Onco genes and Tumor Sup pressors

GPER-Targeted, 99mTc-Labeled, Nonsteroidal Ligands Demonstrate Selective Tumor Imaging and In Vivo Estrogen Binding

Tapan K. Nayak1,2, Chinnasamy Ramesh3, Helen J. Hathaway1,4, Jeffrey P. Norenberg2,4, Jeffrey B. Arterburn3,4, and Eric R. Prossnitz1,4

Abstract

Our understanding of estrogen (17β-estradiol, E2) receptor biology has evolved in recent years with the discovery and characterization of a 7-transmembrane-spanning G protein–coupled estrogen receptor (GPER/GPR30) and the development of GPER-selective functional chemical probes. GPER is highly expressed in certain breast, endometrial, and ovarian cancers, establishing the importance of noninvasive methods to evaluate GPER expression in vivo. Here, we developed 99mTc-labeled GPER ligands to demonstrate the in vivo status of GPER as an estrogen receptor (ER) and for GPER visualization in whole animals. A series of 99mTc(I)-labeled nonsteroidal tetrahydro-3H-cyclopenta[c]quinolone derivatives was synthesized utilizing pyridin-2-yl hydrazine and picolylamine chelates. Radioligand receptor binding studies revealed binding affinities in the 10 to 30 nmol/L range. Cell signaling assays previously demonstrated that derivatives retaining a ketone functionality displayed agonist properties, whereas those lacking such a hydrogen bond acceptor were antagonists. In vivo biodistribution and imaging studies performed on mice bearing human endometrial and breast cancer cell xenografts yielded significant tumor uptake (0.4–1.1%ID/g). Blocking studies revealed specific uptake in multiple organs (adrenals, uterus, and mammary tissue), as well as tumor uptake with similar levels of competition by E2 and G-1, a GPER-selective agonist. In conclusion, we synthesized and evaluated a series of first-generation 99mTc-labeled GPER-specific radioligands, demonstrating GPER as an estrogen-binding receptor for the first time in vivo using competitive binding principles, and establishing the utility of such ligands as tumor imaging agents. These results warrant further investigation into the role of GPER in estrogen-mediated carcinogenesis and as a target for diagnostic/therapeutic/image-guided drug delivery.

Implications: These studies provide a molecular basis to evaluate GPER expression and function as an ER through in vivo imaging. Mol Cancer Res; 12(11); 1635–43. ©2014 AACR.

Introduction

Estrogens mediate profound effects throughout the body and regulate physiologic and pathologic processes in both women and men. The lower prevalence of many diseases in premenopausal women compared with postmenopausal women or age-matched men is widely attributed to the presence of 17β-estradiol (E2), the predominant and most potent endogenous estrogen (1–3). E2, although commonly recognized as the female sex hormone, also has critical roles in additional normal physiologic processes within the nervous, immune, vascular, muscular, skeletal, and endocrine systems (2, 4–8). In addition, E2 signaling plays an important role in various pathologic conditions and disorders, including cancer, cardiovascular diseases, hypertension, osteoporosis, cognitive and behavioral alterations, neurodegenerative diseases, as well as metabolic and immune disorders (2, 4–8). However, elucidating the exact role(s) of E2 in these processes is often complicated by the existence of several types of E2 receptors (ERs) and multiple modes of cellular signaling mechanisms that span time frames from seconds to hours, or even days (6, 9). The actions of E2 have traditionally been ascribed to one of the two closely related classical nuclear hormone receptors, ERα and ERβ, which are best characterized for regulating gene expression (10, 11), and their membrane-localized...
variants. Recent studies have revealed the contribution of a novel G protein-coupled estrogen receptor GPER (previously GPR30), which belongs to the family of seven-transmembrane G protein-coupled receptors, to many of the rapid cellular and biologic responses to E2 (6, 12–14). GPER is expressed in numerous tissues and the scope of research into its many functions has increased dramatically over the last decade (15–23); nevertheless, isolated reports have failed to observe GPER-mediated estrogenic responses in the uterus or the mammary gland or GPER-dependent estrogen binding in cell-based systems (24–26).

