Near-Infrared Fluorescence Imaging and Quantification of Anti-Angiogenic Therapy using an $\alpha_v\beta_3$ Integrin-Targeted Agent

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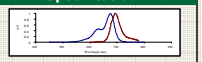
1 Abstract

Integrins are transmembrane cell surface receptors which mediate signal transduction, cell-to-cell interaction and cell-to-extracellular matrix adhesion. These processes lead to cell migration, invasion and extravasation, all key components in angiogenesis, tumorigenesis and metastasis. Integrins have thus been hailed as clinically-relevant biomarkers of pathological conditions such as inflammation and tumor progression. Integrin $\alpha_v\beta_3$ is significantly upregulated in tumor cells and activated endothelial cells during angiogenesis, but not in quiescent endothelium. The aim of this study was to non-invasively image and quantify $\alpha_v\beta_3$ receptor binding using a specific, targeted near-infrared (NIR) fluorescent agent, IntegriSense™ 680 (PerkinElmer) and fluorescence molecular tomography (FMT) imaging (FMT 2500™, PerkinElmer). We developed an optical imaging agent for in vivo detection of $\alpha_v\beta_3$ using a low molecular weight peptidomimetic antagonist coupled to a NIR fluorochrome. Binding specificity was determined by cell attachment to vitronectin using $\alpha_v\beta_3$ overexpressing HEK293 cells and competition studies. The pharmacokinetic profile was assessed in mice by measuring plasma fluorescence at different times after intravenous injection with the agent. Integrin expression in tumors was quantified in both mouse breast tumors and human colorectal tumor xenografts implanted in nude mice and the quantified fluorescence signal strongly correlated with tumor size. In addition, this agent was used as a mechanistic biomarker for anti-angiogenic therapeutic efficacy. As such, integrin agent administration in mice with established tumors allowed the non-invasive and real-time quantification of integrin signal decrease (60-65%) following treatment with the anti-angiogenic drug Avastin. Histology confirmed the expected localization of the agent within the tumors. This study illustrates the potential of NIR fluorescence agents and fluorescence tomographic imaging to non-invasively quantify the underlying biology of angiogenic processes in real time, crucial in the development and monitoring of anti-cancer therapies.

2 Integrin-Targeted *In Vivo* Imaging Agent

The integrin-targeted agent, IntegriSense™ 680 (PerkinElmer) was synthesized by converting the small molecule, non-peptide $\alpha_v\beta_3$ antagonist, compound 5f (Coleman et al.), to the 3-cyano derivative, reducing the derivative to the 3-aminomethyl analog and coupling the resulting compound with VivoTag®-S680 (PerkinElmer), an amine-reactive near-infrared fluorochrome, designed to allow maximal tissue penetration and minimal absorption by physiological absorbers such as hemoglobin or water. The absorption and emission spectra in aqueous solution were found to be 674 nm/692 nm and the ε = 2.2 x 105 $M^{-1}cm^{-1}$.

Property	Specification
Absorbance and emission spectra in 1 x PBS	
MW	1432 g/mol
Fluorescence excitation[1]	675 nm
Fluorescence emission[1]	693 nm
Absorbance	675 nm ± 5 nm
Appearance	Dark blue solid, soluble in water or aqueous buffer

1 *Absorbance and fluorescence maxima of IntegriSense680 in 1X PBS*

3 *In Vitro* Binding

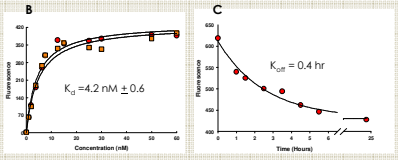
A: HEK293 cells stably transfected with $\alpha_v\beta_3$ (HEK293- $\alpha_v\beta_3$) were incubated with varying concentrations of IntegriSense at 4 ºC for 30 min, lifted with trypsin-EDTA, washed in serum-free medium, added to microtiter wells coated with vitronectin, and allowed to attach at 37 ºC for 2 hr in a humidified incubator; non-attached cells were gently washed away. Attached cells were quantified by colorimetric detection of hexoaminidase enzymatic activity in a microplate reader (Molecular Devices) and the IC_{50} calculated.

