Supplemental Information for

A high affinity near infrared fluorescent probe to target bombesin receptors

Molecular Imaging and Biology

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[1] contributed equally to this work
Short Title: NIRF-bombesin probes

Full Paper

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The material presented herein includes analytical data to demonstrate purity and identity of the compounds studied, additional images demonstrating renal/urinary excretion, pilot studies on normal inbred mice demonstrating pancreatic uptake, additional data removed from the primary figures to enhance clarity of the presentation, and the structures of two dyes that were used in literature compounds discussed and compared in the paper.

Materials and Methods

The synthesis of the peptides studied was uneventful and without significant safety hazards. The HPLC traces in Fig s1 demonstrate purity to ≥95%, with single peaks demonstrated in each case for the final molecules. The HPLC procedure and column details are in the paper.

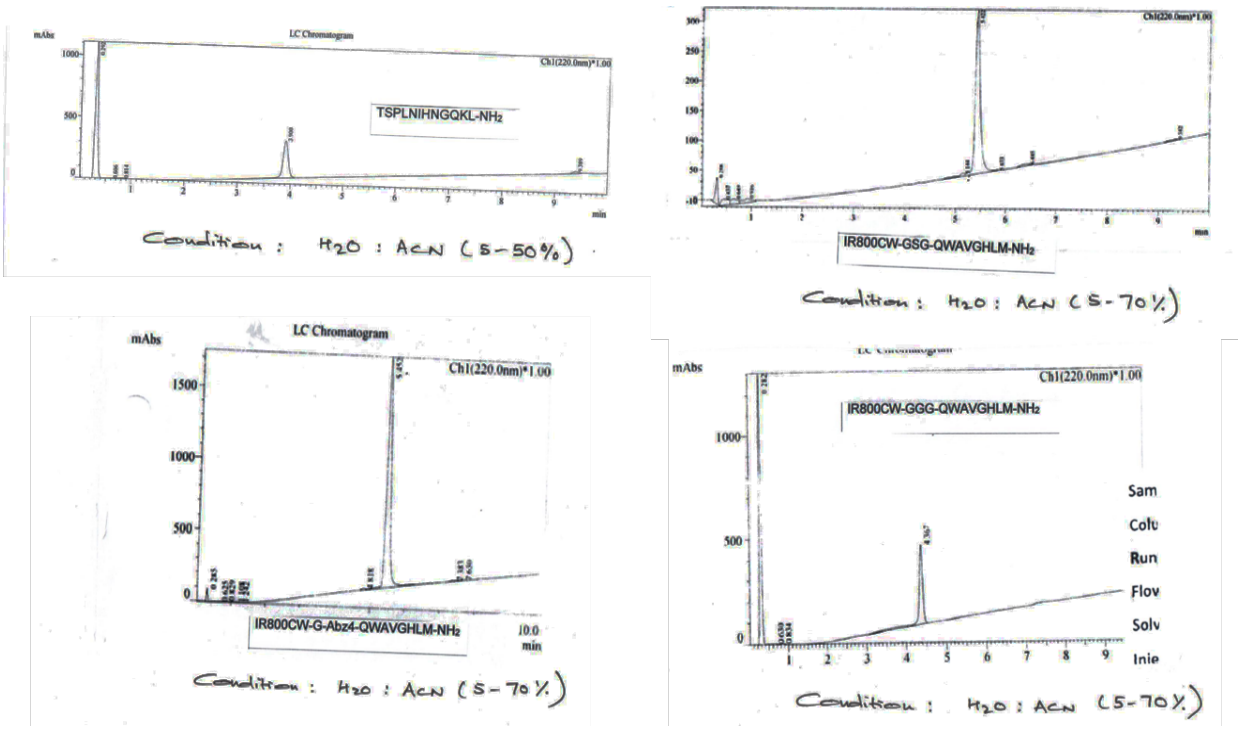


Fig s1. HPLC traces of the four compounds synthesized, clockwise from upper left: HN1, IR800-GSG-t-BBN, IR800-GGG-t-BBN and IR800-G-Abz4-t-BBN. The nomenclature used at the time the traces were collected contained the additional letters, CW, to describe the dye. These two letters were later deleted.

