

Supplementary materials and methods

1. Preparation of biotinylated GV1001

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 122.69 mg, 0.64 mmol) and N,N-diisopropylethylamine (174.49 mg, 1.35 mmol) were dissolved in the presence of GV1001 peptide (1 g, 0.54 mmol), N-(*tert*-butoxycarbonyl-2,2-(ethylenedioxy)diethylamine (158.92 mg, 0.64 mmol) and hydroxybenzotriazole (HOBt, 86.48 mg, 0.64 mmol) containing dimethylformamide (DMF) 10 ml at room temperature (RT) overnight. The product was concentrated in vacuo. *Tert*-butylcarbamate-protected compound was reacted with dichloromethane:trifluoroacetic acid = 1:1 (v/v) solution for 1 h at RT, and the resident solvent containing removed protecting group was eliminated in vacuo. The crude products, biotin (134.37 mg, 0.55 mmol), EDC (105.44mg, 0.55 mmol), HOBt (74.32 mg, 0.55 mmol) and N,N-diisopropylethylamine (149.93 mg, 1.16 mmol) were redissolved in 10 ml DMF at RT overnight. The product was separated using dialysis bag (MWCO 2000, Spectrum Labs, Rancho Dominguez, CA, USA), and the solvent was removed in vacuo.

2. Fluorescence-based ligand binding assay

HEK293-pcDNA3.1(+)(mock-transfected cells) and HEK293-GnRHR cells were cultured on 100 mm² plates and 80% confluent cells were harvested in 500 μ l cold PBS using a cell scraper. The cells were passed through a 25 G needle 10 times and homogenized with homogenization buffer containing 100 mM KCl, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.2), 1 mM EGTA plus protease inhibitors, and centrifuged at 720 \times g for 5 min to remove unbroken cells. The samples were centrifuged at 10,000 \times g and the supernatants were ultracentrifuged (Optima-XE100, Beckman Coulter, Inc., Fullerton, CA, USA) at 100,000 \times g for 1 h. Pellets containing the membrane fractions were resuspended with cold-sample buffer containing 10 mM Tris-HCl (pH 7.2), 250 mM sucrose, 1 mM EGTA plus protease inhibitors. The suspensions were mixed with FITC-conjugated GV1001 (0.3 and 3 nM) in TEDG buffer 0.3 ml (10 mM Tris-HCl, pH 7.6, 1.5 mM EDTA, 1 mM Dithiothreitol, 10 % glycerol) for 1 h at 4 $^{\circ}$ C. To separate the free ligand in the mixture, the mixture was 3 times washed with washing buffer

(50 mM Tris base, pH 7.9-8.0). The FITC-labeled GV1001 bound pellet was resuspended with sample buffer and analyzed using fluorometer (Berthold Technologies, Bad Wildbad, Germany).

3. Protein structure homology modeling of GnRHR

Based on the previous report on the homology modeling of human GnRH, the three-dimensional (3D) structure of bovine rhodopsin regulator receptor (accession: GI: 197107530, PDB: 1F88, chain A) (Janovick, et al. 2009; Lu, et al. 2005) was chosen as a template in this study. Homologous template protein sequences with known 3D structure were identified through Basic Local Alignment Search Tool (BLAST) program and the distance geometry, simulated annealing and energy minimization approaches were used to build the 3D model of GnRHR by employing GENO3D (www.geno3d-pbil.ibcp.fr), an web server for structure modeling (Dwivedi, et al. 2015). Finally, after structural and energy minimization evaluation, Model_6 was identified as more accurate than others with maximum homology to the known 3D structure. In order to gain reasonable docking model of GV1001 with hGnRH, another homology model typically was developed using Swiss Model (www.swissmodel.expasy.org). For this studies, hGnRH (accession: P30968) was used as a template protein to produce apelin receptor protein (accession: 5VBL_B GI: 1200169791) with 22% sequence identity (Maudsley, et al. 2004; Millar and Pawson 2004). Best model_1 based on highest number of residues present in the allowed region of Ramachandran plot was selected.

4. Protein-protein interaction studies

Protein–protein docking was performed by ZDOCK v3.5 module of Discovery Studio (DS; Accelrys, USA). 3D-structure of GV1001 (as a multiple conformer ensemble) was acquired under CHARMM force field and resampled under in-house sampling method (Kim, et al. 2017). To maximize the effect of tentative core residues, 16-mer GV1001 (seq: ‘EARPALLTSRLRFIPK’) was cut to 10-mer (seq: ‘ALLTSRLRFI’). ZDOCK docking score contains pairwise shape complementarity scoring function of each docking pose. In the present study, Pose-427 (from Model_6, 1st homology model) and Pose-31 (from Model_1, 2nd homology model) showed the best and highest ZDOCK docking score and was

thus selected for further studies (Singh, et al. 2013).

