

# Development of a near infrared fluorescent labeled agent for assessing bombesin receptor expression in cancer.

## Abstract

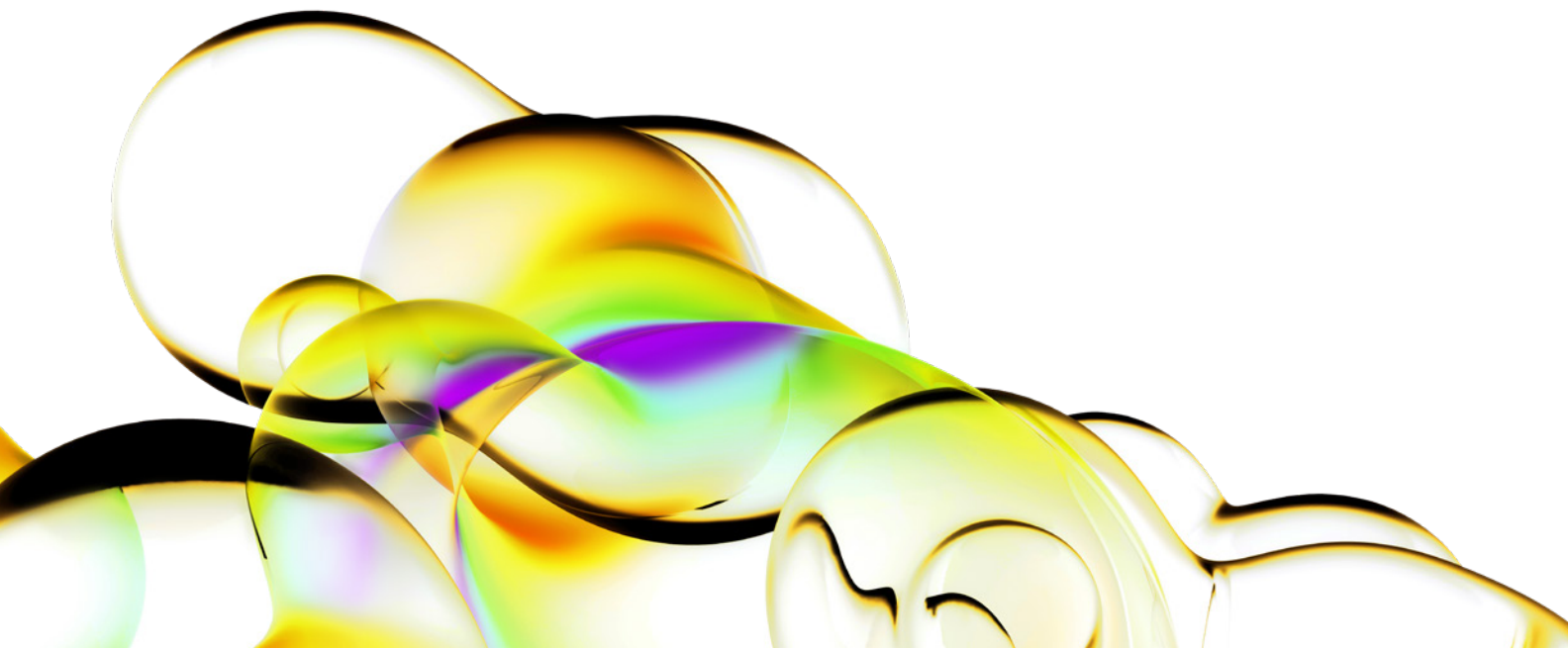
Bombesin-like peptides and their G-protein coupled receptors have been shown to play a role in cancer and are overexpressed in a variety of tumors, including prostate, breast, lung, CNS, gastric, colon, and renal cell carcinomas. We developed a novel near infrared (NIR) fluorescent imaging agent, IVISense™ Bombesin Receptor 680 (BRS 680), comprising a 7-amino acid bombesin peptide analog, an NIR fluorophore (ex/em 665/691 nm) and a pharmacokinetic modifier to improve its plasma availability (plasma  $t_{1/2}$  = 30-45 min). This agent can be used to target and quantify upregulation of bombesin receptors (BBR) *in vivo* associated with tumor proliferation. Selectivity of agent binding was confirmed by blockade of BRS 680-labeling of human colorectal HT-29 cells with excess unlabeled native bombesin peptide. BBR-expressing tumors were readily imaged in nude mice, showing that BRS 680 has the potential to serve as an *in vivo* real-time early indicator of tumor growth and chemotherapy efficacy.