Maldi-TOF parent ion peak data in Table s1 match the theoretical data for the intended products of the syntheses. These are the same samples used for the HPLC traces in Fig. s1 that demonstrated the predominance of a single species. Together with the synthetic method, we conclude from the data in Fig. s1 and Table s1 that the samples were adequately pure and composed of the intended molecules.

Table s1. Names, sequences and analytical data for peptide containing molecules.

Compound	MW[a, b] Calc / found	HPLC RT[c] (min)	Sequence
Bombesin (BBN)	1620.9 / ND	ND	Pyr-QRLGNQWAVGHLM-NH_2
IR800-G-Abz4-t-BBN	2100.77 / 2100.80	5.45	IR800-G-Abz4-QWAVGHLM-NH_2
IR800-GGG -t-BBN	2095.8 / 2095.84	4.36	IR800-GGG-QWAVGHLM-NH_2
IR800-GSG -t-BBN	2125.79 / 2125.76	5.22	IR800-GSG-QWAVGHLM-NH_2
HN1	1320.5 / 1320.61	3.90	TSPLNIHNGQKL-NH_2

[a] By MALDI-TOF. [b] ND, not determined on commercial BBN. [c] All synthesized peptides were > 95% by relative peak area. Maldi-Tof mass spectra were performed on a Bruker ultrafleXtreme™ (Bruker, Breman, Germany) mass spectrometer operated in reflection, positive ion mode with a N_2 smartbeam II™ laser. Laser power was used at the threshold level required to generate signal. Accelerating voltage was set to 28 kV. The instrument was calibrated with the Peptide Calibration Standard II (Bruker Daltonics) covering mass range ~700 Da - 3200 Da. α–cyano-4-hydroxy-cinnamic acid was used as the matrix, prepared as a saturated solution in 50% ACN/ 0.1% TFA (in water). Allotments of 1 μL of matrix and 1 μL of sample were thoroughly mixed; 0.5 μL of this was spotted on the target plate and allowed to dry.

Results

Animal Imaging

Nude male mice, four to six weeks old, CrTac:NCr-$_{Foxn1}^{nu}$ homozygous (Taconic Farms Inc.) with or without PC-3 xenografts were injected while conscious via tail vein with 5 – 10 nmol of test compound in 100 μL of injection buffer (5% ascorbic acid containing 0.025% w/v EDTA, 0.2 mg/mL selenomethionine, and 0.2% human serum albumin in phosphate buffered saline (PBS) adjusted to pH 5.8 with NaOH). Animals were observed from 1 - 5 h post injection of compounds and again at 24 h post injection. Other than bladder accumulation the whole body images out to 5 h were little different from one another. The Fig s2-3 show evidence of high intensity bladder at both early and 24 h post administration. The bladder could often be seen in both dorsal and ventral images, but more prominently in ventral images. Evidence of excretion of what was seen in bladder is shown in Fig. s2 Left, taken with the Fluobeam imager. No significant signal was observed in animals that had no fluorescent dye injection.

