Bioorganic & Medicinal Chemistry Letters 23 (2013) 687–692

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Heterobivalent dual-target probe for targeting GRP and Y1 receptors on tumor cells

Ajay Shrivastava [†], Shu-Huei Wang [†], Natarajan Raju [†,‡], Izabela Gierach [§], Haiming Ding, Michael F. Tweedle [*]

Department of Radiology, The Ohio State University, Columbus, OH 43210, United States

ARTICLE INFO

Article history:
Received 17 October 2012
Revised 20 November 2012
Accepted 26 November 2012
Available online 5 December 2012

Keywords:
Heterobivalent dual-target probe
Gastrin-releasing peptide receptor
Y1 receptor
Neuropeptide Y
Imaging probe
DO3A
AMBA
BVD15
Hormone peptide
Bombesin
Neuromedin B
Breast cancer
Prostate cancer

ABSTRACT

Receptor targeting ligands for imaging and/or therapy of cancer are limited by heterogeneity of receptor expression by tumor cells, both inter-patient and intra-patient. It is often more important for imaging agents to identify local and distant spread of disease than it is to identify a specific receptor presence. Two natural hormone peptide receptors, GRPR and Y1, are specifically interesting because expression of GRPR, Y1 or both is up-regulated in most breast cancers. We describe here the design and development of a new heterobivalent peptide ligand, truncated bombesin (t-BBN)/BVD15-DO3A, for dual-targeting of GRPR and Y1, and validation of its dual binding capability. Such a probe should be useful in imaging cells, tissues and tumors that are GRPR and/or Y1 positive and should target radioisotopes, for example, ^{68}Ga and/or ^{177}Lu, to more tumors cells than single GRPR or Y1 targeted probes. A GRP targeting ligand, J-G-Abz4-QWAVGHLM-NH_2 (J-G-Abz4-t-BBN), and an Y1 targeting ligand, INP-K[ε-J-(α-DO3A-ε-DGa)-K]-YRLRY-NH_2([ε-J-(α-DO3A-ε-DGa)-K]-BVD-15), were synthesized and coupled to produce the heterobivalent ligand, t-BBN/BVD15-DO3A. Competitive displacement binding assays using t-BBN/BVD15-DO3A against ^{125}I-Tyr^4-BBN yielded an IC_{50} value of 18 ± 0.7 nM for GRPR in T-47D cells, a human breast cancer cell line. A similar assay using t-BBN/BVD15-DO3A against porcine ^{125}I-NPY showed IC_{50} values of 80 ± 11 nM for Y1 receptor in MCF7 cells, another human breast cancer cell line. In conclusion, it is possible to construct a single DO3A chelate containing probe that can target both GRPR and Y1 on human tumor cells.

The use of Positron Emission Tomography (PET) for tumor imaging and management has become routine for imaging hyper-metabolic tumors due to the development of hybrid PET/CT machines, ^{18}F-FDG (fluoro-deoxyglucose) distribution centers, and improved reimbursement of imaging procedures.[1] Although ^{18}F-FDG is an effective tumor imaging agent, it has had limited usefulness in breast cancer[2] and prostate cancer.[3] In breast cancer, it does not differentiate tumors from acute and chronic inflammation, physiological lactation, and benign focal breast masses, including silicon granuloma, fat necrosis, fibroadenoma, and post-surgical changes.[2] In prostate cancer, ^{18}F-FDG is accumulated by normal, inflamed and infected prostate, and lacks the ability to differentiate benign prostatic hyperplasia from severe forms of cancer.[3] Other metabolic imaging agents like ^{18}F- or ^{11}C-acetate, and ^{18}F or ^{11}C-choline have similar limitations in imaging prostate cancers.[3] There is thus a need for receptor targeted and more specific PET imaging agent(s) that can differentiate breast and prostate tumor tissues from non-tumor tissues.

An effective approach to breast and prostate tumor imaging is to target hormone peptide receptors on tumor cells using radio-labeled peptides[4,5] based upon the native agonists. Compared to antibodies, the strengths of this approach derive from the small size (<5 kDa) of the molecules that results in short circulation times and rapid tumor permeation. Other advantages include high binding affinity for the receptor, and the ability to use chemical synthesis to tune the small molecules chemically[6] to modulate the weaknesses, such as greater renal retention, limited bioavailability and undesirable normal tissue agonistic activity. A useful attribute is the option of using exactly the same probe for imaging and radiotherapy by changing the radio-metal (e.g., ^{68}Ga/^{90}Y,

Abbreviations: GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; NPY, Neuropeptide Y; Y1, Neuropeptide Y1 receptor; DGa, diglycolic acid; J, 8-amino-3,6-dioxaoctanoic acid; Abz4, 4-aminobenzoic acid; DO3A, 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid; ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl.

* Corresponding author. Tel.: +1 44 614 247 4427.
E-mail address: Michael.Tweedle@osumc.edu (M.F. Tweedle).
† These authors contributed equally to this work.
‡ Current address: Enzo Life Sciences, Inc., 10 Executive Blvd, Farmingdale, NY 11735, United States.
§ Current address: Chemical Abstracts Services, 2540 Olentangy River Rd, Columbus, OH 43210, United States.

