Supplemental Methods

Cell Culture

Supplemental Table 1. Overview of the human derived BC cell lines with corresponding molecular classification, ER-, PR-, HER2-status and GRP-R mRNA level. Cell lines were obtained from the department of Medical Oncology, Erasmus MC (Rotterdam, The Netherlands).

Cell lines	Molecular classification	ER	PR	HER2	mRNA GRP-R level
SUM44PE	Luminal	+	+	-	0.0183
MCF7	Luminal	+	+	-	0.0117
T47D	Luminal	+	+	-	0.0305
UACC812	Luminal	+	+	++	0.0065
BT474	Luminal	+	+	++	0.0492
CAMA-1	Luminal	+	-	-	0.0009
SUM52PE	Luminal	+	-	-	0.0241
HCC1806	Triple negative	-	-	-	0.0001
Hs578t	Triple negative	-	-	-	0.0017

Table was based on Riaz et al. (1); ER = Estrogen receptor, PR = Progesterone receptor, HER2 = Human epidermal growth factor receptor 2, GRP-R = Gastrin releasing peptide receptor.

Internalization assay

Cells were seeded in 12-well plates, 300,000 cells/well/1 mL. The next day adhering cells were washed with PBS (GIBCO/Life Technologies) and incubated with 10^{-9} M ^{111}In-AMBA (without or with 10^{-6} M Tyr^4-bombesin) in internalization medium (RPMI1640, 20 mM HEPES and 1% bovine serum albumin (Sigma-Aldrich) for 1 h at 37˚C. Cellular uptake was stopped by removing the supernatant and washing twice with cold PBS. The membrane-bound radiopeptide fraction was separated by 10 min treatment with acid solution (50 mM Glycine and 100 mM NaCl, pH=2.8) and collected. The residual cell fractions were lysed

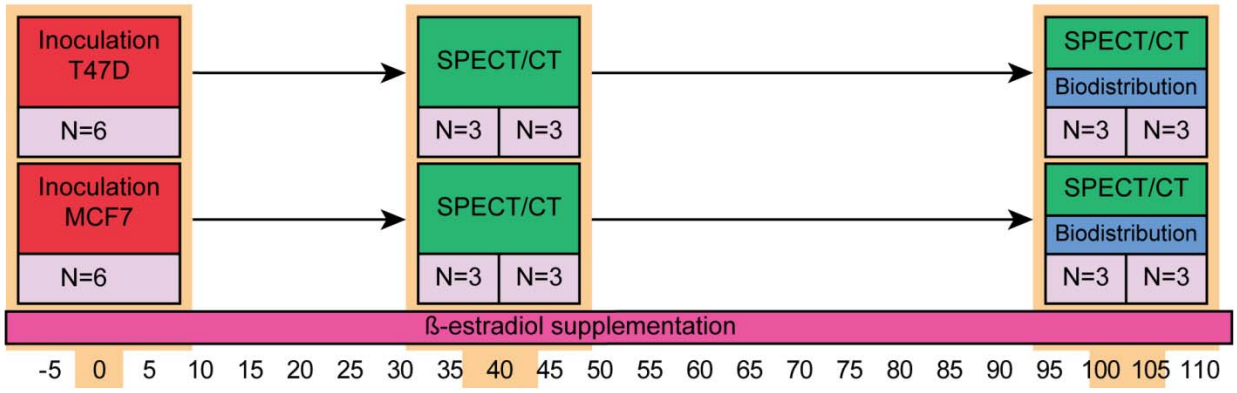
with 0.1 M NaOH. Membrane bound and internalized fractions were counted in a γ-counter (1480 WIZARD automatic gamma counter, PerkinElmer). Counting time was 60 sec per sample with an isotope-specific energy window and a counting error not exceeding 5%.

SRB protocol

Fixed cells were stained for 20 min at room temperature using 500 µL of 0.5% sulforhodamine B (SRB) (Sigma-Aldrich) dissolved in 1% acetic acid (Merck Millipore). After repeated washing with 1% acetic acid, plates were left to dry at 37˚C. Bound SRB was released by adding 1 mL 10 mM tris-(hydroxymethyl)aminomethane. The absorbance was measured at 510 nm using the Wallac Victor 1420 (PerkinElmer).

In-vivo NanoSPECT/CT imaging of BC xenograft-bearing mice with GRP-R radioligands

Multi-pinhole mouse collimators with 9 pinholes (1.4 mm diameter) per head were used, acquisition was performed with a matrix of 256x256 and 20 projections (90 sec/projection). Reconstruction and quantification was performed using In-vivo Scope software 2.0. Bladder uptake was masked. Tumors and organs were counted in a γ-counter (PerkinElmer), after decay of radioactivity (13 d) to avoid dead time artifacts. Counting time was 60 sec per sample with an isotope-specific energy window and a counting error not exceeding 5%.