GPER protein is (over) expressed in approximately 50% of all breast cancers and correlates with clinical and pathologic biomarkers of poor outcome, such as tumor size and the presence of metastases, regardless of ER status (27). Additional studies have found that GPER protein is overexpressed in ovarian cancer where it is associated with lower survival rates (28, 29). Similarly, GPER is overexpressed in tumors where E2 and progesterone receptors are downregulated and in high-risk endometrial cancer patients with lower survival rates (30, 31). GPER is also widely expressed in cancer cell lines isolated from diverse organs as well as primary tumors of the thyroid, lung, prostate, pancreas, and testicular germ cells, in addition to the breast, endometrium, and ovaries (6, 12, 32–34).

Importantly, in patients with breast cancer treated only with tamoxifen, GPER protein expression increased and survival was markedly reduced in patients with initial GPER-positive tumors, suggesting that patients with breast cancer who have high GPER protein expression should not be treated exclusively with tamoxifen (35). Cellular effects of tamoxifen via GPER were further demonstrated through tamoxifen-mediated stimulation of tumor cell proliferation and migration (36–38). Thus, although anti-estrogens such as tamoxifen, fulvestrant, and raloxifene function as ER α/β antagonists, they act as GPER agonists (39–42), stimulating proliferation and other cellular activities via the GPER-mediated transactivation of EGFR (43).

Several radiopharmaceuticals have been developed for the noninvasive imaging and assessment of ER status (44–48). The most successful E2 radiopharmaceutical 160-[18F]fluorostriadiol-17β (FES) is under clinical investigation and to date has produced promising results in PET imaging of ER-expressing tumors, in particular for the evaluation of responsiveness of breast tumors to anti-estrogen drugs such as tamoxifen (47, 49, 50). However, FES binds to all subtypes of the classical ERs and furthermore does not differentiate between classical ERs and GPER. Therefore, to identify potentially aggressive forms of cancer, as well as for risk stratification of patients about to undergo endocrine therapy, the assessment of GPER status is likely to be vital.

Therefore, with the ultimate goal of noninvasive assessment of GPER expression in patients, we developed a series of nonsteroidal 99mTc-labeled agents based on the previously reported GPER-selective agonist G-1 and GPER antagonists G15 and G36 (51–53). Using competitive binding and radiotracer principles, the primary objectives of this study were to characterize GPER-selective 99mTc-labeled agents for demonstrating the status of GPER as an ER in vitro and in vivo. In vivo biodistribution and competition binding studies with E2 and G-1 were performed in mice bearing ERα/β-negative and GPER-expressing type II human endometrial carcinoma HeC50 tumors and ERα/β- and GPER-positive human breast adenocarcinoma MCF7/HER2-18 tumors. Our results demonstrate not only that GPER functions in vivo as an E2-binding receptor, but also that GPER-selective 99mTc-labeled ligands can be used to visualize breast and endometrial tumors in vivo.

Materials and Methods

Chemical synthesis of nonsteroidal GPER-specific ligands

Synthetic derivatives of the GPER-targeting tetrahydro-3H-cyclopenta[e]quinoline scaffold possessing different chelating heterocyclic aminocarboxylate ligands with demonstrated capacity for the formation of neutral tricarbonylrhenium(I) and tricarbonyltechnetium(I) complexes at the C8 position were prepared as previously described (54). Ligand compounds containing a pyridin-2-yl-hydrazineylthioanic acid group (1, 3, 4), or pyridin-2-yl-methylaminothioanic acid (2), were prepared as the tert-butyl esters with nitrogen groups protected as tert-butoxycarbonyl derivatives, and deprotected with trifluoroacetic acid in dichloromethane at ambient temperature before labeling. The nonradioactive tricarbonylrhenium(I) complexes (5-Re - 8-Re) were prepared as previously described (54).