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.11.110

$^{68}Ga/^{177}Lu$) labels. There are multiple receptors that are being individually targeted using this approach including somatostatin (sst1–sst5), gastrin-releasing peptide (BB2/GRPR), cholecystokinin B/gastrin (CCK_2/CCK-B), glucagon-like peptide-1 (GLP-1), $\alpha_v\beta_3$-integrin, melanocortin 1 (MC1R), neurotensin (NTR1), Neuropeptide Y receptor (Y1), luteinizing hormone-releasing hormone (LHRH-R), neurokinin 1 (NK-1) and chemokine (CXCR4) receptors.[4] There are two peptide receptors, GRPR and Y1, that are specifically interesting because the expression of GRPR, Y1 or both was up-regulated in most breast cancers.[7] In 77 frozen primary breast tumor tissues tested, 50 (65%) were GRPR positive, 45 (58%) were Y1 receptor positive and 63 (82%) were positive for either GRPR, Y1, or both receptors. Apart from breast cancers, Y1 is also present in prostate cancer cells,[4,8,9] ovarian tumors, renal cell carcinoma and gastrointestinal stromal tumors (GIST) among others.[10] Similarly, GRPR are present in prostate, breast, pancreas, gastric, small cell lung cancer, colorectal cancer[4,5,11–13] and in the neovasculature of ovarian and bladder cancers.[14,15] These data strongly suggest that there may be advantages to developing a dual-target target probe that can bind both GRPR and Y1. A further consideration is the economics of the drug development process, now costing >$100 million for an imaging drug, that favors a dual-target probe over two separate single target probe development projects.[16,17] Popularity of dual-target probes is increasing, as several heterovalent ligands targeting two different domains of one receptor[18–22] or two different receptors have recently appeared.[23–35]

A heterobivalent dual-target probe, t-BBN/BVD15-DO3A, to recognize both GRPR and Y1 is described herein. The probe combines AMBA, a ligand for two cancer specific GRPR subtypes[36] and BVD15, a ligand for Y1 receptor,[37] a cancer-specific subtype of the Neuropeptide Y receptor family. Bombesin is a 14 amino acids (aa) long natural ligand of GRP receptors.[38] AMBA comprises amino acids 7–14 of bombesin (t-BBN), a 4-aminobenzoyl linker (Abz4), and 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (DO3A) for radio-labeling,[36] and binds to two GRPR receptor subtypes, neuromedin B (NMB) and BB2, expressed in cancers preferentially to the GRP receptor subtype, BB3, expressed in a few normal tissues.[36] BVD15 is a ligand for Y1 that can be modified at the fourth lysine position with DO3A for radio-labeling.[37] BVD15 is known to have some binding to other members of Y1 receptor family, Y2 and Y4, but [Lys^4(DOTA)]BVD15 is shown to be Y1 selective.[37] The heterobivalent probe, t-BBN/BVD15-DO3A, is capable of competing with the natural ligands, ^{125}I-BBN and ^{125}I-NPY, with reasonable affinities and can recognize both GRPR and Y1 on human breast tumor cells.

Bombesin (BBN, #20665, >95% pure, MW 1620.9) and Neuropeptide Y (NPY, #22465, >95% pure, MW 4271.8) were obtained from AnaSpec. Human ^{125}I-Tyr^4-BBN (#NEX258050UC) and porcine ^{125}I-NPY (NEX222050UC) were obtained from Perkin–Elmer. Other peptides were synthesized using solid phase peptide synthesis methods.[46] All the intermediates and final compound were purified using reverse phase high-performance liquid chromatography (HPLC) and characterized by NMR and mass spectrometry. Sequences of all the compounds tested are shown in the Table 1 (details of experiments are described in the References and Notes Section). AMBA analog (MW estimated 1545.8, MW found 1545.76), BVD15-DO3A (MW estimated 1607.9, MW found 1607.92), and t-BBN/BVD15-DO3A (MW estimated 3239.7, MW found 3239.39) were 98%, 96%, and 98% pure.

The T-47D cells are known to overexpress human GRPR[39–41] and were used for GRPR competition assays. The T-47D cells do not express very much human Y1 (tested but data not shown). The T-47D cells were pre-blocked with 1 μM NPY to prevent any Y1 influence on GRPR binding. Bombesin and AMBA analogs (Table 1) were used as controls to compare the affinity of heterobivalent dual-target probe, t-BBN/BVD15-DO3A (Table1), with the natural GRP ligand, BBN, and AMBA (based upon a truncated BBN, t-BBN) that binds cancer specific GRPR subtypes, NMB and BB2. The IC_{50} values of BBN and its AMBA analog were 2 ± 0.1 nM and 3.6 ± 0.3 nM. The IC_{50} value of t-BBN/BVD15-DO3A was 18 ± 0.7 nM (Fig. 3) in the same assay. The MCF7 cells express Y1 receptor[37] and were used for Y1 receptor competition assays. The MCF7 cells do not express very much human GRPR (tested but data not shown). The MCF7 cells were pre-blocked with 1 μM BBN to prevent any GRPR influence on Y1 binding. NPY and BVD15-DO3A (Table 1) were used as controls to compare the affinity of t-BBN/BVD15-DO3A, with the natural NPY ligand (binds all Y1–Y5 receptors) and its monomeric BVD15 component (preferentially binds cancer specific Y1). The IC_{50} value of NPY and BVD15-DO3A were 2.6 ± 1.2 and 9.8 ± 1.2 nM (Fig. 4). In the same assay, the IC_{50} value of t-BBN/BVD15-DO3A was 80 ± 11 nM (Fig. 4). The experiment shown here was done with 1 μM BBN to avoid any consequences of undetectable or low GRPR on NPY expression. In the same experiment, we also obtained IC_{50} of NPY without pre-blocking with BBN (4.7 ± 1.9 nM) to compare with BBN blocking and found no significant effect (data not shown in Fig. 4). To confirm Y1 binding of t-BBN/BVD15-DO3A, IC_{50} of t-BBN/BVD15-DO3A was also done in SK-N-MC (human neuronal epithelioma) cells (74 ± 5 nM) which are known to express only Y1 receptor (data not shown).[42,43] Binding of t-BBN/BVD15-DO3A to Y2 and Y4 receptor was not explored because BVD15 modified at Lys-4 position, [Lys^4(DOTA)]BVD15, has been shown to be Y1 selective.[37] Finally, our data clearly shows that t-BBN/BVD15-DO3A can bind both GRPR and Y1.