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## Materials and methods

### Fluorescent agents

The IVISense Bombesin Receptor 680 (BRS 680) fluorescent agent is specific for bombesin receptors (BBRs) expressed in many types of cancer. This agent was designed to detect and image BBR expression of tumors implanted in *NU/NU* mice. The imaging dose for this agent was 2 nmol/25 g mouse.

Table 1: Basic properties of BombesinRSense 680 fluorescent tumor imaging agent.

	IVISense Bombesin Receptor 680
Agent Type	BBR-targeted agent
Molecular Weight	24,000 gmol <sup>-1</sup>
Ex/Em	665/691 nm
Blood Half-life	t <sub>1/2</sub> 30-45 min
Tissue Half-life	~ 96-120 h

**Agent summary.** MW/size, excitation/emission [Ex/Em], and blood/tissue pharmaco-kinetics were determined in multiple independent studies. Plasma half-life was measured by blood collection from mice at different times post-intravenous injection. Blood samples were measured for fluorescence levels in a fluorescence microplate reader. Tissue half-lives were determined by time course FMT® 4000 imaging of tumors.

### Flow cytometry and microscopy

Human colorectal carcinoma HT-29 cells (BBR positive) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were routinely cultured in McCoy's 5A medium with 10% fetal bovine serum and 1% penicillin-streptomycin in 75 cm<sup>2</sup> flasks. Exponentially growing cells between passages 1-10 were used for all experiments. Cells were incubated with BRS 680 (1 µM) for 5-15 min. Cells were analyzed by flow cytometry (Becton Dickinson LSRII) and fluorescence microscopy using appropriate lasers and filters appropriate for detection of 680 nm wavelength. Cell nuclei were stained with DAPI (blue).

Frozen tissue sections were obtained 24 h after *in vivo* agent intravenous (i.v.) injection (2 nmol per mouse) or labeled *ex vivo* by incubation of 10 micron frozen tumor tissue sections with 1 µM BRS 680 for 15 min. Some control sections were stained with a rabbit polyclonal antibody against GRPR (BB2 receptor) for 30 min, followed by a FITC-conjugated secondary antibody (goat anti rabbit polyclonal secondary antibody). Sections were imaged by fluorescence microscopy using DAPI as a nuclear counterstain.

### HT-29 tumor models

Six to eight week-old female *NU/NU* mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in a pathogen-free animal facility with water and low-fluorescence mouse chow (Harlan Tekland, Madison, WI). Handling of mice and experimental procedures were in accordance with Revvity IACUC guidelines and approved veterinarian requirements. HT-29 (ATCC, Manassas, VA) and HT-29-luc2 (Revvity, Waltham, MA) cell lines were maintained according to supplier instructions. To induce tumor growth, mice were injected subcutaneously either in the dorsal flank or upper chest with 5x10<sup>6</sup> HT-29 or HT-29-luc2 cells, yielding tumor masses within a few weeks (Figure 1).

### In Vivo imaging and analysis

Tumor-bearing mice were anesthetized using inhaled isoflurane, injected IV with BRS 680, and positioned for imaging in either the IVIS® Spectrum or the FMT 4000 *in vivo* imaging system (both systems from Revvity, Waltham, MA). Fluorescence images were captured at a variety of time points post-agent injection as described in Figure legends.

### Imaging data analysis

The collected FMT fluorescence images were reconstructed by FMT system software (TrueQuant™ v4.0, Revvity Waltham, MA) for the quantification of three-dimensional fluorescence signal within the tumors. Three-dimensional regions of interest (ROI) were drawn encompassing the relevant flank or chest regions. IVIS Spectrum data were analyzed by Living Image® software (Revvity, Waltham, MA) and analyzed for 2D epifluorescence.