Radiosynthesis of 99mTc(I)-labeled nonsteroidal GPER-specific ligands

The organometallic aqua ion labeling agent [99mTc(CO)3(H2O)3]+ was prepared by adding 3.7 GBq of freshly eluted Na+99mTcO4 to the Isolink kit (Tyco healthcare, Mallinckrodt) as previously described (55). The alkaline [99mTc(CO)3(H2O)3]+ mixture was then neutralized to pH 7 with acetic acid. The synthetic chelates 1–4 were dissolved in ethanol and 10 μg of each derivative was added to the prepared [99mTc(CO)3(H2O)3]+ mixture. The reaction mixture was stirred for 2 hours at room temperature. Alternatively, the mixture was heated to 80°C for 30 minutes as an alternative rapid radiosynthesis approach. Inorganic impurities from the Isolink kit, aqua ions of 99mTc (if any) and excess ligand were separated using solid phase extraction (SPE). SPE was performed using C-18 SepPak Plus cartridges (Waters). The impurities and excess ligand were eluted with 4 × 0.5 mL fractions of the weak solvent (50% ethanol in water). Elution of the final nonsteroidal GPER-specific 99mTc-labeled product was performed with 4 × 0.5 mL fractions of the strong solvent (100% ethanol). High-performance liquid chromatography (HPLC) was performed to assess radiochemical purity and specific activity. To assess radiochemical purity and specific activity, 10 μL of the final product was diluted in 200 μL of HPLC grade ethanol (JT Baker), and 10 μL of the diluted sample was injected on a reverse-phase C-18 column.
(JT Baker) using HPLC grade ethanol and HPLC grade water as previously described (55). Stability, transfection, and partition coefficient studies were performed as previously described (55).

Cell culture and receptor binding studies
ERα/β-negative/GPER-positive human endometrial carcinoma Hec50 cells (42, 56) and ERα/β-positive/GPER-positive human breast adenocarcinoma MCF7/HER2-18 cells (57) were cultured in DMEM medium supplemented with FBS (10%), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown as a monolayer at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Competition binding studies of the corresponding Re-labeled agents were performed using an 125I-labeled GPER-specific radioligand 1-[6-[4-(2-ethyl-1H-cyclopenta[c]quinolin-8-yl)-ethoxy]oxy]-3-(3-iodo(125)phenyl)-urea (8 in ref. 58) as previously described (58).

Animal and tumor models
GPER-targeted 99mTc-labeled agents were evaluated in mice bearing Hec50 or MCF7/HER2-18 tumors. Hec50 cell tumors were generated by injecting 3 to 4 million Hec50 cells subcutaneously in 8-week-old female ovariectomized athymic, Crl:Nu/Nu- nuBR "athymic nude" mice (Harlan Inc.). MCF7/HER2-18 tumor models were similarly generated by injecting 4 to 6 million cells subcutaneously. One day before injection of cells, a 60-day release E2 (1.7 mg) pellet (Innovative Research of America) was implanted subcutaneously. After 8 to 10 weeks, palpable tumors were observed and the pellet was removed. After a 1-week recovery period, biodistribution and imaging studies were performed. All protocols were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center (Albuquerque, NM).

Histologic staining of tumors
Sections (5 µm) from paraffin-embedded approximately 10-week Hec50 cell tumors were prepared for IHC using an affinity purified polyclonal antibody raised against the carboxy-terminus of GPER as previously described (31). Briefly, sections were deparaffinized in CitriSolv (Fisher), followed by rehydration in increasing H2O:ethanol solutions. Antigen retrieval was carried out by microwaving slides in 0.01 mol/L sodium citrate buffer (pH 6.0) for 25 minutes, followed by incubation in fresh 2% H2O2 for 10 minutes. Permeabilization and blocking were carried out by incubating the slides for 30 minutes in 200 µL 0.1% Triton X-100 in PBS containing 3% BSA in a humid chamber. Slides were then incubated with the GPER carboxy-terminal antibody diluted to a final protein concentration of 2 µg/mL in 3% normal goat serum for 1 hour. Following washes, bound antibody was detected using goat anti-rabbit IgG conjugated to horseradish peroxidase (diluted 1:250 in 3% normal goat serum, 45 minutes), which was detected with 3,3-diaminobenzidine tetrahydrochloride (Sigma).