Imaging of breast and prostate cancers actually suffers from two problems. First, the sensitivity of PET/CT for detection of lesions <1 cm is poor. PET/CT has made significant advances over PET in this regard, and MRI/PET is poised to further increase the sensitivity for detection of small lesions.[1] The second problem is specificity for malignancy, and is better addressed with a new probe. For example, in early triage of breast cancer, FDG is not used because its high false positive rate creates inefficiencies in time and costs as false leads are followed up in a triage where time to treatment is important. A reasonable goal for a new PET/CT probe will therefore be to perform at greater specificity for cancer versus normal (e.g., inflamed) tissue. Dual-target binding of our bivalent probe is so intended: the AMBA analog binds to GRPR subtypes, NMB and BB2 with no or minimal BB3 binding,[36] and the BVD15 analog binds specifically to Y1.[37]

Table 1
Sequences of peptide compounds

Compound	Molecular weight	Sequence
Bombesin analog	1620.9	Pyr-QRLGNQWAVGHLM-NH_2
AMBA analog	1555.85	DO3A-Dab4-Abz4-QWAVGHLM-NH_2
Human NPY	4271.8	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY-NH_2
BVD15-DO3A	1607.9	INPK(DO3A)YRLRY-NH_2
t-BBN/BVD15-DO3A	3240.7	INP-K[ε-Aoa-K(α-DO3A-ε-DGa-Aoa-G-Abz4-QWAVGHLM-NH_2)]-YRLRY-NH_2

Abbreviations used are Pyr (pyroglutamic acid), DO3A (1,4,7,10-tetraazadodecaundecane-1,4,7,10-tetraacetic acid), Dab4 (1,4-diamino-L-butyric acid), Abz4 (4-aminobenzoic acid), and DGa (diglycolic acid).

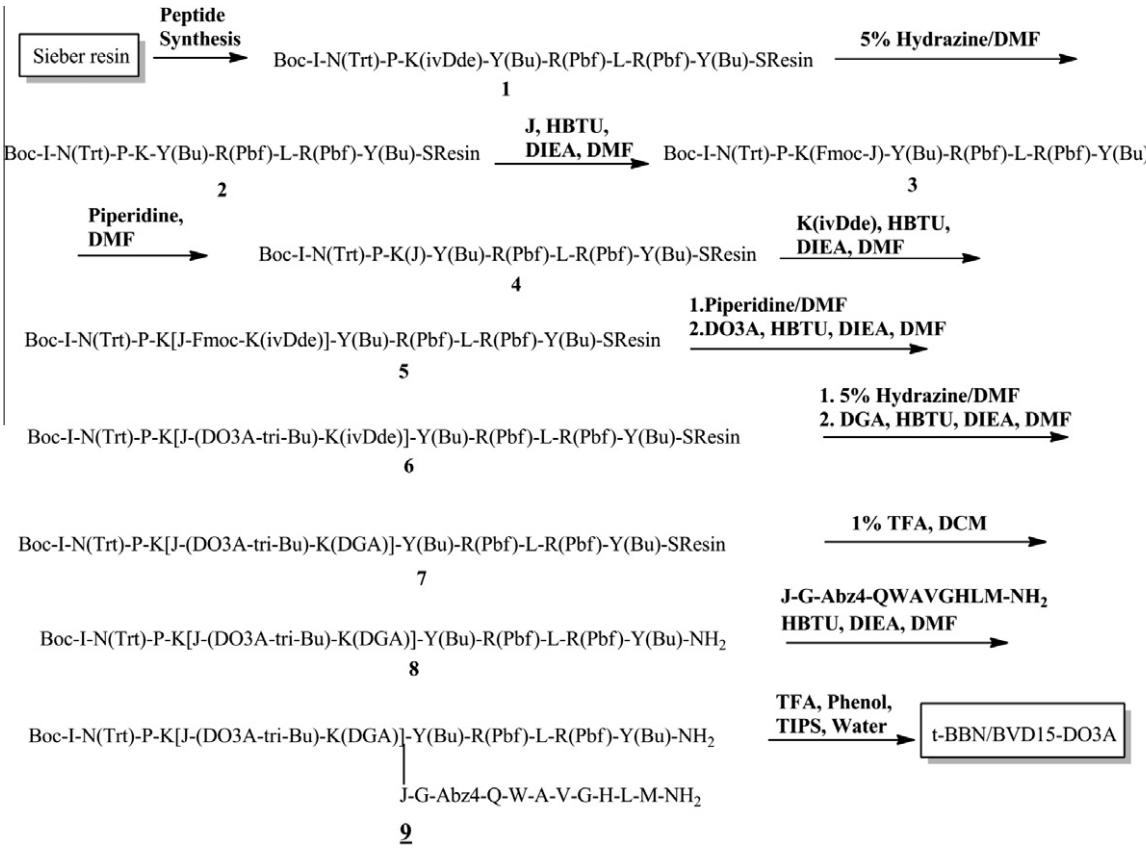

Figure 1.