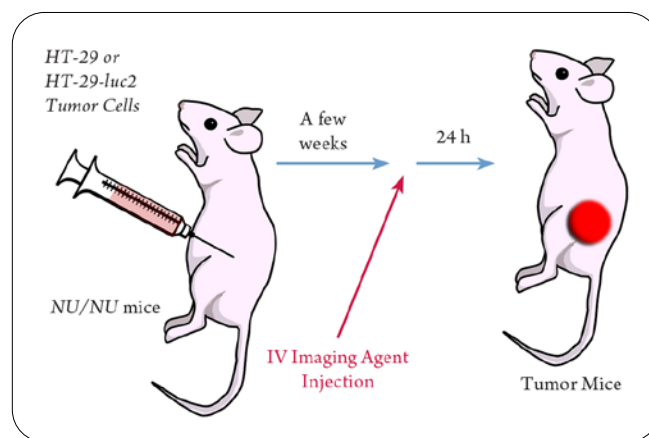


Figure 1: Tumor xenograft model.

## Introduction and results

Bombesin receptors (BBRs), which play a role in a variety of physiological systems in mammals, have been the subject of investigation for the past two decades because of the overexpression of the three characterized receptor types (BB1R, BB2R, and BB3R) in several prevalent solid tumors, including prostate, breast, small cell lung, and gastrointestinal cancers. The bombesin family of peptide ligands for these BBRs, first described in the 1970s in amphibian skin, include the mammalian analogs, Neuromedin B (NMB), gastrin-releasing peptide (GRP), and phyllolitorins. Receptors are increased in cancer as a marker of proliferation, and bombesin peptides serve as autocrine growth factors, signaling either calcium mobilization or proliferation in tumor cells (findings particularly attributed to GRP signaling through BB2R). Several radio-labeled analogs of these peptides have been used for PET or SPECT imaging in preclinical cancer research as well as in imaging of patients. Cytotoxic analogs of bombesin, as well as bombesin receptor antagonists, are being explored as novel therapeutic approaches.

Revvity developed a NIR fluorescent agent for detection of upregulation of BBRs *in vivo*, comprising a 7-amino acid bombesin peptide analog, an NIR fluorophore (ex/ em 665/691 nm) and a pharmacokinetic modifier. The NIR- labeled bombesin peptide (BombesinRSense 680 [BRS 680]) effectively labeled HT-29 human colon adenocarcinoma cells, and the specificity of this binding was confirmed by control experiments using competitive blockade with unlabeled excess bombesin. Fluorescence microscopy confirmed the expected BRS 680 localization and uptake of fluorescence. *In vivo* and *ex vivo* imaging (FMT 4000 and IVIS Spectrum), showed significant accumulation within tumors, peaking at 24 h following intravenous injection of 2 nmol of the agent, decreasing thereafter with a tissue half- life of ~5 days. Other tissues known to express BBRs (e.g. pancreas, skin) also showed preferential accumulation of BRS 680, and the short half-life in blood was in agreement with high signal in the kidneys, suggesting rapid clearance via the kidneys and bladder. In summary, BRS 680 selectively targets BBR expression, allowing both imaging *in vitro* and the non-invasive real-time NIR imaging and quantification *in vivo*.

## Microscopy and flow cytometry

Figure 2 shows that BBR-positive HT-29 cells can be effectively labeled with BRS 680 with only a short 5 minute incubation. The fluorescent signal of labeled cells was readily quantified by flow cytometry as ~40-fold increase in fluorescence (Figure 2A). In contrast, a peptide-scrambled version of BRS 680 (Scrambled 680 Control) showed only background cell labeling. BRS 680 binding specificity was confirmed by prior blockade with native unlabeled bombesin; the ability of BRS 680 to label HT-29 cells was inhibited ~90%. Results were confirmed by fluorescence microscopy, and Figure 2B illustrates the strong labeling of tumor cells as assessed by fluorescence microscopy.

