A Near-Infrared Fluorescent Transferrin Agent for Quantitative Imaging of Transferrin Receptor Expression in Tumor Xenografts

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Abstract

Transferrin (TF) transports iron to all tissues, particularly those with highly metabolic cells like tumors because of their high demand for iron (for heme synthesis and as cofactors of non–heme enzymes). Given the overexpression of TF receptors (TR) on malignant cells, targeting TF has been a successful approach as a strategy for pharmacological intervention in both cancer diagnosis and therapy by using fluorescent-labeled TF variants for sensitive and receptor-mediated internalization in tumors. While most of the imaging agents targeting TR have involved the use of radiochemicals, in this study we generated a novel TR-targeted agent using near infrared fluorophore labeled transferrin, Transferrin-Vivo™ 750 (TFV750). This approach offered a non-radioactive alternative and exploited the favorable characteristics of optical imaging in the near infrared wavelengths, allowing efficient penetration of photons through living tissue and minimizing interference from tissue autofluorescence. The agent consists of recombinant human transferrin coupled to a fluorophore (VivoTag® 750, excitation 750 nm/ emission 770 nm) and a pharmacokinetic modifier designed to improve its plasma availability (plasma T½ = 10 hours). In vitro, TFV750 shows dose-dependent binding to HT-29 colorectal tumor cells and frozen tumor xenograft sections as quantified by flow cytometry and fluorescence microscopy, respectively. Specificity of binding was confirmed by near complete blockade of the cell labeling upon prior incubation with an excess of unlabeled transferrin. In vivo, TR upregulation in tumors was quantified following intravenous injection of 2 nmol of the TF agent into nude mice bearing HT-29 tumor xenografts. Imaging performed by fluorescence molecular tomography (FMT) at 2, 6 and 24 h post-injection revealed a peak of 25-40 pmol of fluorescent signal at 6-24 h, the time range at which optimal signal-to-noise ratios were achieved. The in vivo biodistribution profile was determined by harvesting tissues from these tumor bearing mice at 24 h, showing a favorable distribution dominated by tumor signal as compared to other tissues. In vivo targeting specificity was validated by injecting mice with a 645 nm labeled version of the TF agent and determining appropriate co-localization in frozen tumor tissue sections with anti-TR antibody staining. The results from these studies indicate that the newly developed TFV750 provides a useful tool for near infrared fluorescent-based optical tumor imaging both in vitro and in vivo.

1 Description of the Agent

A. Physiochemical properties

<table>
<thead>
<tr>
<th>Property</th>
<th>Specification</th>
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<tbody>
<tr>
<td>MW</td>
<td>~106,000 g mol⁻¹</td>
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<tr>
<td>Fluorescence emission</td>
<td>770 nm</td>
</tr>
<tr>
<td>Absorbance</td>
<td>750 nm</td>
</tr>
<tr>
<td>Purity</td>
<td>&gt;90 %</td>
</tr>
<tr>
<td>Appearance</td>
<td>Blue solid</td>
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</tbody>
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1. Absorbance and fluorescence maxima in 1% PBS.
2. Assessed by UV/Vis, measuring absorbance at 750 nm.

B. Absorbance/emission spectra

A. Recombinant human transferrin was conjugated with PEGs and a near infrared fluorophore (VivaTag® 750) and purified to provide an imaging agent designed to target transferrin receptors on the surface of cells. B. Transferrin-Vivo™ 750 (TFV750) has a complex absorbance profile with peaks at 680 and 750 nm, and the agent fluoresces at a wavelength maximum of 770 nm.

2 In Vitro Validation of Transferrin Receptor Targeting

HT-29 tumor cell labeling with TFV750

A. Flow cytometry histogram B. Titration and blockade

C. Fluorescence microscopy

A. HT-29 cells (human colon cancer tumor line) were incubated with 1 μM TFV750 for 1 h at RT and assayed by flow cytometry, showing effective uptake in the tumor cells. B. Titration of TFV750 showed the dose dependence of cell uptake, and prior incubation with 100 μM unlabeled transferrin blocked uptake of 1 μM TFV750. C. Generation of cellular microscopy images required the use of a 645 nm fluochrome-labeled transferrin to more sensitively detect cellular fluorescence by microscopy. Transferrin 645 was incubated with cells for 1 h at room temperature under the conditions described in B, with and without blockade by excess unlabeled transferrin.

3 Non-invasive Imaging of Tumor Transferrin Receptor Uptake with TFV750

A. Cross-platform imaging of Hepa tumor flank xenografts

B. HT-29 tumor xenografts – Agent kinetics

A. Dorsal view of a representative mouse bearing Hepa tumor xenografts on the flanks is shown from a cohort injected with 2 nmol TFV750 and imaged 24 h later on both the IVIS Spectrum CT and FMT 4000. Both tomographic and epifluorescence readouts for each system were used. B. HT-29 tumor bearing mice were imaged at different times post-TFV750 injection and elevated iron metabolism was quantified in both the tumors and liver, as expected.

4 TFV750 Biodistribution and Pharmacokinetics

A. Tissue epifluorescence B. Tissue biodistribution and plasma PK

TFV750 was injected IV (2 nmol) and tissues were collected 24 h later for assessment of tissue biodistribution. A. Epifluorescence images of excised tissues shows predominant tumor, liver, and kidney signal. B. Quantification of epifluorescence images, with data inset representing plasma pharmacokinetics assessed from serial bleed of mice measured on a microplate fluorimeter.

5 Agent Localization and Validation

A. Multiplex tomographic tumor imaging of TFV750 & AngioSense 680EX

B. Ex vivo HT-29 tumor tissue localization of Transferrin-645

C. Vasculature

D. Tumor TIR Uptake

A. HT-29 tumor xenografts were imaged after co-injection of TFV750 and a vascular imaging agent, AngioSense 680EX, to confirm that signal for these two different imaging agents localized differently in tumor tissue. A representative tumor-bearing mouse was imaged tomographically by FMT 4000. B. Specificity of tumor localization of transferrin receptor targeting was determined by dosing mice IV with 645 nm fluorochrome-labeled Transferrin (as a surrogate for TFV750) that allows sensitive detection by fluorescence microscopy. Hoechst 33342 was injected 5 minutes prior to animal termination (to label vasculature), and tissues from injected, tumor-bearing mice were excised and flash frozen. Ten μm sections were also stained with FITC-labeled anti-transferrin receptor antibody, and the acquired images confirm non-colocalization with the vasculature and good co-localization with TIR staining.

Summary

Iron is essential for cellular proliferation, and transferrin receptors are required for iron transport into the cell. In particular, highly metabolic cancer cells have an increased need for iron, and as a result one can detect upregulation of transferrin receptors in a variety of different tumor types.

Modifications of recombinant transferrin molecules for imaging, using MRI, PET, and SPECT reporters, have provided useful tools for imaging in preclinical animal models of cancer. Optical imaging of near infrared fluorescent-labeled transferrin (Transferrin-Vivo 750) offers a safe and easy means for non-invasive preclinical imaging, in real time and in vivo. The results presented here further demonstrate the imaging and validation of this agent in vitro, in vivo, and ex vivo in two different tumor models and using two different optical imaging systems, the FMT 4000 and the IVIS Spectrum CT.

References