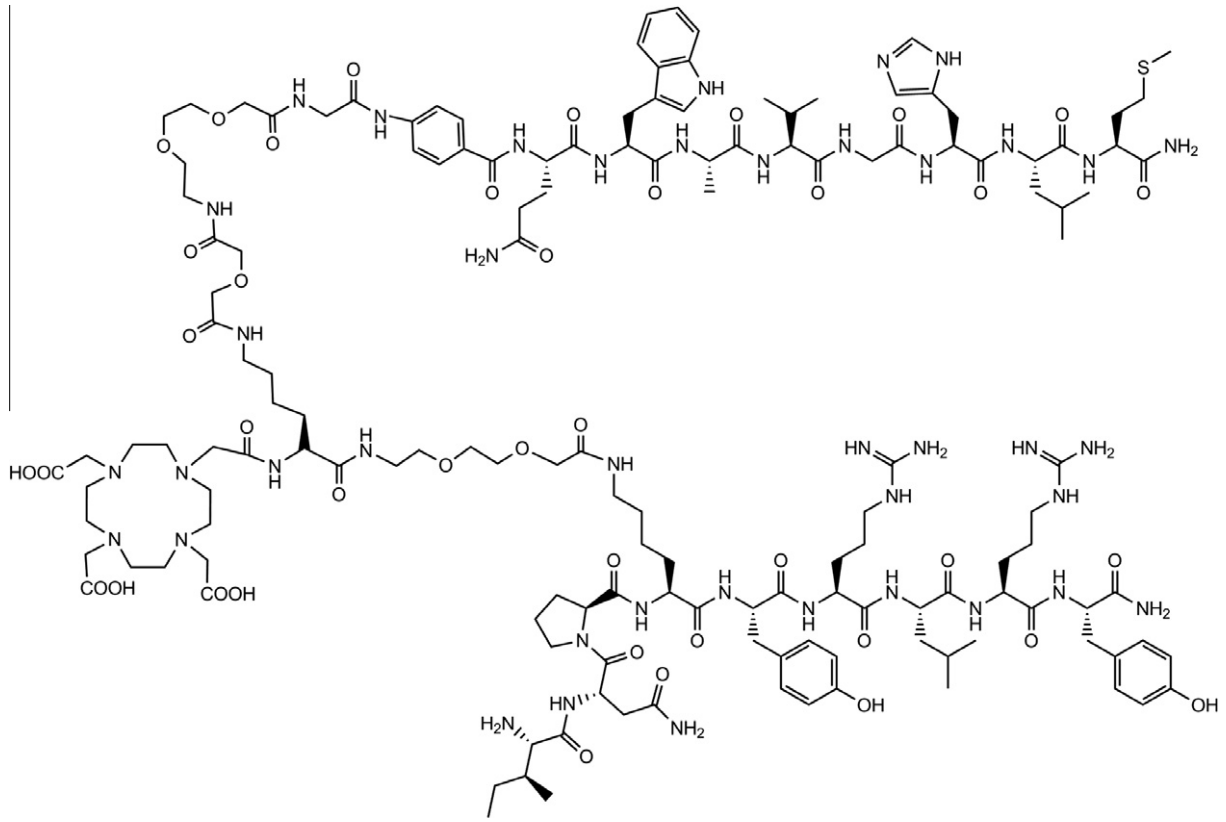

Figure 2.

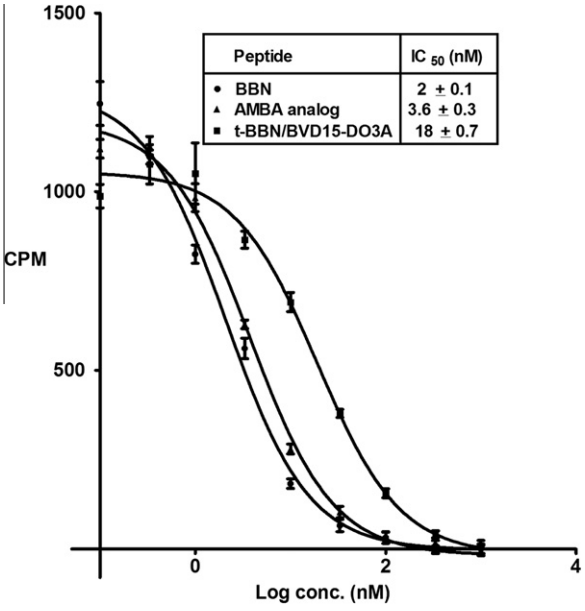

Figure 3.

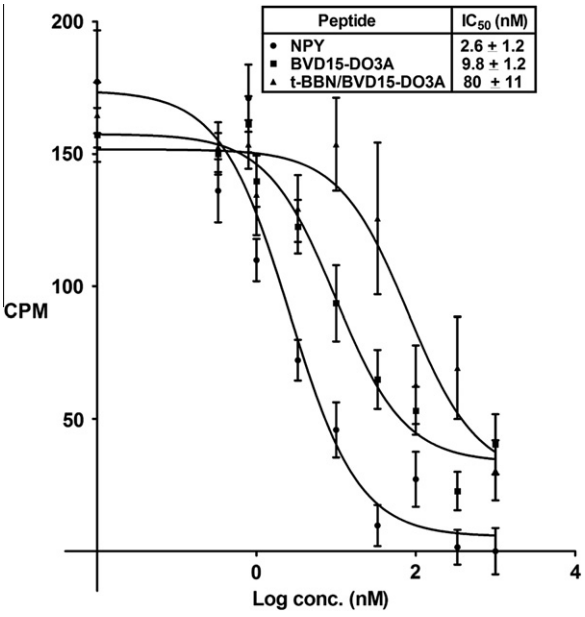

Figure 4.