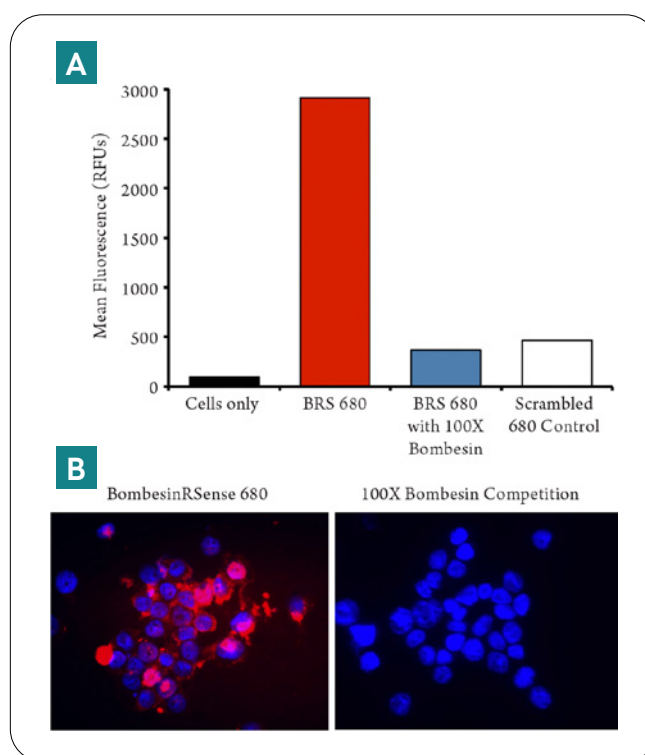


Figure 2: HT-29 cells were incubated with 1  $\mu$ M BRS 680 or negative control agent (scrambled peptide,) for 5 min at RT, with or without prior incubation with 100  $\mu$ M of native, unlabeled Bombesin (15 min at 37  $^{\circ}$ C) to compete for binding. Cells were rinsed and imaged by both flow cytometry (A.) and by fluorescence microscopy (B.). Red signal is BRS 680, and blue signal represents DAPI nuclear counterstain.

## *In Vivo* imaging: Cross-validation with bioluminescence

Figure 3 highlights the *in vivo* ability of BRS 680 to detect BBR expression in subcutaneously implanted colorectal tumor xenografts in a manner similar to the detection of tumor luciferase expression. Three different mice, bearing three different levels of tumor burden, showed clear bioluminescence signal defining the tumor localization

(Figure 3A) with signal to background ratios exceeding 100. The same mice, imaged for 2D epifluorescence by IVIS Spectrum 24 h after injection with BRS 680, showed the same tumor masses by virtue of their expression of BBRs (Figure 3B). These NIR fluorescence imaging results confirmed the appropriate localization of signal, with good

tumor definition and ~3.5 fold target to background ratios. In addition, BRS 680 detected the normal, expected BBR expression in the pancreas. The optimal imaging time in this study was 24 h post-injection, with a tissue residence half-life of approximately 96 h (not shown).

