In Vivo Quantification of Integrin-Targeted and Protease-Activated Imaging Agents in Response to Anti-Angiogenic Therapy using Quantitative Fluorescence Tomography

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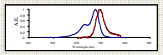
1 Abstract

Integrins are transmembrane cell surface receptors which mediate signal transduction, cell-to-cell interaction and cell-to-extracellular matrix adhesion, key processes involved in angiogenesis, tumorigenesis and metastasis. Integrins have thus been hailed as clinically-relevant biomarkers of pathological conditions such as inflammation and tumor progression. The integrin $\alpha_v\beta_3$ is significantly upregulated in tumor cells and activated endothelial cells during neoangiogenesis. To date, it has not been possible to strictly quantify integrin levels *in vivo* with existing optical imaging technologies. What is more, the simultaneous quantification and *in vivo* localization of integrins and of a distinct biomarker has proved unfeasible. The aim of this study was to simultaneously and non-invasively image and quantify signal of $\alpha_v\beta_3$ receptor binding and the signal of a cathepsin-activatable imaging agent using a specific, targeted near-infrared (NIR) fluorescence agent and Fluorescence Molecular Tomography – or FMT™ (FMT 2500™ Quantitative Tomography System, PerkinElmer). We developed a fluorescent imaging agent (IntegriSense®, PerkinElmer) for *in vivo* detection of $\alpha_v\beta_3$ using a low molecular weight peptidomimetic antagonist coupled to a NIR fluorochrome. The dissociation constant Kd, as determined by binding to $\alpha_v\beta_3$-overexpressing HEK293 cells, was found to be 4.8 ± 10 nM. The pharmacokinetic profile was assessed in mice by measuring plasma fluorescence at different times after intravenous injection with the agent and found to fit a two-compartmental model and calculated to be $t(1)_{1/2}$= 6 min and $t(2)_{1/2}$= 210 min. Integrin expression in tumors was quantified in both mouse breast tumors and human rhabdomyosarcoma tumor xenografts implanted in nude mice, and the quantified fluorescent signal strongly correlated with tumor size (r^2=0.87). In addition, this agent was used as a mechanistic biomarker for anti-angiogenic therapeutic efficacy. Integrin agent administration in mice with established tumors allowed the non-invasive and real-time quantification of integrin signal decrease (54%, p=0.017 Avastin-treated *versus* vehicle control) following treatment with the anti-angiogenic drug Avastin. This treatment had no significant effect on the cathepsin-cleavable NIR agent. Interestingly, the simultaneous imaging of these two agents revealed different patterns of distribution reflecting underlying differences in integrin and cathepsin biology during tumor progression. These results underscore the potential of non-invasive quantitative fluorescent tomography (FMT) imaging and imaging agents in improving and providing more refined approaches for pre-clinical and translational cancer detection and monitoring of treatment.

2 Integrin-targeted Imaging Agent

The integrin-targeted agent (IntegriSense® 680, PerkinElmer) was synthesized by converting the small molecule, non-peptide a_vb_3 antagonist, compound 5f (Coleman *et al.*), to the 3-cyano derivative, reducing the derivative to the 3-aminomethyl analog and reacting the resulting compound with VivoTag™ 680 (PerkinElmer), an amine-reactive near-infra-red 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-1cm-1. The molecular weight as calculated by LC/Mass was 1430.4 for C67H82N8O17S5; found 1431.5 M+1.

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

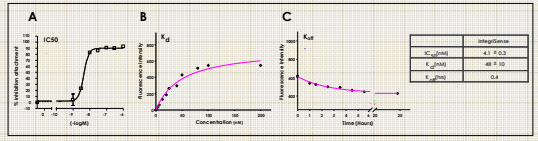
Absorbance and fluorescence maxima of IntegriSense 680 in 1X PBS

3 *In Vitro* Binding

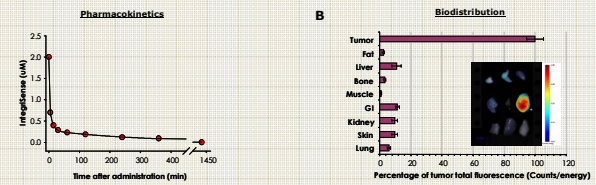
A: IC_{50}: HEK293 cells stably transfected with $\alpha_v\beta_3$ (HEK293-$\alpha_v\beta_3$) were incubated in the presence of varying concentrations of IntegriSense at 4ºC for 30 min. Cells were trypsinized, washed and added to vitronectin-coated microtiter wells, and allowed to attach at 37ºC for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

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

C: K_{off}: HEK293-$\alpha_v\beta_3$ were incubated with 100 nM IntegriSense at 4ºC for 30 min, transferred into PBS containing 10 mM of unlabeled compound (parent compound). The amount of agent 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.