Similar approaches of creating heterobivalent dual-target probes have been used for different reasons. We have used it previously to target two different domains of a single target receptor to increase affinity.[18–20] Heterobivalent probes targeting two epitopes of VEGFR-2, a receptor tyrosine kinase involved in angiogenesis, were created with affinities >100-fold higher than either corresponding monovalent peptide or homodimers of the monovalent peptides. A heterobivalent probe targeting c-Met, a receptor tyrosine kinase involved in angiogenesis and cancer metastasis, was created with affinities 250-fold higher than corresponding monovalent peptides or homodimers of the monovalent peptides. Ultrasound bubbles coated with our heterobivalent peptide against VEGFR-2 are in a phase I clinical trials, as BR55, for ultrasound imaging of neoangiogenic blood vessels.[44] Others have used heteromultivalent probes to simultaneously target two different receptors on the same cell[24,31–33,35] or two different receptors in the same tumor environment.[23,25–30,34] Heterobivalent ligands targeting two different receptors on the same cell can simultaneously bind both receptors generating higher affinity than corresponding monovalent peptides for one of the receptors, and greater avidity for cells expressing both receptors.[24,31–33,35] Heterobivalent ligands targeting two different receptors in the same tumor had similar or lower affinity than corresponding monovalent peptides for two targeted receptors and did not simultaneously bind both receptors, but still showed higher tumor affinity in vivo.[23,25–30,34] It was hypothesized that increased tumor avidity is due to increased local ligand concentration and the availability of two different receptors for binding,[34] although receptor interaction or pharmacokinetics differences are possible contributing variables.

The goal of the current study was to create a heterobivalent dual-target probe, t-BBN/BVD15-DO3A, for imaging and radiotherapy of cells, tissues and tumors positively expressing receptors, GRPR, Y1 or both. We conclusively showed that t-BBN/BVD15-DO3A is capable of binding both GRPR and Y1 on two different breast cancer cells, T-47D (ductal carcinoma) and MCF7 (adenocarcinoma). Therefore, t-BBN/BVD15-DO3A is a potentially useful candidate for imaging and radiotherapy of GRPR and Y1 receptor positive tumors, a class including both breast and prostate cancers. The IC_{50} values of t-BBN/BVD15-DO3A for GRPR and Y1 receptors were significantly higher than the IC_{50} value of the monomeric components, t-BBN and BVD15. A similar phenomenon was observed previously with the RGD-BBN heterobivalent ligand but that ligand still worked well in vivo.[34] A similar phenomenon might be expected in GRPR and Y1 positive tumors using t-BBN/BVD15-DO3A.

Our work provides a proof of concept for dual-target probe targeting of GRPR and Y1 receptors. Further characterization of t-BBN/BVD15-DO3A will require radio-labeling with ^{68}Ga for imaging, ^{177}Lu for radiotherapy, in vitro cell binding and in vivo tumor animal studies with radio-labeled material, determining whether t-BBN/BVD15-DO3A is an agonist or antagonist, and the kinetics of its receptor binding.

Acknowledgment

The authors acknowledge Ms. Keisha Milum for maintaining the cell cultures and reviewing the manuscript.

References and notes

1. Jadvar, H.; Alavi, A.; Gambhir, S. S. *J. Nucl. Med.* **2009**, *50*, 1820.
2. Adejolu, M.; Huo, L.; Rohren, E.; Santiago, L.; Yang, W. T. *AJR Am. J. Roentgenol.* **2012**, *198*, W304.
3. Jadvar, H. *J. Nucl. Med.* **2011**, *52*, 81.
4. Fani, M.; Maecke, H. R. *Eur. J. Nucl. Med. Mol. Imaging* **2012**, *39*, S11.
5. Laverman, P.; Sosabowski, J. K.; Boerman, O. C.; Oyen, W. J. *Eur. J. Nucl. Med. Mol. Imaging* **2012**, *39*, S78.
6. Khan, I. U.; Beck-Sickinger, A. G. *Anticancer Agents Med. Chem.* **2008**, *8*, 186.
7. Reubi, J. C.; Gugger, M.; Waser, B. *Eur. J. Nucl. Med. Mol. Imaging* **2002**, *29*, 855.
8. Magni, P.; Motta, M. *Ann. Oncol.* **2001**, *12*, S27.
9. Ruscica, M.; Dozio, E.; Boghossian, S.; Bovo, G.; Martosriano, V.; Motta, M.; Magni, P. *Endocrinology* **2006**, *147*, 1466.
10. Korner, M.; Reubi, J. C. *Peptides* **2007**, *28*, 419.
11. Reubi, J. C.; Wenger, S.; Schmuckli-Maurer, J.; Schaer, J. C.; Gugger, M. *Clin. Cancer Res.* **2002**, *8*, 1139.
12. Reubi, J. C. *Endocr. Rev.* **2003**, *24*, 389.
13. Reubi, J. C.; Korner, M.; Waser, B.; Mazzucchelli, L.; Guillou, L. *Eur. J. Nucl. Med. Mol. Imaging* **2004**, *31*, 803.
14. Fleischmann, A.; Waser, B.; Reubi, J. C. *Cell. Oncol.* **2007**, *29*, 421.
15. Fleischmann, A.; Waser, B.; Reubi, J. C. *Endocr. Relat. Cancer* **2009**, *16*, 623.
16. Nunn, A. D. *Invest. Radiol.* **2006**, *41*, 206.
17. Nunn, A. D. *J. Nucl. Med.* **2007**, *48*, 169.
18. Pillai, R.; Marinelli, E. R.; Swenson, R. E. *Biopolymers* **2006**, *84*, 576.
19. Shrivastava, A.; von Wronski, M. A.; Sato, A. K.; Dransfield, D. T.; Sexton, D.; Bogdan, N.; Pillai, R.; Nanjappan, P.; Song, B.; Marinelli, E.; DeOliveira, D.; Luneau, C.; Devlin, M.; Muruganandam, A.; Abujoub, A.; Connelly, G.; Wu, Q. L.;