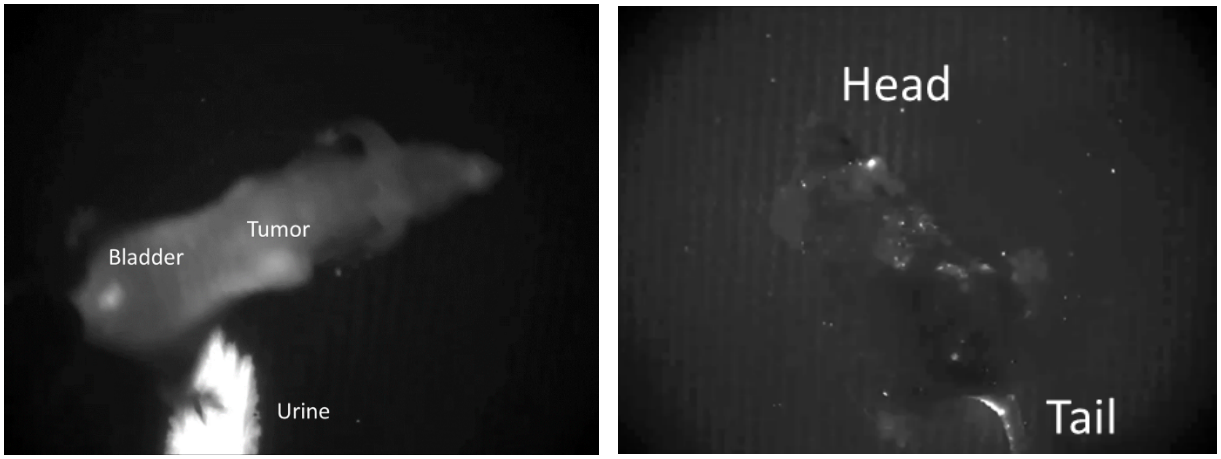


Fig s2. Left: IR800CW-G-t-BBN 10 nmol dose in a 24 h post administration PC-3 tumor mouse using Fluobeam. This is a dorsal image (a single frame of a video) of a living, unanaesthetized mouse. The tumor, bladder and urine excreted onto the imaging pad indicate the presence of the fluorescent label. Right: Some residual fluorescence in skin is obvious when compared to the image on the right. Right: Untreated live mouse housed 24 h with treated mice post administration of the IR800-G-Abz4-t-BBN. This demonstrates that residual fluorescent drug in images in Fig s2 is not external.

We took the whole body images shown in the paper after skinning the mice to lessen the background signal from skin.

Images in Fig s3 further demonstrate accumulation in bladder of IR800-G-t-BBN. At 24 h many mice showed evidence of probable coprophagy with bright fecal pellets and gastrointestinal tracks.

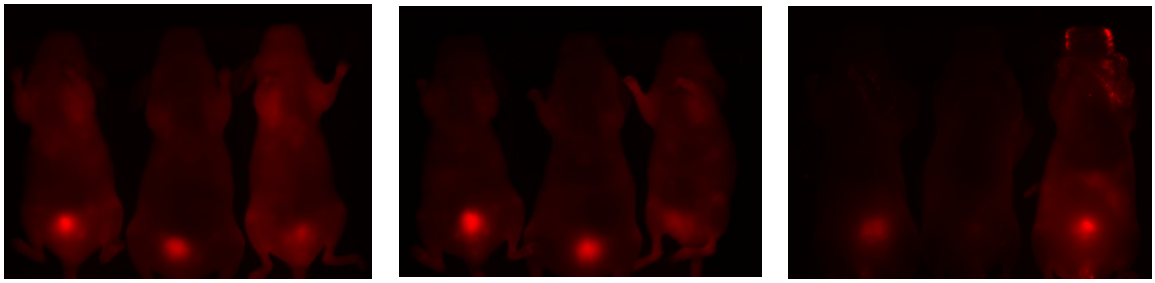


Fig s3. Left to right CRi images at 1, 4, and 24 h post administration of IR800-G-t-BBN.

In an initial investigation we sought to investigate the uptake differences of the strongest and weakest GRP-R binding peptides, the Abz4- and GGG-linked peptides, respectively. We injected 5 – 10 nmol of the compounds into non tumor bearing mice as described in the Material and Methods section of the paper. We compared the pancreas and other tissues because it is known that pancreas contains a high quantity of receptor. There is probably some excretion via the hepatobiliary route as liver is brighter than spleen. The gastrointestinal tract is expected to be bright through

copraphagy after 24 h, akthough we did not prove that this was the source. All animals were fed with chlorophyl free chow.