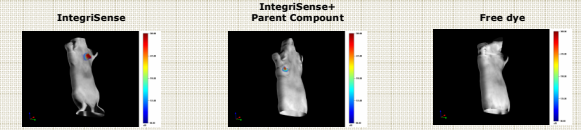


4 Pharmacokinetic and Biodistribution Profile

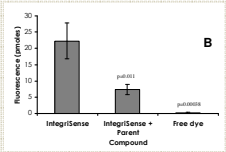


A. BALB/c mice were injected i.v. with IntegriSense 680 (2 nmoles). Blood was collected at various times, plasma obtained by centrifugation, and fluorescence read using a fluorescence microplate reader. **B.** Human rhabdomyosarcoma A673 tumor-bearing nude mice were injected i.v. with IntegriSense (2 nmoles) and sacrificed 24 hrs later. Organs were excised and imaged on the FMT 2500™ quantitative tomography system using the reflectance mode. Regions of interest (ROI) were drawn around each organ using the FMT software and the mean fluorescence (Counts/Energy) determined for each organ and normalized to the mean fluorescence of the tumors (set to 100%). Shown are Mean ± SEM. Insert shows a representative image of the fluorescence detected in different organs, * tumor.

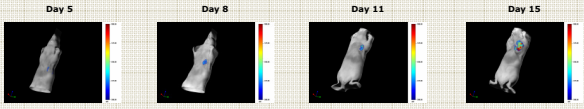
5 Integrin-Targeted Agent Specifically Detects Tumor-Associated Integrins: Quantification with FMT *In Vivo* Imaging



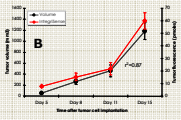
Nu/Nu mice were injected subcutaneously bilaterally in the mammary fat pads with human rhabdomyosarcoma A673 cells. Mice were randomized according to tumor volume and injected i.v. with 2 nmoles of IntegriSense in the absence or presence of the unlabeled parent compound (100 nmoles) which acts as a competitor, and imaged 24 hrs later by FMT. Control mice were injected with 2 nmoles of free dye. *In vivo* imaging was performed using FMT 2500™ under gas anesthesia. **A.** Representative volume rendering projections taken at the same color gating from mice injected with IntegriSense, co-injected with IntegriSense and parent compound or injected with free dye. **B.** 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.



6 IntegriSense Signal Strongly Correlates with Tumor Volume

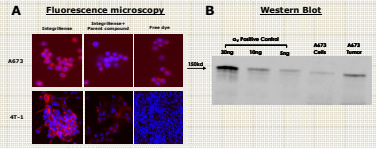


Nu/Nu mice were injected subcutaneously in the mammary fat pads with mouse breast carcinoma 4T1 cells. At different times thereafter, mice were injected i.v. with IntegriSense and imaged 24 hrs later by FMT. **A.** Representative volume rendering projections taken at the same gating from mice injected with IntegriSense 5, 8, 11 and 15 days after tumor injection. **B.** Tumors were measured with calipers and tumor volume calculated as 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 ROIs around each tumor. A strong correlation was seen between tumor volume and IntegriSense signal (r^2 = 0.87).

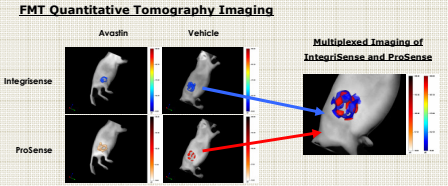


7 Specific Binding of Integrin-Targeted Agent to Tumor Cells

A. Tumor cells (A673 or 4T-1) were cultured in the presence of IntegriSense (20 nM), Integrisense and parent compound (200 nM) or free dye (20 nM) for 30 min at 37ºC. Fluorescence microscopy images show endocytosis of IntegriSense (in red, nuclei counterstained with DAPI in blue) effectively blocked by incubation with parent compound, and incubation with free dye showing little or no detectable uptake. **B.** Western blot analysis of the expression of the α_v subunit in A673 tumors *ex vivo* confirming that A673 tumors have significant levels of α_v subunit.



8 Integrin-Targeted Imaging Agent can be used to Assess Therapeutic Efficacy *In Vivo*



A673 tumor-bearing mice (implanted in the flank) were 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. Seven days later, mice were injected i.v. with 2 nmoles IntegriSense and 2 nmoles Prosense®750 (PerkinElmer), and were imaged 24 hrs later.

A. Representative isosurface rendering projections of a mouse treated with Avastin and a mouse treated with vehicle. Note the differential localization of IntegriSense (blue) and ProSense (red) within the same tumor. **B.** Avastin significantly inhibited tumor growth as assessed by calculated tumor volume derived from caliper measurements (left). A significant decrease in IntegriSense signal of 63% (p=0.007), but not ProSense signal (p=0.13) was observed 1 week after treatment (right).

9 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 the non-invasive imaging of disease status and progression. In breast and rhabdomyosarcoma tumors, this agent detects the integrin avb3 localized in the tumor. Pairing of an integrin antagonist treatment with IntegriSense 680 provides a mechanistic biomarker approach for assessing target coverage. Furthermore, treatment with Avastin showed quantitative changes in integrin imaging with as little as 1 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 tomographic imaging 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.

10 References

Coleman PJ et al. Nonpeptide $\alpha_v\beta_3$ 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).

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