Conley, G.; Chang, Q.; Tweedle, M. F.; Ladner, R. C.; Swenson, R. E.; Nunn, A. D. *Protein Eng. Des. Sel.* **2005**, *18*, 417.
20. Shrivastava, A.; Nunn, A. D.; Tweedle, M. F. *Curr. Pharm. Des.* **2009**, *15*, 675.
21. Tweedle, M. F. *Contrast Media Mol. Imaging* **2006**, *1*, 2.
22. Tweedle, M. F. *Acc. Chem. Res.* **2009**, *42*, 958.
23. Jackson, A. B.; Nanda, P. K.; Rold, T. L.; Sieckman, G. L.; Szczodroski, A. F.; Hoffman, T. J.; Chen, X.; Smith, C. J. *Nucl. Med. Biol.* **2012**, *39*, 377.
24. Josan, J. S.; Vagner, J.; Handl, H. L.; Sankaranarayanan, R.; Gillies, R. J.; Hruby, V. J. *Int. J. Pept. Res. Ther.* **2008**, *14*, 293.
25. Li, Z. B.; Wu, Z.; Chen, K.; Ryu, E. K.; Chen, X. *J. Nucl. Med.* **2008**, *49*, 453.
26. Liu, Z.; Huang, J.; Dong, C.; Cui, L.; Jin, X.; Jia, B.; Zhu, Z.; Li, F.; Wang, F. *Mol. Pharm.* **2012**, *9*, 1409.
27. Liu, Z.; Niu, G.; Wang, F.; Chen, X. *Eur. J. Nucl. Med. Mol. Imaging* **2009**, *36*, 1483.
28. Liu, Z.; Wang, F. *Curr. Pharm. Biotechnol.* **2010**, *11*, 610.
29. Liu, Z.; Yan, Y.; Chin, F. T.; Wang, F.; Chen, X. *J. Med. Chem.* **2009**, *52*, 425.
30. Liu, Z.; Yan, Y.; Liu, S.; Wang, F.; Chen, X. *Bioconjug. Chem.* **2009**, *20*, 1016.
31. Vadas, O.; Rose, K. *J. Pept. Sci.* **2007**, *13*, 581.
32. Vadas, O.; Rose, K. *Adv. Exp. Med. Biol.* **2009**, *611*, 505.
33. Vagner, J.; Xu, L.; Handl, H. L.; Josan, J. S.; Morse, D. L.; Mash, E. A.; Gillies, R. J.; Hruby, V. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 1685.
34. Yan, Y.; Chen, X. *Amino Acids* **2011**, *41*, 1081.
35. Zhang, Y.; Xiao, L.; Chordia, M. D.; Locke, L. W.; Williams, M. B.; Berr, S. S.; Pan, D. *Bioconjug. Chem.* **2010**, *21*, 1788.
36. Lantry, L. E.; Cappelletti, E.; Maddalena, M. E.; Fox, J. S.; Feng, W.; Chen, J.; Thomas, R.; Eaton, S. M.; Bogdan, N. J.; Arunachalam, T.; Reubi, J. C.; Raju, N.; Metcalfe, E. C.; Lattuada, L.; Linder, K. E.; Swenson, R. E.; Tweedle, M. F.; Nunn, A. D. *J. Nucl. Med.* **2006**, *47*, 1144.
37. Guerin, B.; Dumulon-Perreault, V.; Tremblay, M. C.; Ait-Mohand, S.; Fournier, P.; Dubuc, C.; Authier, S.; Benard, F. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 950.
38. Schroeder, R. P.; van Weerden, W. M.; Bangma, C.; Krenning, E. P.; de Jong, M. *Methods* **2009**, *48*, 200.
39. Ma, L.; Yu, P.; Veerendra, B.; Rold, T. L.; Retzloff, L.; Prasanphanich, A.; Sieckman, G.; Hoffman, T. J.; Volkert, W. A.; Smith, C. *J. Mol. Imaging* **2007**, *6*, 171.
40. Parry, J. J.; Andrews, R.; Rogers, B. E. *Breast Cancer Res. Treat.* **2007**, *101*, 175.
41. Prasanphanich, A. F.; Retzloff, L.; Lane, S. R.; Nanda, P. K.; Sieckman, G. L.; Rold, T. L.; Ma, L.; Figueroa, S. D.; Sublett, S. V.; Hoffman, T. J.; Smith, C. J. *Nucl. Med. Biol.* **2009**, *36*, 171.
42. Chatenet, D.; Cescato, R.; Waser, B.; Erchegyi, J.; Rivier, J. E.; Reubi, J. C. *EJNMMI Res.* **2011**, *1*, 21.
43. Wahlestedt, C.; Regunathan, S.; Reis, D. J. *Pharmacol. Lett.* **1992**, *50*, PL7.
44. Pochon, S.; Tardy, I.; Bussat, P.; Bettinger, T.; Brochot, J.; von Wronski, M.; Passantino, L.; Schneider, M. *Invest. Radiol.* **2010**, *45*, 89.
45. Larhammar, D.; Blomqvist, A. G.; Yee, F.; Jazin, E.; Yoo, H.; Wahlestedt, C. *J. Biol. Chem.* **1992**, *267*, 10935.
46. *Notes: Peptide synthesis:* monomeric peptides were synthesized using Fmoc strategy. For each mmol of the amine on the resin, protected amino acid 4.0 mmol was activated with 4.0 mmol of the appropriate coupling agent like HATU/HBTU and 8.0 mmol of DIEA for 5 min and the activated acid was transferred to the amine on the solid phase and the vessel was shaken for 90.0 min. The final products and the protection groups were released from the resin (process repeated twice, 10 mL) using a cocktail containing trifluoroacetic acid, phenol, tris-isopropylsilane and water in a ratio of 95:2:2:1 and precipitated into methyl-*tert*-butyl ether. The heterobivalent dual-target target probe synthesis was accomplished using a multistep process. A scheme for synthesis of t-BBN/BVD15-DO3A is shown in Figure 1. As a first step, Sieber amide resin (0.5 mmol/g) was loaded on to a reaction vessel, placed on an ABI 433A automated peptide synthesizer (0.4 g, 0.2 mmol) and the synthesis was carried out using the standard peptide synthesis protocol until the last amino acid was added (intermediate **1**). The resin was then transferred to another reaction vessel and shaken with 10.0 mL of 5% hydrazine in *N*,*N*-dimethylformamide or DMF (volume/volume or v/v) for 10.0 min. The resin was drained and the above