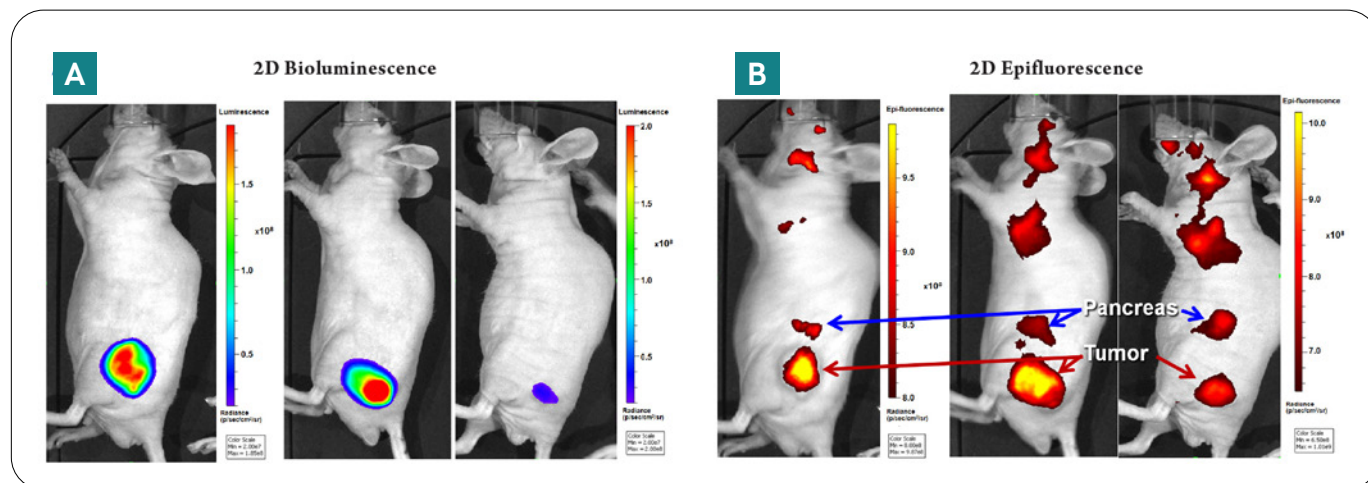


Figure 3: *NU/NU* mice were implanted with human colorectal HT-29 luc tumors, injected IV with 2 nmol BRS 680, and imaged by IVIS Spectrum CT 24 h later; (A.) bioluminescence and (B.) epifluorescence. The same three mice per group were imaged to show tumor specific localization of BRS 680. Red and blue arrows indicate tumor masses and pancreatic tissue, respectively. Upper torso signal may be attributed to either lymph nodes or salivary glands. Tumor signal as assessed by BRS 680 correlates strongly with bioluminescence intensity ( $r^2 = 0.91$ ).

### In Vivo imaging: timecourse optimization

To establish a full range of imaging datasets for defining optimal imaging time and the kinetics of agent clearance, HT-29-bearing mice were injected with 2 nmol of BRS 680 and imaged 15 and 30 minutes, 1 and 4 h, and 1, 2, 5, and 12 days post injection. As shown in Figure 4, most of the fluorescent signal is in circulation prior to 4 h, yielding poor tumor definition by epifluorescence imaging (Figure 4A). Tomographic imaging by FMT detected and quantified this early heart signal, providing a kinetic imaging profile in good agreement with the established plasma pharmacokinetics

(Figures 4A and B). The kinetic changes of liver fluorescence also closely agreed with heart imaging data, suggesting that the apparent liver signal was due to signal within the blood vessels of the liver rather than due to agent extravasation into liver tissue. The optimal imaging time was 24 h post-injection, with a tissue residence half-life of approximately 96-120 h. These data generated from a single tumor-bearing mouse were in close agreement with averages of all 4 mice within the study group (data not shown).