Biodistribution and SPECT/CT imaging studies
Conscious tumor-bearing mice were injected in the tail vein with selected GPER-targeted 99mTc-labeled agents. To

<table>
<thead>
<tr>
<th>Table 1. Time-dependent uptake of 99mTc-G derivatives</th>
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<td>Brain</td>
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<td>Urinary bladder</td>
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<td>Hec50 tumor</td>
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*Receptor blocking studies were performed by coinjecting 5 µg G-1 with the radiotracer.
Figure 1. Chemical structures of GPER-targeted derivatives 1-4 and the corresponding metal (M) chelates 5-8. Synthetic derivatives possessing heterocyclic aminocarboxylate chelate ligands: pyridin-2-yl-hydrazinylethanoic acid (1,3,4); and pyridin-2-yl-methylaminoethanoic acid (2). Neutral complexes (5-Re - 8-Re) and (5-99mTc - 8-99mTc) isolated from labeling with Re\(^{3+}\)(CO)\(_3\) and 99mTc\(^{3+}\)(CO)\(_3\) in aqueous ethanol, respectively.

Statistical analysis

All numerical data were expressed as the mean of the values ± SEM. GraphPad Prism version 5 was used for statistical analysis and analysis of competitive binding data, which was performed using a single fit model. A P value ≤ 0.05 was considered statistically significant.

Results

Radiochemistry

The 99mTc- and Re-labeled derivatives of 1-4 (Fig. 1) were prepared using the tricarbonyl approach. The \([99mTc(CO)\(_3\)]\(^{3+}\)\) intermediate was prepared with a radiochemical purity of >95% and mixed with the corresponding chelating ligands and stirred at room temperature for 2 hours. Radiolabeling yields were high with more than 95% of the \([99mTc(CO)](H_2O)\(_3\)]\(^{3+}\) incorporated into the derivatives 1, 2 (Supplementary Fig. S1A and S1B, respectively), and 3 to generate 5-99mTc, 6-99mTc and 7-99mTc, and more than 85% incorporation into derivative 4 (Supplementary Fig. S1C) to produce 8-99mTc. On the basis of the elution profile of the 99mTc generator and the transient equilibrium between the parent and the daughter radionuclide, the calculated specific activity of the final products ranged from 22.8 to 41.6 TBq/mmol with radiochemical purities exceeding 95%. Typical radiochemical yields after purification ranged from 60% to 75%. Although the rate of radiolabeling using the \([99mTc(CO)](H_2O)\(_3\)]\(^{3+}\) intermediate was increased with heating at 80°C for 30 minutes, which allows access to radioligands with increased specific activity, HPLC analysis revealed the presence of trace degradation products and the radiochemical purity was less than 70% under these conditions. The higher radiolabeling yields and increased purity obtained using the room temperature procedure were advantageous for the subsequent in vivo studies.
Stability, transchelation, and partition coefficient studies

All of the $^{99m}$Tc-labeled derivatives demonstrated good stability (more than 95%) in mouse plasma and PBS buffer after incubation at 37°C for 24 hours. Overall, less than 10% transchelation was observed upon incubation with a solution of 1 mmol/L cysteine or 1 mmol/L histidine at 37°C for 24 hours. The radiogands $^{6}$-$^{99m}$Tc, $^{7}$-$^{99m}$Tc, and $^{8}$-$^{99m}$Tc exhibited log $P_{(o/w)}$ values of 4.6 ± 0.3, 5.0 ± 0.1, 4.9 ± 0.1, and 5.5 ± 0.1 (mean ± SEM from at least three determinations).

