptake specificity of cyp-2GlcN was tested by competitive uptake study at 24 h post-injection. Thirty minutes prior to i.v. probe injection, the mice were preinjected with D-Glc, or D-ClcN, or GlcNAc intraperitoneally. At 24 h after injecting the probe, the mice were sacrificed, and the major organs were excised and collected for *ex vivo* biodistribution analysis (Fig. 7). The results of this study showed that the *in vivo* uptake of the probe was not inhibited by D-Glc. These results are similar to those described earlier for the effects of D-Glc on probe uptake by PC3-luc cells in culture. This indicates that the probe transport was facilitated not only by glucose metabolism, but by some other complex metabolic processes in the tumor. Interestingly, the *in vivo* uptake of cyp-2GlcN was inhibited by GlcNAc, while in *in vitro* experiments, GlcNAc had no effect on cyp-2GlcN uptake. The inhibition of cyp-2GlcN uptake *in vitro* was negligibly small, probably due to the low GlcNAc to cyp ratio, compared to the much higher GlcNAc to cyp ratio used for *in vivo* experiments. GlcNAc is generated during lysosomal degradation of certain glycoproteins and glycosaminoglycans by lysosomal enzymes and recycled by the cell [29]. It was reported that lysosomes possess a highly specific transport system for *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine [30]. We assume that the lysosomal transport and trafficking were partially blocked by pre-injection of *N*-acetyl-D-glucosamine *in vivo*. As a result, the cypate-glucosamine NIRF probe accumulation in the cells was reduced not only in the tumor but also in organs with a rich reticuloendothelial system. The uptake of the probe in prostate tumors was significantly enhanced by pre-injection of GlcN. As we proposed earlier, *in vivo* results suggest that cyp-2GlcN uptake is through the GlcN-sensing transporters that could be activated by GlcN IP pre-injection. It is well known from the literature that infusion of D-GlcN can decrease insulin-stimulated Glc uptake and stimulate the hexosamine biosynthesis pathway [31, 32]. As a result of these changes, the cell might signal increased uptake of GlcN and glucosamine-linked probes.

The findings in this study support the initial hypothesis that two GlcN conjugated to NIRF dye will improve targeting properties of the NIRF probe and have potential for metabolic imaging. GLUT1 and GLUT4 have similar affinities for glucose and GlcN, and extracellular concentration of glucose is higher than GlcN, even during glucosamine infusions [19, 33]. At least two mechanisms have been reported for the cellular utilization of GlcN [19, 34, 35]. GlcN is carried into cells by GLUTs and phosphorylated to glucosamine-6-phosphate by hexokinase activity of glycolytic pathway. The second pathway is transcriptional in which the regulatory products derived from glucosamine-6-phosphate are involved in translocation, transcription, and insulin action cascade. Overexpression of different GLUT types may lead to higher or lower specificity of GlcN-based NIRF probes in different types of cancers. GlcN-containing NIRF probes seem to be good candidates for metabolic imaging of tumors. Unfortunately, the transport mechanism of GlcN-conjugated NIRF probes is still unclear. GlcN and Glc may share some of the same carriers and enzymes for transport and metabolic processing in tumor tissue. *In vivo* cellular uptake studies of technetium-labeled Glc analogs [36, 37] have shown similarities with uptake characteristics of GlcN-linked NIRF probes, especially with respect to insensitivity to Glc. *In vivo* cellular uptake of FDG on the other hand is quite sensitive to Glc concentration. This suggests that the transport of GlcN-linked NIRF probes and technetium-labeled Glc may involve a common mechanism (s). Furthermore, D-GlcN has been reported to inhibit growth of many types of cancer cells *in vivo* and *in vitro* [38-40], and thus has potential to expand the usage of GlcN-based NIRF probes from cancer diagnosis and monitoring of cancer treatment to theranostics of cancer.