B: HEK293- $\alpha_v\beta_3$ cells were incubated with varying concentrations of IntegriSense as described above. The amount of probe bound to integrins on HEK293- $\alpha_v\beta_3$ cells was determined by flow cytometry (FACSCalibur, BD Biosciences). Data was analyzed using FlowJo software and K_d values calculated using SigmaPlot 10.

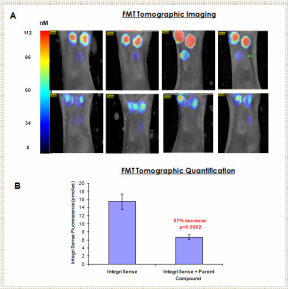
C: Cells were incubated with 100 nM IntegriSense at 4 ºC for 30 min and transferred into PBS containing 10 mM of unlabeled compound (parent compound). The amount of probe bound to integrins on HEK293- $\alpha_v\beta_3$ cells was determined by flow cytometry before mixing with parent compound and at various times after mixing. Data was analyzed using FlowJo software and K_{off} values calculated using SigmaPlot 10.

A

	IC50 (nM)	
	IntegriSense	Parent Compound
$\alpha_v\beta_3$	4.1 ± 0.3	0.82 ± 0.2
$\alpha_v\beta_5$	33	1.3 ± 0.2
$\alpha_{IIb}\beta_3$	39	>10,000
$\alpha_5\beta_1$	> 2,000	>10,000



4 Integrin-Targeted Agent Specifically Detects Integrins in a Mouse Breast Cancer Model

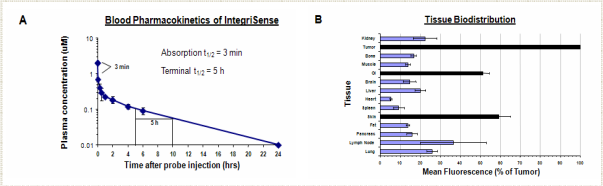


Nu/Nu mice were injected subcutaneously bilaterally in the mammary fat pads with mouse breast carcinoma 4T1 cells. One week later, mice were randomized according to tumor volume as measured with calipers and injected intravenously (i.v.) with 4 nmoles of the IntegriSense agent in the absence or presence of the parent compound which acts as a competitor, and imaged 24 hrs later by FMT.

(A) Representative maximum projection slices were taken at the same color gating from scans of 4 mice injected with IntegriSense 680 (top) and mice co-injected with IntegriSense 680 + 200 nmoles of parent compound (bottom). Corresponding background 3D regions of interest (ROIs) are shown (green arrow).

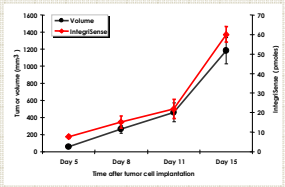
(B) Images were reconstructed using the FMT software and the total amount of fluorescence (pmol) was quantified in specific ROIs for each tumor. Co-injection with the parent compound resulted in a significant decrease in IntegriSense signal.

5 Pharmacokinetic and Biodistribution Profile



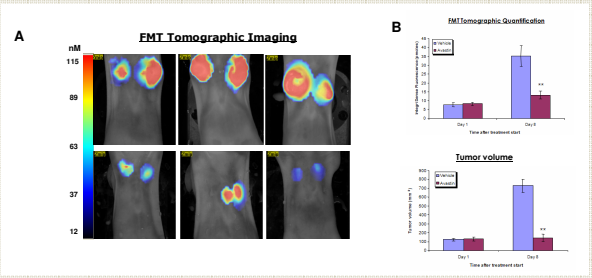
A. CD1 mice were injected i.v. with IntegriSense 680 (4 nmoles). Blood was collected at various times post-injection and plasma obtained by centrifugation. Plasma samples were diluted 1:2 in DMSO and the fluorescence was read using a fluorescence plate reader. **B.** For assessment of biodistribution, 4T1 tumor-bearing mice were injected i.v. with IntegriSense (4 nmoles) and sacrificed 24 hrs later for tissue analysis. Organs were excised, and imaged on a 2D fluorescence reflectance system (Kodak 2000 MM). Mean fluorescence (Relative Fluorescence Units) was determined by drawing a region of interest around each tissue and normalizing the values to those obtained for tumors (set to 100%). Shown are Mean + SEM.