procedure was repeated two more times. The resin was then washed with DMF (3 × v) (intermediate **2**) and taken to the next step. A solution of Fmoc-J (Fmoc: 9-fluorenylmethyloxycarbonyl, 0.385 g, 1.0 mmol, in 2.0 mL of DMF) was activated with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate or HBTU (0.378 g, 1.0 mmol) and *N*,*N*-diisopropylethylamine or DIEA (0.26 g, 2.0 mmol). The solution was stirred for 5.0 min, transferred to the vessel containing the resin from above (intermediate **2**) and the remaining activated acid was transferred to the vessel with 5.0 mL more of DMF. The coupling was carried out for 60.0 min by shaking on a mechanical shaker; resin was drained by positive pressure of nitrogen and then thrice washed with DMF, to obtain intermediate **3**. The resin in the vessel from intermediate 3 was de-protected using the standard protocol and washed three times with DMF to obtain intermediate **4**. The above intermediate resin was coupled to Fmoc-K(ivDde)-OH (ivDde:1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-3-methylbutyl) employing a standard coupling procedure as described for intermediate **3** to obtain intermediate **5**. The intermediate **5** was de-blocked to remove the Fmoc protecting group and washed for subsequent coupling to DO3A-tri-*t*-butyl acid under standard coupling conditions for 1 h, drained and washed with DMF to obtain intermediate **6**. The resin was shaken with 5% hydrazine in DMF for 10 min and then drained. The procedure was repeated two more times and then washed thrice with DMF to yield the free amine on the resin as intermediate **7**. The resin was suspended in 10.0 mL of anhydrous pyridine, diglycolic acid anhydride (0.464 g, 4.0 mmol) was added followed by 4-dimethylaminopyridine or DMAP (0.122 g, 1.0 mmol) and then it was shaken for 20 h at rt. The resin was drained, washed with DMF (10 times containing 1% DIEA, v/v) and dichloromethane or DCM (3 times). The resin was suspended in 10.0 mL of cleavage cocktail (1% trifluoroaceticacid or TFA, 1% triisopropylsilaneor TIPS, 98% DCM; v/v/v) and shaken for 30.0 min. The solution from the resin was drained in a mixture of *N*-methylmorpholine or NMM in DCM, (10% NMM, 90% DCM, v/v; 50.0 mL). The above procedure was repeated nine more times, filtrates were combined and evaporated under reduced pressure to about 5.0 mL. The residue was then diluted with water (50.0 mL) and extracted with 5 × 20.0 mL of DCM. The combined organic layers were washed with water (3 × 30.0 mL) and dried with Na_2SO_4. The solution was filtered and evaporated under reduced pressure to yield the required acid (intermediate **8**) as a colorless solid. Analytical HPLC of the acid indicated that the purity was about 75%. As a second step, J and a glycine spacer were attached to Abz4-QWAVGHLM-$NH_2$to synthesize J-G-Abz4-QWAVGHLM-NH_2 or J-G-Abz4-t-BBN. Initially, Fmoc-PAL-PEG-PS Resin (Applied Biosystems Inc., 1.0 g, 0.19 mmol) was used on an ABI 433A automated synthesizer until the sequence Fmoc-Abz4-Q(Trt)-W(Boc)-A-V-G-H(Trt)-L-M-resin was built. The resin was then transferred to another peptide reaction vessel and the protecting group (Fmoc-) was removed under standard conditions. The resulting amine on the resin was coupled to Fmoc-G-OH using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate or HATU as the coupling agent twice. The resin was then washed, deprotected and coupled to Fmoc-J-OH manually in two steps to provide J-G-Abz4-QWAVGHLM-NH_2. In the first step, Fmoc-G-OH was coupled with fully protected Abz4-Q(Trt)W(Boc)AVGH(Trt)LM-NH_resin to afford G(Fmoc)-Abz4-Q(Trt)W(Boc)AVGH(Trt)LM-NH_resin under the HATU conditions and this operation was repeated twice. The resin was then manually washed, deprotection (Fmoc) from G residue under 20% piperidine and then was subsequently coupled with Fmoc-J-OH, under HBTU conditions. Eventually the peptide was released from the resin using a cocktail containing trifluoroacetic acid, phenol, tris-isopropylsilane and water in a ratio of 95:2:2:1 and precipitated into methyl-*tert*-butyl ether discussed previously. The precipitate was filtered and the crude solid was purified on preparative HPLC [Shimadzu preparatory purification unit (LC8A)] using C18 column (10 μm, 50 × 250 mm, 60 min runtime at 100 mL/min) with water (0.1% TFA): MeCN (0.1% TFA)-10–100% solvent. Finally, the fully protected acid intermediate **8** (60.0 mg, 0.02 mmol) was dissolved in DMF (100 μL), and HATU (8.0 mg, 0.04 mmol) and NMM (8.0 mg, 0.08 mmol) were added