Conclusion

In summary, both cyp-GlcN and cyp-2GlcN NIRF probes have demonstrated good tumor-targeting properties in prostate cancer cells *in vitro* and in live mice. The probe constructed with two GlcN, cyp-2GlcN, showed the highest uptake with prolonged half-life *in vivo*. The uptake of cyp-2GlcN and cyp-GlcN was tumor specific, active, and likely mediated by GlcN-recognizing transporters. The uptake mechanism is being explored further for developing cypate-GlcN-based probes for *in vivo* applications. This study showed that the addition of a second GlcN can significantly change NIRF probe characteristics, resulting in better and prolonged tumor contrast and improved pharmacokinetics. This general idea of probe design can be applied to future NIRF probe development in cancer theranostics.

Acknowledgments

This work was supported by grants from DOD USAMRMC W81XWH-10-1-0767 and NIH/NCRR RCMI 2G12 RR003048.

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Fig. 1. Structures of near-infrared fluorescence probes: cypate (*cyp*), cypate-D-(+)-glucosamine (*cyp-GlcN*), and D-(+)-glucosamine-cypate-D-(+)-glucosamine (*cyp-2GlcN*).

Fig. 2.

Cellular uptake and localization of glucosamine NIRF probes. The cells were incubated with the different GlcN-linked probes for 1 h at 37°C to examine the tendency of the NIRF probes to accumulate in the cytoplasm of PC3-luc prostate cancer cells. (The *horizontal bar* in the photomicrographs represents 20 μm).

Fig. 3.

Quantitative analysis of cellular uptake of the different NIRF probes in medium (*control*) and in presence of different sugars (1 mM D-GlcN, 5 mM D-GlcN, 5 mM L-Glc, 25 mM L-Glc, 5 mM D-Glc, 25 mM D-Glc, 25 mM Glc). The uptake of the cyp-2GlcN was significantly higher than the uptake of the cyp-GlcN and cyp. The uptake of the cyp-2GlcN and cyp-GlcN was not inhibited by D-Glc, L-Glc. D-GlcN caused a statistically significant increase in the uptake of the cyp-2GlcN and cyp-GlcN; * $p < 0.05$.

Fig. 4.

In vivo dynamic imaging study of targeting specificity of cyp-2GlcN to PC3-luc prostate tumor xenografts. Representative superimposed photography and color-coded fluorescence *in vivo* images of human PC3-luc prostate tumor-bearing mice are shown at selected time points before and after intravenous administration of cyp-2GlcN (10 nmol). In order to show the tissue contrast at different time points, the window/level was adjusted for each picture individually. The absolute image intensity for the later time point is lower than that of the earlier time.

Fig. 5.

Kinetics of *in vivo* targeting characteristics of cypate and glucosamine-based probes in PC3-luc prostate tumor xenografts. Typical time course of *in vivo* fluorescence intensities (average radiance) after i.v. administration of 10 nmol of cyp, cyp-GlcN, and cyp-2GlcN is shown for the first 50, 24, and 10 h of the *in vivo* imaging, respectively. The autofluorescence signal was measured just before injection (*zero time point in the time axis*). Cyp-2GlcN demonstrated higher maximum fluorescence intensity and longer residence lifetime (see *insert on the first panel*) than cyp-GlcN and cyp. The *insert* represents the maximum *in vivo* fluorescence intensities per day over 26 days.

Fig. 6.

Ex vivo study of biodistribution of molecular probes in PC3-luc prostate tumor-bearing mice. The *top two panels* represent the fluorescence intensities (average radiance) at 24 h (*left panel*) and 500 h (*right panel*) after systemic intravenous injection of the cyp-2GlcN, cyp-GlcN, and cyp. The *bottom row* of the histograms show the corresponding fluorescence intensities normalized to the muscle signal. Cyp-2GlcN had the best tumor-targeting ability and retention characteristics. Tumor uptakes of cyp-2GlcN and cyp-GlcN were four and twofold higher than that of cyp at 24 h post-injection.

Fig. 7.

Competitive uptake study of cyp-2GlcN. *Left panel* represents the biodistribution at 24 h after i.v. injection of cyp-2GlcN in mice pre-injected with D -Glc, D -GlcN, and GlcNAc. *Right panel* represents the corresponding fluorescence intensities normalized to the fluorescent signal from the muscle. The uptake of the cyp-2GlcN was not inhibited by D -Glc. The uptake of cyp-2GlcN was inhibited by GlcNAc and enhanced by D -GlcN.