Radioigand receptor binding studies

Competition binding studies on the corresponding stable isotopic Re-labeled derivatives were performed on ER$\alpha$/β-negative and GPER-expressing human endometrial carcinoma HeC50 cells using an $^{125}$I-labeled GPER-specific derivative as the radiotracer (Fig. 2). The mean IC$\text{50}$ values of tricarbonylrhenium(I)-labeled derivatives $^{5}$-Re, $^{6}$-Re, $^{7}$-Re, and $^{8}$-Re, were 16.3, 11.3, 12.5, and 29.2 mmol/L, respectively (see Table 3 for summary).

Biodistribution in tumor-bearing animals

Biodistribution studies were performed in ovariectomized athymic (NCr) $\text{nu/nu}$ female mice bearing HeC50 and MCF7/HER2-18 cell tumor xenografts by injecting 1.5 MBq $^{99m}$Tc-labeled derivatives in the tail vein of conscious mice. The 13% to 16% and 15% to 18% ID/g liver uptake of $^{5}$-$^{99m}$Tc and $^{6}$-$^{99m}$Tc, respectively, were up to 1.5–2-fold lower than the 21% to 28% ID/g liver uptake of $^{7}$-$^{99m}$Tc (Table 1), despite the similar log $P$ values of $^{6}$-$^{99m}$Tc and $^{7}$-$^{99m}$Tc, suggesting a correlation between the presence of the ethanone moiety in the linker with decreased liver uptake. Three hours postinjection (PI), the 1.05% ID/g tumor uptake for $^{5}$-$^{99m}$Tc was more than 1.5–2-fold more...
than 0.40% ID/g tumor uptake for 7-99mTc and 0.69% ID/g for 6-99mTc. Overall, all three derivatives exhibited comparable receptor binding affinity and specificity, yet showed different uptake values for target and nontarget organs. At 24 hours PI, washout of the tracer was observed in tumor and nontarget organs except the liver (Supplementary Table S1).

All of the evaluated 99mTc-labeled derivatives demonstrated receptor specificity when GPER-blocking studies were performed by coinjecting 5 μg G-1 with the corresponding radiotracer (Fig. 3). GPER-mediated uptake was observed in the adrenals, uterus, mammary tissue, and the Hec50 and MCF7/HER2-18 tumors (Fig. 3; Table 2). Of the 99mTc-labeled derivatives evaluated, 7-99mTc exhibited the lowest target/blood ratio, whereas 5-99mTc exhibited the highest target/blood ratio (Table 3). However, all of the 99mTc-labeled derivatives evaluated were rapidly metabolized as the collected urine showed the presence of radiometabolites. At 1 hour PI, approximately 30% of 5-99mTc was found in the urine. The remaining radioactive species were hydrophilic radiometabolites (Supplementary Fig. S2A). At 2 hours PI, 5-99mTc was completely metabolized (Supplementary Fig. S2B). At 4 hours PI, most of the radiometabolites were excreted and the only radioactive species circulating in the blood on HPLC analysis of the plasma sample was the injected radiotracer (Supplementary Fig. S2C). Similarly, for 6-99mTc, at 2 hours PI most of the radioactive species were hydrophilic radiometabolites. The excretion of the three radiotracers ranged from 8% to 14% ID at 3 hours PI.