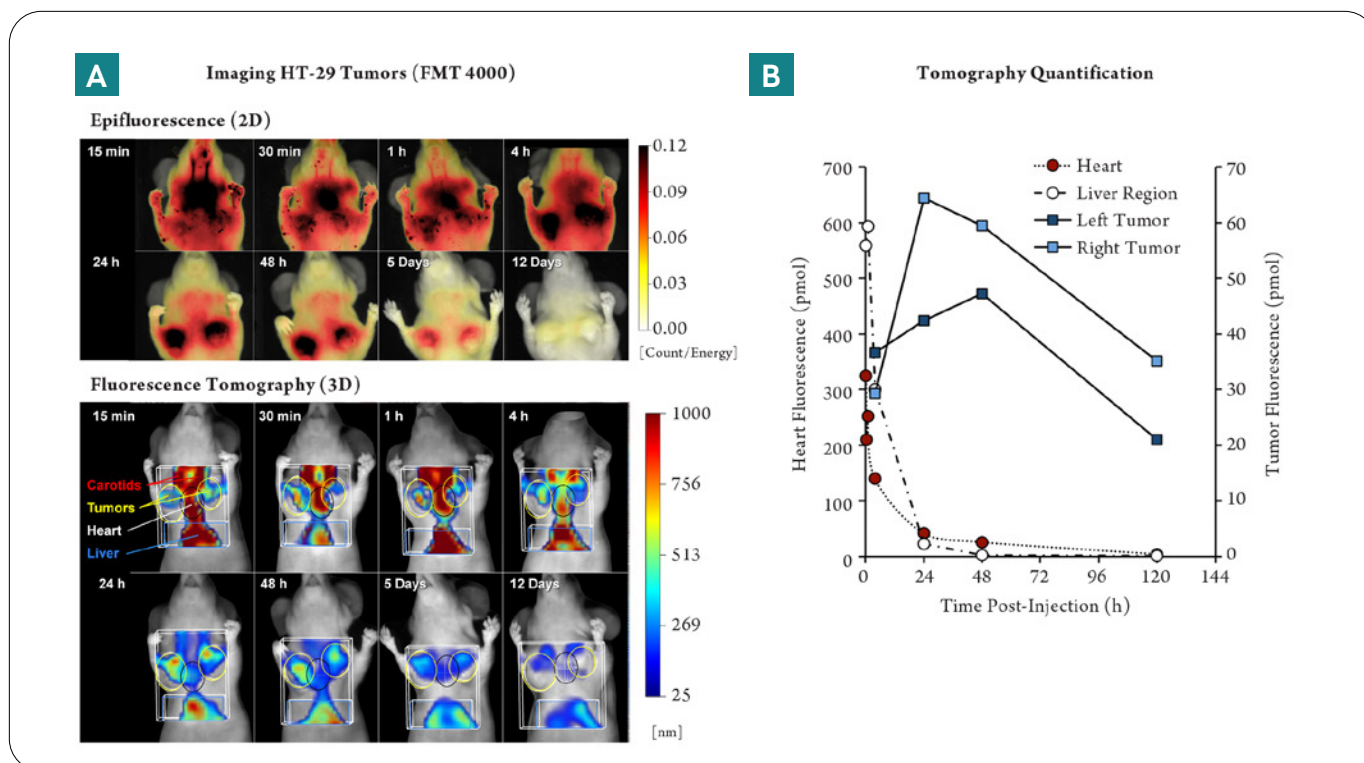


Figure 4: NU/NU mice were implanted subcutaneously with human colorectal HT-29 tumors (2 tumors per mouse). When tumors reached the desired volume, mice were injected IV with 2 nmol BRS 680 and imaged tomographically (FMT 4000) at times ranging from 15 minutes to 12 days. (A.) Shown is 1 representative mouse imaged at all timepoints by both epifluorescence (upper panels) and fluorescence tomography (lower panels). (B.) Tomographic imaging datasets were used to quantify kinetic fluorescence changes in the upper liver region and the heart as well as targeted accumulation in the tumors.

### In Vivo multiplex imaging

The BRS 680 imaging agent can be paired with other imaging agents to better provide a more complete view of the complex biology of a tumor. For example, in Figure 5, BRS 680 (which provides a measure of increased proliferative status of the tumor) was co-imaged with IVISense Vascular 750 probe (which shows areas of increased vascular leak). As expected for this pair of agents, there is a good degree of overlap in localization with some other regions of differential localization. Tumor metabolism and proliferation are expected to be higher in well perfused tumor regions. However, vascular leak is also generally high in regions around the tumor where you see less BRS 680 accumulation.

### In Vivo biodistribution and pharmacokinetics

To better understand the *in vivo* performance of BRS 680, normal mice were injected with the agent and blood was collected at a variety of times spanning 24 h. Changes in the fluorescent signal in the blood over time revealed a circulation half-life of 30-45 minutes (Figure 6A inset). This relatively short half-life is supported by the observed

increased fluorescence in the kidneys of HT-29-bearing NU/NU mice at 24 h post-injection (Figure 6A). Most tissues showed low fluorescent signal at 24 h, except for pancreas and skin, supporting studies showing expression of BBRs in pancreatic acinar tissue and in skin mast cells. Naturally, HT-29 tumors also showed increased BRS 680 signal in agreement with data in Figures 3, 4, and 5. Epifluorescence images of excised organs and tissues (Figure 6B) shows clearly the brighter signal in these tissues collected from a representative mouse.