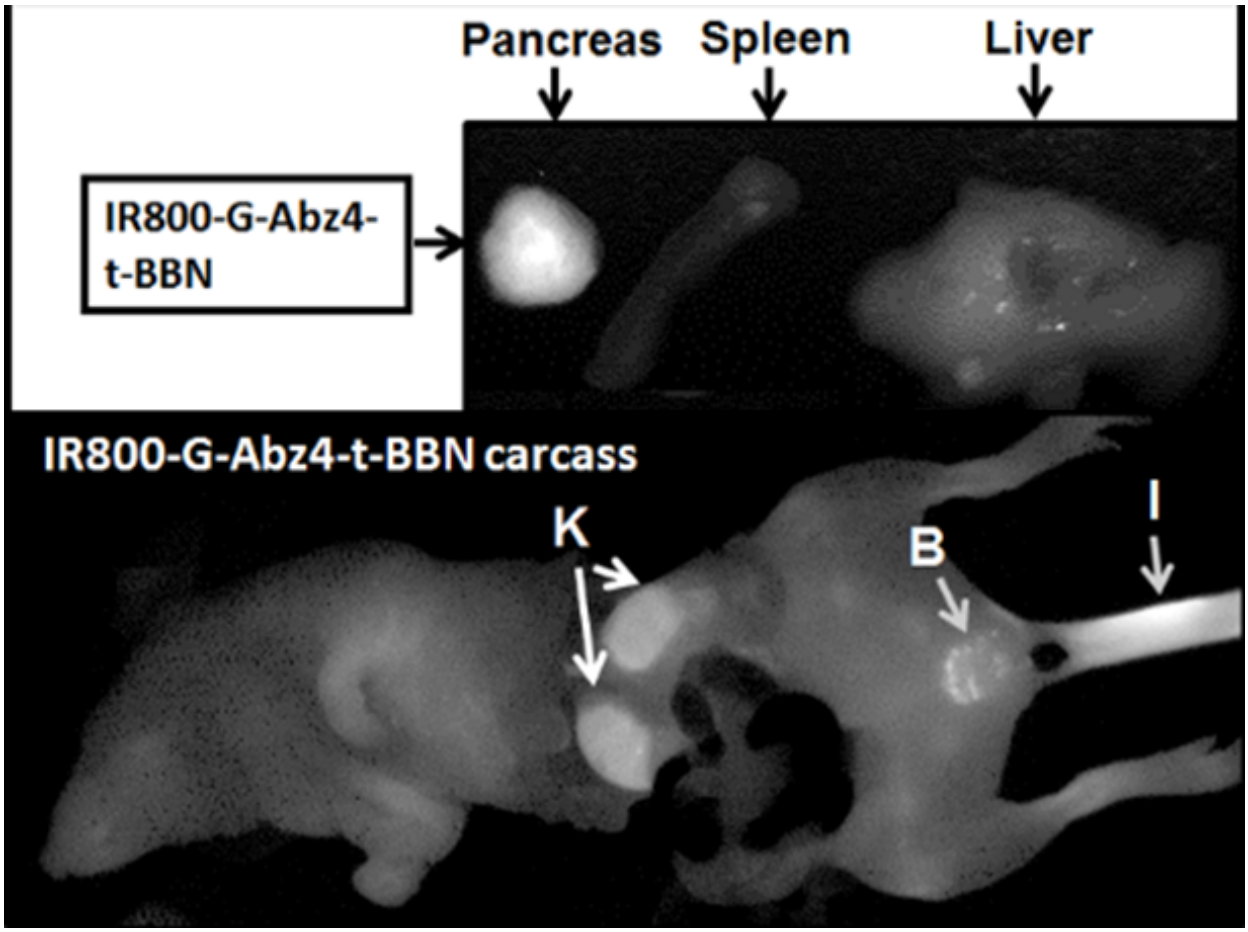


Fig. s4 IR800CW-G-Abz4-t-BBN concentrates in pancreas and kidney in nude mice and does not concentrate in liver or spleen. The dose was 10 nmol and the time of euthanasia was 24 h. The abdomen has been opened to expose the organs that would otherwise be covered by skin and far less visible. The carcass image is not the same size as the pancreas, liver and spleen images. K = kidneys, B = bladder, I=injection site. The very bright gastrointestinal track was unmixed from the image using the CRi software. CRi allows multiple colors to be used interchangeably. We used white here in this early image but used red in the paper to differentiate CRi and Fluobeam images easily.