6 Integrin Agent Signal Strongly Correlates with Tumor Volume



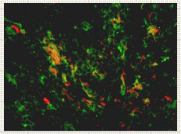
4T1 tumor-bearing mice were injected i.v. with 4 nmoles of IntegriSense 680 at different times after tumor implantation and imaged 24 hrs later by FMT. Tumor dimensions were measured with calipers and values were used to calculate tumor volume [mm^3 = (length x width^2)/2]. Images were reconstructed using the FMT software and the total amount of fluorescence (pmol) was quantified in specific 3D regions of interest around each tumor. A strong correlation was seen between tumor volume and IntegriSense signal (r^2 = 0.87).

7 Anti-Angiogenic Treatment: An Integrin-Targeted Imaging Agent can be Used to Assess Therapeutic Efficacy in a Tumor Xenograft Model *In Vivo*



Twelve days after human colon carcinoma A673 tumor cell injection in mice, tumor volumes were measured and mice randomized into 2 groups: Avastin or Vehicle. Mice in the Avastin group received 2 mg/kg Avastin (bevacizumab, Genentech, CA) i.p. 2x per week, while mice in the Vehicle group received PBS instead. At the start of the treatment, or 7 days later, mice were injected i.v. with 4 nmoles of the IntegriSense and imaged 24 hrs post-probe injection by FMT. **(A)** Representative maximum projection slices taken at the same color gating from 3 mice treated with vehicle only (top) and 3 mice treated with Avastin for 1 week (bottom). **(B)** Images were reconstructed and the total amount of fluorescence was determined in specific 3D regions of interest around each tumor (left). FMT quantitative tomography results were compared to calculated tumor volume measurements of (right). A significant decrease in IntegriSense signal of 63% was observed 1 week after treatment ($p<0.001$), correlating with a decrease in tumor volume.

8 Distribution of Integrin-Targeted Agent in Breast Tumors



Immediately following the imaging session, mice were sacrificed and tumors were excised and snap frozen in OCT for fluorescence microscopy. The distribution of NIR fluorescence was determined using a fluorescence microscope (Carl Zeiss MicroImaging). Digital images were captured using appropriate filters for FITC and for the near-infrared agent. Endothelial cells were detected using a monoclonal anti-CD31 FITC-conjugated antibody (green) and the distribution of IntegriSense is shown in red. Note that IntegriSense co-localizes with some blood vessels (yellow) but also targets tumor cells.

7 Summary

Integrins are a family of transmembrane glycoproteins which play a crucial role in the pathogenesis of various diseases, including cancer, and as such represent viable biomarkers for the progression of these diseases. We have developed IntegriSense™ 680, an integrin-targeted molecular imaging agent that allows for the non-invasive imaging of disease status and progression. In breast and colorectal tumor imaging, this agent detects the integrin $\alpha_v\beta_3$ localized in the tumor. Pairing of an integrin antagonist treatment with IntegriSense provides a mechanistic biomarker approach for assessing target coverage. Further, in a breast cancer model, treatment with Avastin showed quantitative changes in integrin imaging with as little as one week of treatment. The ability to spatially and temporally visualize and quantify tissue integrin levels *in vivo* using this targeted fluorescent agent and quantitative FMT imaging approach will greatly improve the ability to assess integrin expression during tumor development and metastasis, to develop novel anti-integrin therapies and to monitor treatment efficacy longitudinally.

8 References

Coleman PJ et al. Nonpeptide αvβ3 Antagonists. Part11: Discovery and preclinical evaluation of potent $\alpha_v\beta_3$ antagonists for the prevention and treatment of osteoporosis. J. Med. Chem. 47, 4829-4837 (2004).

Stupack, D.G. The biology of integrins. Oncology (Williston Park). 21, 6-12 (2007).

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