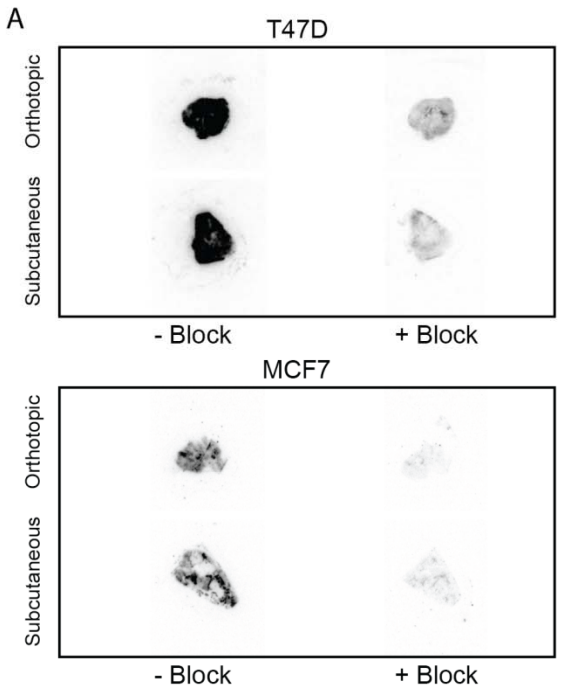


Supplemental Figure 1. Time line of in-vivo experiments. Seven days before orthotopic and subcutaneous tumor cell inoculation until the end of the experiment, animals were orally supplemented with ß-estradiol (4 mg/L). Animals were inoculated with either T47D or MCF7 BC cell lines subcutaneously and orthopically (in the 4^{th} left mammary fat pad). SPECT/CT scans were performed at two time points after inoculation t1 (40±3 d) and t2 (100±3 d), 4 h post injection of ~35 MBq/200 pmol ^{111}In-JMV4168 and 300 μg phosphoramidon. Following the second scan animals were euthanized, tumors and organs were collected and weighed. After decay of radioactivity (13 d) to avoid dead time artifacts, samples were counted in a gamma counter.

Statistics

Pearson's chi-squared exact test and a logistic regression were used to analyze the correlation between the extent of GRP-R expression and ER status of the human BC specimens. For correlation between ER, PR and HER2 status and internalization of ^{111}In-AMBA by the BC cell lines a univariate ANOVA test was used. mRNA GRP-R levels and internalization of ^{111}In-AMBA were correlated using Spearman's statistical test. A two tailed t-test was used to analyze the in-vitro cytotoxicity assay, and to determine differences in radiotracer uptake between T47D- and MCF7 xenografts and orthotopic and subcutaneous tumors.

Supplemental Results



B

T47D

	- Block (%AD) Average (±SD)	+ Block (%AD) Average (±SD)	Delta (%AD) Average (±SD)
Orthotopic	2.3 (±0.3)	0.1 (±0.1)	2.2 (±0.4)
Subcutaneous	2.0 (±0.1)	0.1 (±0.0)	1.9 (±0.1)

MCF7

	- Block (%AD) Average (±SD)	+ Block (%AD) Average (±SD)	Delta (%AD) Average (±SD)
Orthotopic	0.9 (±0.3)	0.1 (±0.1)	0.8 (±0.2)
Subcutaneous	0.7 (±0.2)	0.1 (±0.0)	0.7 (±0.1)

Supplemental Figure 2. A) In-vitro autoradiography of T47D and MCF7 xenografts displayed in figure 4 of the article. Tumors were excised, snap frozen and counted in the γ-counter for biodistribution purposes. When radioactivity had completely decayed, 10 µm tumor slices were incubated with 10^{-9} M ^{111}In-JMV4168, without (-block) or with (+block) 10^{-6} M of unlabeled Tyr^4-bombesin for specificity control. B) Quantification of the autoradiography results calculated as percentage of added dose (%AD). Results were quantified using OptiQuant Software (Perkin Elmer) and expressed as arbitrary digital light units/mm^2. Autoradiography results confirmed higher binding in T47D xenografts (orthotopic: 2.2±4%AD, subcutaneous: 1.9±0.1%AD) compared to MCF7 xenografts (orthotopic: 0.8±0.2%AD, subcutaneous: 0.7±0.1%AD). Binding of ^{111}In-JMV4168 was specific since low or no binding was observed when GRP-R was blocked by adding an excess of Tyr^4-bombesin (0.1±0.1% for orthotopic tumors and 0.1±0.0% for subcutaneous tumors).

1. Riaz M, van Jaarsveld MT, Hollestelle A, et al. miRNA expression profiling of 51 human breast cancer cell lines reveals subtype and driver mutation-specific miRNAs. *Breast Cancer Res.* 2013;15:R33.