A detailed in vivo study of GPER ligand specificity was performed using 5-99mTc in which receptor binding was blocked by coinjecting 5 μg of either G-1 or E2. The 5-99mTc agent exhibited specific binding to target organs at 1, 2, and 3 hours PI, suggesting limited nonspecific uptake of a radiometabolite in the target organs (Fig. 3D). Furthermore, at 3 hours PI, the level of inhibition of 5-99mTc uptake was similar when either G-1 or E2 was coinjected, demonstrating 5-99mTc binding specificity (with respect to both E2 and G-1) to GPER in target organs such as mammary tissue and the uterus. This GPER selectivity was further demonstrated in ERα/β- and GPER-positive human breast adenocarcinoma MCF7/HER2-18 tumor-bearing mice (Table 2) where similar levels of blocking were observed with coinjection of either G-1 or E2 (Table 2), suggesting that binding sites for 5-99mTc were equally accessible to both G-1 and E2.

Imaging studies

Imaging studies were carried out after intravenous injection of 19 MBq 5-99mTc via the tail vein of Hec50 endometrial tumor-bearing mice. Whole body SPECT/CT (60 s/projection) imaging studies, carried out under 1.5% to 1.7% isoflurane using a temperature-controlled bed (36–38°C), revealed high liver, gall bladder, and intestine uptake. To better visualize the tumor, a limited field of view SPECT image (200 s/projection) was obtained from live mice. The GPER-expressing tumor, which shows intense intracellular expression of GPER (Fig. 4A), was clearly visualized at 3 hours (Fig. 4B). Image quantification revealed no significant washout of the tracer from the tumor for up to 6 hours PI (1.10 ± 0.27% ID at 3 hours PI vs. 1.23 ± 0.16% ID at 6 hours PI). More than 70% ID was in the liver, gall bladder, and the intestines at 3 and 6 hours PI.

Discussion

Roles for the membrane-bound GPER in resistance to endocrine therapy and aggressive forms of breast, ovarian, and endometrial carcinoma have been reported (6, 29, 31, 35, 59, 60). The GPER-selective ligands G-1, G15, and G36 have been extensively used (with >180 publications) to elucidate the roles and functions of this receptor in normal physiology and pathology (6, 37, 39, 52). Although consensus supports the involvement of GPER in many estrogenic responses, a small number of conflicting reports have suggested that GPER may not function as an ER in vivo or in vitro (24, 26, 61). To further address the functions of GPER in vivo, we developed radiolabeled GPER-specific agents and performed in vivo competition binding studies using radiotracer principles to demonstrate the expression profile of GPER in various tissues and in different ERα/β- and/or GPER-expressing tumor xenograft cancer models. We also evaluated whether these radiolabeled GPER-specific agents can be used for noninvasive GPER visualization of tumors.

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**Table 3.** Comparison of selected chemical and biologic characteristics of 99mTc-labeled GPER-targeted agents

<table>
<thead>
<tr>
<th>GPER-targeted agent</th>
<th>LogP&lt;sub&gt;o/w&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Tumor/blood (3-h PI)</th>
<th>Tumor uptake (% ID/g, 3-h PI)</th>
<th>Liver uptake (% ID/g, 3-h PI)</th>
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<tbody>
<tr>
<td>5-99mTc</td>
<td>4.6 ± 0.3</td>
<td>16 (8.6–31)</td>
<td>0.76</td>
<td>1.05 ± 0.09</td>
<td>13.38 ± 0.60</td>
</tr>
<tr>
<td>6-99mTc</td>
<td>5.0 ± 0.1</td>
<td>11 (6.4–20)</td>
<td>0.59</td>
<td>0.69 ± 0.09</td>
<td>16.91 ± 2.97</td>
</tr>
<tr>
<td>7-99mTc</td>
<td>4.9 ± 0.1</td>
<td>13 (6.9–23)</td>
<td>0.24</td>
<td>0.40 ± 0.03</td>
<td>27.95 ± 0.91</td>
</tr>
<tr>
<td>8-99mTc</td>
<td>5.5 ± 0.1</td>
<td>29 (18–48)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