### Detection of BBR expression in frozen tumor tissue sections

To further confirm the specificity of BRS 680 binding and localization within tumors, we performed studies using frozen HT-29 tumor xenograft sections. Figure 7A shows tumor sections which were incubated *ex vivo* with BRS 680 in the presence or absence of 100X excess of native unlabeled bombesin as a receptor blocker. BRS 680 signal in unblocked tissue showed a heterogeneous distribution of signal within the tissue, however excess native bombesin blocked this tissue labeling completely.



Tumor tissues were also collected from mice 24 h after receiving an IV injection of BRS 680. Serial 10 micron sections were prepared to determine whether the pattern of signal generated by the agent *in vivo* correlated with ex vivo staining of serial sections with anti-BBR antibody. Figure 7B shows excellent co-localization of BRS 680 and ex vivo anti-BBR staining, supporting the contention that *in vivo* BRS 680 localizes selectively to BBR-expressing cells.

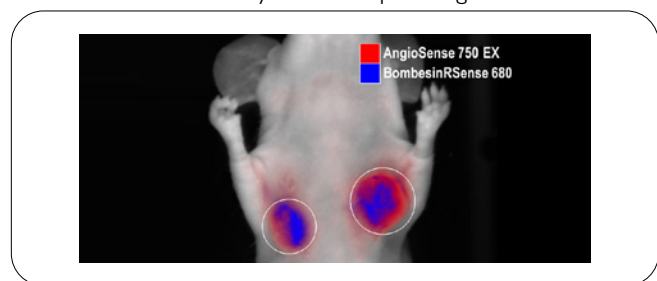


Figure 5: *NU/NU* mice implanted subcutaneously with human colorectal HT-29 tumors were injected intravenously with 2 nmol IVISense Bombsin Receptor 680 and 2 nmol of IVISense Vascular 750 EX. Epifluorescence imaging by FMT 4000 at 24 h post IV injection shows overlapping intratumoral distribution of these two imaging agents.

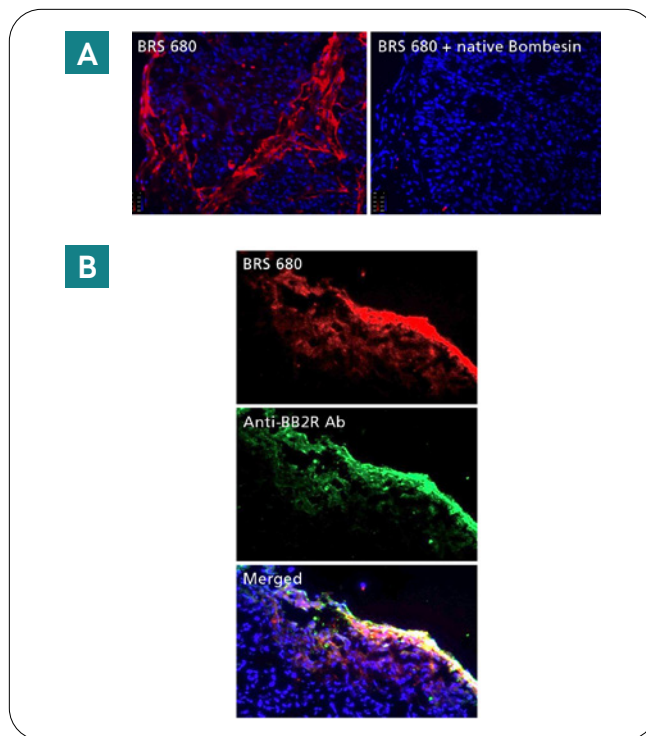


Figure 7. (A.) To assess binding specificity of BRS 680 tumors were removed from HT-29-tumor bearing mice, and frozen sections (10 micron thickness) were incubated with 1  $\mu$ M BRS 680 for 15 min. at RT, without or with previous incubation (for 15 min. at 37 °C) with 100  $\mu$ M native bombesin, rinsed and imaged by fluorescence microscopy using DAPI as a nuclear counterstain. (B.) To confirm proper tumor localization of BRS 680 *in vivo*, HT-29 tumor bearing mice were injected with 2 nmoles BRS 680. Tumors were excised 24 h later and 10 micron sections were compared to serial sections stained with a rabbit polyclonal antibody against GRPR (BB2 receptor) Sections were imaged by fluorescence microscopy using DAPI as a nuclear counterstain.