with stirring; the activation was continued for 30 min. The amine, J-Gly-Abz4-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (25.0 mg, 0.02 mmol) in 100 μL of DMF (pH adjusted to 8.0 with NMM) was transferred to the acid and stirring was continued for 20 h. The reaction mixture was diluted with *t*-butyl methyl ether, and the precipitate was filtered, washed with water and air dried. Fully protected peptide **9** (crude; 80.0 mg) was air dried and subjected to cleavage with 10.0 mL of cocktail reagent B for 3 h. All the volatiles were removed under reduced pressure and the residue was triturated with dry ether; the clear ether layer was decanted and discarded. This step was repeated two more times and the residue was then dissolved in water and filtered. The filtrate was loaded on to a preparative HPLC column and purified. Fractions with >90% purity were pooled and checked for the product by MS. The fractions with the required mass and purity >90% were pooled and freeze dried to yield the product as a colorless fluffy solid, 16.0 mg (4% yield). The chemical structure of t-BBN/BVD15-DO3A is shown in Figure 2. *Cell culture:* the GRPR competition assays were done using T-47D human breast cancer cells (ductal carcinoma; American Type Culture Collection, cat#HTB-133). T-47D cells were maintained in DMEM high glucose supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% Pen Strep (Gibco, Life Technologies) and sub-cultured using 0.25% trypsin once per week using standard cell culture techniques. The Y1 receptor competition assays were done using MCF7, another human breast cancer cell (adenocarcinoma; American Type Culture Collection, cat#HTB-22). MCF7 cells were maintained in DMEM high glucose containing additional 2 mM glutamine supplemented with 10% fetal bovine serum (Atlanta Biologicals) and 1% Pen Strep (Gibco, Life Technologies) and sub-cultured using 0.25% trypsin acid twice per week using standard cell culture techniques. Cells were seeded in 96-well plate at 30,000 cells per well in 200 μL of cell culture medium for competition assays. *GRPR competition binding:* cell culture medium was removed 24 h after plating and cells were washed twice with binding buffer (RPMI 1640, 20 mM HEPES, 0.1% BSA w/v, 0.1 mg/mL bacitracin) at room temperature (rt). Cells were placed in a 4 °C refrigerator for 30 min for slow cooling. All the subsequent steps used ice cold solutions and incubations were performed at 4 °C. Cells were pre-blocked with 70 μL of 1 μM NPY (AnaSpec, cat#22465) in binding buffer for 30 min to reduce any binding to Y1 receptors in T-47D cells. Peptide mixtures for the competition assay were prepared using constant concentration of ^{125}I-Tyr^4-BBN (0.22 μCi/mL), constant concentration of NPY (1 μM) and varied (0.1 nM–1 μM) concentration of competing peptide. Cells were incubated with 70 μL of peptide mixtures in each well for 1 h. Each concentration of competing peptide was tested in triplicate. After 1 h, cells were washed five times with ice cold wash buffer (25 mM HEPES, 150 mM NaCl, pH 7.4). Cells were lysed by adding 200 μL of 1 N NaOH kept at 37 °C twice to each well, solutions were transferred to tubes and radioactivity was measured using an automatic gamma counter (Perkin–Elmer Wizard II, Model 2480). Data were analyzed using GraphPad Prism 5. Y1 Competition Binding: Cell culture medium was removed 24 h after plating and cells were washed twice with binding buffer (25 mM Hepes, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1% BSA and 0.1 mg/mL bacitracin, pH 7.4) at rt.

Cells were pre-blocked with 70 μL of 1 μM bombesin (AnaSpec, cat#20665) in binding buffer for 30 min at rt to reduce any binding to GRPR in MCF7 cells. Peptide mixtures for the competition assay were prepared using a constant concentration of ^{125}I-NPY (porcine) (0.2229 μCi/mL), constant concentration of BBN (1 μM) and varied (0.1 nM–1 μM) concentration of competing peptide. Cells were incubated with 70 μL of peptide mixtures in each well for 2 h at rt. Each concentration of competing peptide was tested in triplicate. After 2 h, cells were washed five times with the binding buffer at rt. Cells were lysed by adding 200 μL of 1 N NaOH kept at 37 °C twice to each well, solutions were transferred to tubes and radioactivity was measured using an automatic gamma counter (Perkin–Elmer Wizard II, Model 2480). Data were analyzed using GraphPad Prism 5. Porcine ^{125}I-NPY was used instead of human because it is commercially available and is equipotent with human NPY.[45]