<sup>a</sup>Determined by competition binding with the respective Re complex. Values represent best-fit values using a one-site competition analysis with 95% confidence intervals provided in parentheses.
On the basis of our previous studies, we had demonstrated the need for an uncharged neutral agent for intracellular targeting of GPER (62, 63); therefore, we synthesized a series of uncharged neutral GPER-selective agents based on organometallic rhenium(I)/technetium(I) tricarbonyl chemistry (54). Radioligand receptor-binding studies revealed binding affinities in the 10 to 30 nmol/L range, demonstrating minimal to moderate loss of affinity relative to the parent molecules G-1 and G15. Cellular signaling assays previously demonstrated that derivatives retaining a hydrogen bond acceptor ketone functionality in the linker displayed agonist properties whereas those lacking this moiety were antagonists (54). In vivo biodistribution studies revealed further differences associated with the structures of the linkages; the radiolabeled conjugate 7-99mTc with a flexible hydrophobic ethane linkage displayed greater liver uptake and lower tumor uptake than radiolabeled derivatives with an ethane linkage (5-99mTc and 6-99mTc).

In vivo competition studies with excess G-1 and E2 in ERα/β-negative and GPER-positive human endometrial carcinoma Hec50 tumors as well as in ERα/β- and GPER-positive human breast adenocarcinoma MCF7/HER2-18 tumors demonstrated GPER-specific uptake in the mammary gland, uterus, and tumor, thus demonstrating for the first time the status of GPER as an E2-binding receptor in vivo. Although liver and intestine uptake values were high, selective tumor targeting was achieved. SPECT/CT imaging further demonstrated tumor localization and retention of the 99mTc-labeled G derivatives for up to 6 hours postinjection. GPER expression patterns within primary and metastatic breast cancers could play an important diagnostic role because GPER is expressed in approximately 50% of breast cancers, regardless of ER status. GPER overexpression is also correlated with tumor size, Her2 status, and the presence of metastases (27) as well as hormone therapy resistance (35, 60) and recurrence in triple-negative breast cancer (64). In addition, with the recent report that ER-negative Hec50 endometrial tumors respond to E2-stimulation in vivo with enhanced tumor growth via GPER, as evidenced by the inhibition of E2-stimulated tumor growth by the GPER-selective antagonist G36 (42) as well as the poorer outcome for patients with GPER-positive endometrial cancers (31), noninvasive assessment of GPER expression in tumors is also likely to become a critical factor in the diagnosis and selection of potential treatment options in endometrial cancers. In addition to its importance in breast and endometrial cancers, GPER expression has also been associated with poor survival, or recognized as a diagnostic biomarker, in ovarian (29), pancreatic (34), lung (65), testicular germ cell (66), and other cancers.

In conclusion, we have synthesized and evaluated a series of first generation 99mTc-labeled GPER-specific radionuclide imaging agents and demonstrated for the first time the status of GPER as an E2-binding receptor in vivo, using competitive radioligand binding principles with both the endogenous hormone E2 as well as a synthetic GPER-selective ligand G-1. Although we chose 99mTc as the radionuclide for the development GPER-targeted agents due to the widespread availability of this isotope for clinical radiopharmacy, with further structural optimization, 18F- and 11C-labeled GPER-targeted agents could be developed with potentially improved biodistribution and imaging characteristics. Taken together, these studies advance investigations into the roles of GPER in E2-mediated carcinogenesis (67) as well as its clinical application as both a diagnostic and therapeutic target in breast, endometrial, and other cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: T.K. Nayak, H.J. Hathaway, J.P. Norenberg, J.B. Arterburn, E.R. Prossnitz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T.K. Nayak, C. Ramesh, J.P. Norenberg
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.K. Nayak, H.J. Hathaway, J.P. Norenberg, J.B. Arterburn, E.R. Prossnitz
Writing, review, and/or revision of the manuscript: T.K. Nayak, H.J. Hathaway, J.P. Norenberg, J.B. Arterburn, E.R. Prossnitz
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Ramesh, J.P. Norenberg, E.R. Prossnitz
Study supervision: J.P. Norenberg, J.B. Arterburn, E.R. Prossnitz
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