The pilot experiment in Fig s5 was run in non tumor bearing animals to support the hypothesis that the weaker and stronger binding fluorescent labeled peptides would show weaker and stronger binding to the organ known to express the highest concentration of GRP-R, the pancreas. Muscle was chosen as an internal control and samples created the same size. After seeing an apparent difference we justified the preparation of tumor bearing animals for further study.

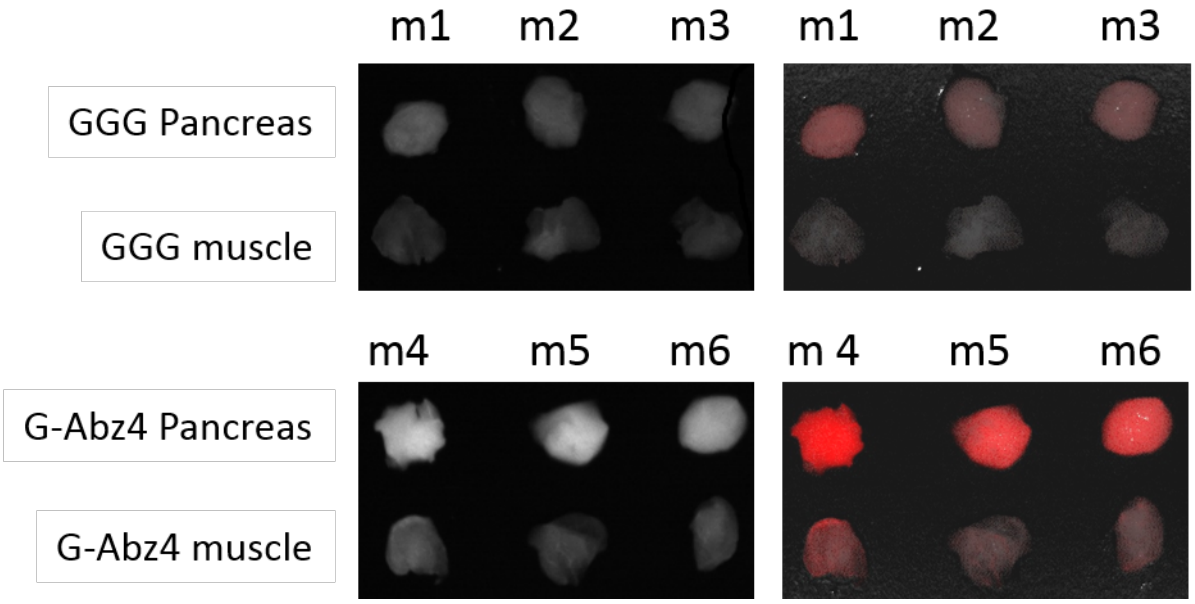


Fig s5. Muscle and pancreas data from three non tumor bearing mice 3 h post administration of 10 nmol of peptide.

Fig s6 adds two further animals to the tumor bearing animal ex vivo imaging study whose partial results are depicted in Fig. 5.