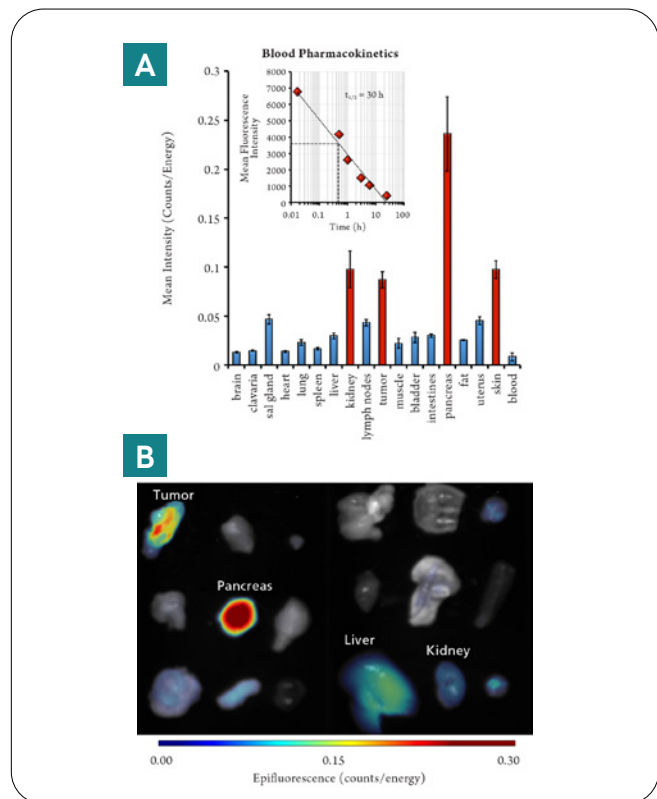


Figure 6: Twelve HT-29-bearing *NU/NU* mice were injected IV with 2 nmol BRS 680. Blood was collected at multiple times, and tissues were removed at 24 h, to assess blood and tissue biodistribution, respectively. (A.) Quantified tissue biodistribution measured by FMT epifluorescence. Inset shows plasma pharmacokinetics measured by microplate assay. (B.) Epifluorescence tissue images using tissues from a representative mouse.

## Conclusions

Cancer is a term used for diseases in which cells spontaneously proliferate out of control and invade other tissues via the blood and lymph systems. Bombesin-like peptides and bombesin receptors (BBRs) have been the subject of investigation for the past two decades because of the association between these molecules and the proliferative status of tumor cells. Overexpression of the three characterized receptor types (BB1R, BB2R, and BB3R) is prevalent in several solid tumors, including prostate, breast, small cell lung, and gastrointestinal cancers.

The ability to detect expression of BBRs non-invasively in animal models of cancer has provided an important tool for biomedical research. Adapting bombesin as a fluorescence imaging tool, however, offers further advantages in *ex vivo* microscopy as well as in the replacement of hazardous radioactive labels. For this reason, we developed a NIR fluorescent imaging agent specific for tumor BBRs by conjugating a NIR fluorescent dye to a bombesin peptide analog. This imaging agent was profiled extensively *in vitro* and *in vivo* for performance and selectivity, offering a useful tool for examining the proliferative status of tumors. The further pairing of IVISense Bombesin Receptor fluorescent probe with Revvity imaging systems, and other novel red and near infrared (NIR) imaging agents, allows the detection and quantification of multiple important biological processes in the context of tumor BBR expression.

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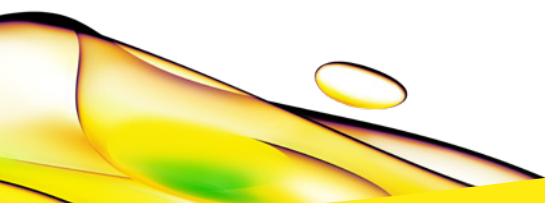
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