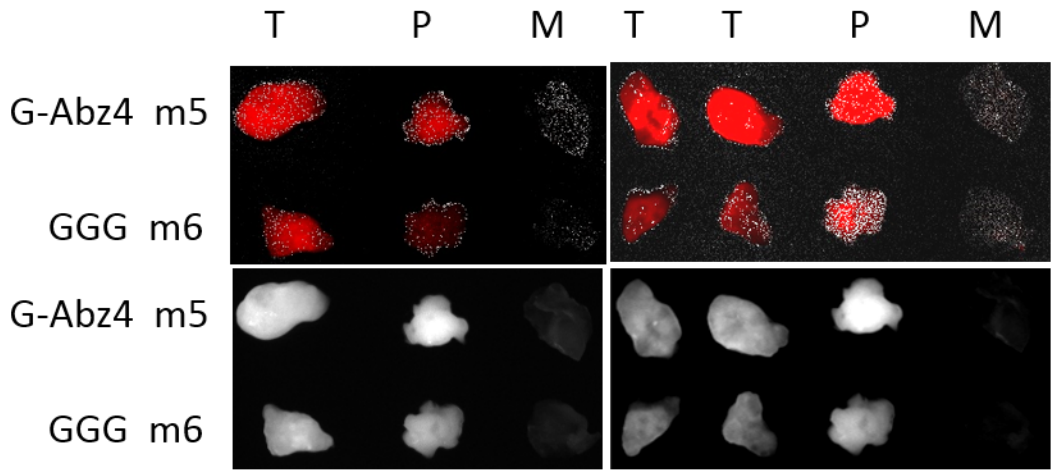
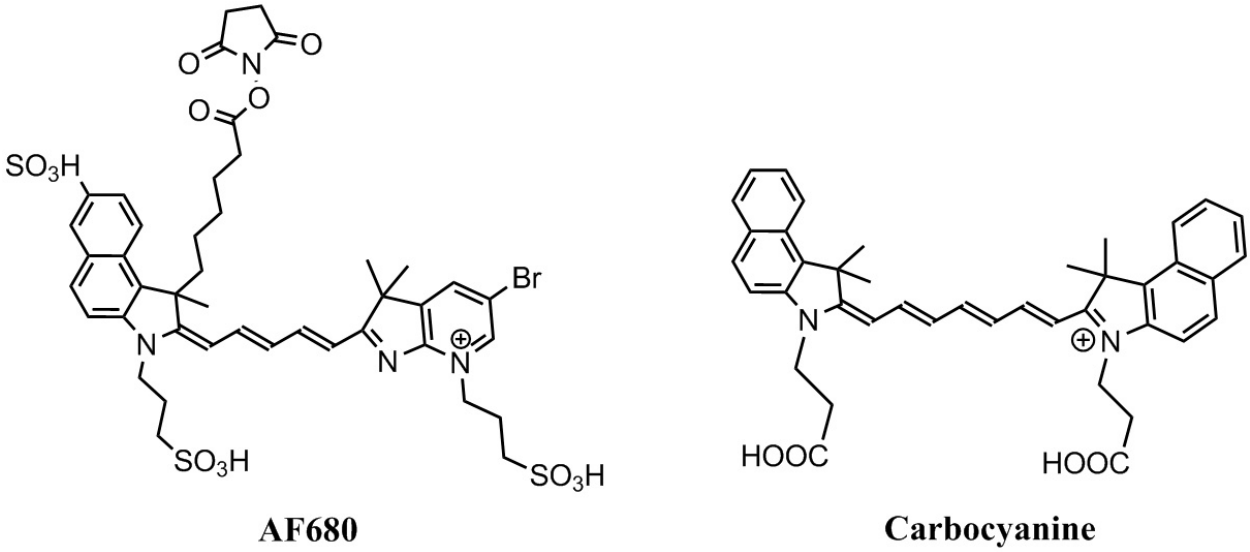


Fig. s6. Two additional animals from the receptor blocking exeriment shown in Fig 5.

Discussion

We discuss the differences in behavior of the two literature fluorescent dyes targeted to GRP-R. The structure of these two cyanine dyes is shown Fig s7. [1-3]



AF680 **Carbocyanine**

Fig. s7. Chemical structures of two cyanine dyes used in labeled peptides in Table 1 and discussed in the paper.

1. Achilefu S, Jimenez HN, Dorshow RB, et al. (2002) Synthesis, in vitro receptor binding, and in vivo evaluation of fluorescein and carbocyanine peptide-based optical contrast agents. J Med Chem 45:2003-2015.
2. Ma L, Yu P, Veerendra B, et al. (2007) In vitro and in vivo evaluation of Alexa Fluor 680-bombesin[7-14]NH2 peptide conjugate, a high-affinity fluorescent probe with high selectivity for the gastrin-releasing peptide receptor. Mol Imaging 6:171-180.
3. Cai QY, Yu P, Besch-Williford C, et al. (2013) Near-infrared fluorescence imaging of gastrin releasing peptide receptor targeting in prostate cancer lymph node metastases. Prostate 73:842-854.