5. Cellular localization of GV1001 in LNCaP cells

LNCaP cells were cultured in 8-chamber glass slide system (Lab-Tek, Nunc, Rochester, NY, USA) for 24 h. 10 μ M FITC-labeled GV1001 peptides were added in culture medium and incubated for 16 h. After two times washing with PBS, cells were fixed with 4% paraformaldehyde for 20 min at RT. Then, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 1 min to visualize the nucleus. After rinsing with PBS, coverslips were mounted and optically imaged with a confocal microscope (Carl Zeiss, Oberkochen, Germany).

6. Analysis of intracellular calcium uptake

Intracellular Ca^{2+} was measured by the fluorometric method employing a fura-2 and digital imaging as described previously (Park, et al. 2016). Briefly, LNCaP and HEK293 cells grown on coverslips were loaded with fura-2 by incubating them in physiological salt solution containing 1 μ M fura-2/AM and 1% bovine serum albumin for 60 min. Coverslips were mounted in a superfusion chamber on the microscope stage and continuously superfused with PSS at a rate of 2 ml/min. All experiments were conducted at 33°C. Cells were imaged with a Nikon Eclipse Ti-U inverted microscope equipped with a S Fluor 40 (N.A. 1.30, oil) objective lens (Nikon, Tokyo, Japan) and an Evolve 512 electron multiplying charge coupled device (EMCCD) camera (Photometrics, Tucson, AZ, USA). Illumination was provided by a Sutter DG-4 filter changer (Sutter Instruments, Novato, CA, USA). The excitation and emission wavelengths used for fura-2 were 340/380 and 535 nm, respectively. Images were acquired and analyzed with a Meta Imaging System (Molecular Devices, West Chester, PA, USA).

7. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed on paraffin-embedded sections using an ApopTaq Peroxidase *in situ* apoptosis detection kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instruction. The positive reaction was visualized with 3,3-diaminobenzidine, and then nuclear counterstaining was performed using methylgreen dye. TUNEL-labeled cells were quantified by the percentage of positive

area per high-power field. A total of 10 high-power fields were analyzed in tumor tissues of individual animal. Data were expressed as the percentage of TUNEL-positive area.

8. Immunohistochemistry

Immunohistochemistry was performed using an antibody against Ki-67 (Santa Cruz Biotechnology, Dallas, TX, USA). For immunohistochemical staining, the tissues were fixed in 10% phosphate-buffered formalin, routinely processed, and then embedded in paraffin. 5 µm-thick sections were cut and placed on glass slides. Before proceeding with the staining protocol, slides were deparaffinized, rehydrated, and then slides submerged in Antigen retrieval solution (Dako, Seoul, South Korea) for 30 minutes at 100 °C. Nonspecific binding was blocked with 3% peroxidase solution followed by 10% normal goat serum. The Pollink-2 HRP Plus Rat-NM DAB detection kit (Golden Bridge International Inc., Mukilteo, WA, USA) was used for immunostaining. The sections were then incubated with anti-Ki-67 antibody in a moist chamber for 1 h. Afterward they were incubated with rat antibody enhancer and polymer-horseradish peroxidase and 3,3-diaminobenzidine substrate. Finally, the sections were counterstained with hematoxylin and processed for mounting with mounting medium. The sections were analyzed by light microscopy and digital image software. Data were expressed as the percentage of positive area per field.

9. RNA extraction and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). PCR was performed using the selective primers for human MMP2 (sense: 5'-ATAACCTGGATGCCGTCGT-3', antisense: 5'-AGGCACCCTTGAAGAAGTAGC-3'), human MMP9 (sense: 5'-GAACCAATCTCACCGACAGG-3', antisense: 5'-GCCACCCGAGTGTAACCATA-3'), human GnRHR (sense: 5'-TTGCCTTTTTAAACCCATGC-3', antisense: 5'-AACATGCTCCAACATTTGTG-3'). MMP2 and MMP9 mRNA quantification was normalized by β-actin (sense: 5'-GATGAGATTGGCATGGCTTT-3', antisense: 5'-GTCACCTTCACCGTTCCAGT-3')

mRNA determination. GnRHR mRNA quantification was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense: 5'-AAGGCTGAGAACGGGAAG-3', antisense: 5'-GCCCCACTTGATTTTGGGA-3'). The SYBR Green real-time PCR amplifications were conducted with MiniOpticon real time PCR detection system (Bio-Rad laboratories Inc., Hercules, CA, USA).

10. Serum testosterone levels

6-week-old male BALB/c mice (Jung Ang Lab Animal Inc., Seoul, South Korea) were subcutaneously injected with vehicle (PBS) or 10 mg/kg/day GV1001 for 7 days. Blood samples were collected 4 h after initial injection and 4 h after last injection. The plasma concentration of testosterone was determined using a testosterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA).

11. GnRHR knockout in LNCaP cell line

GnRHR knockout LNCaP cells were established by using U6-GnRHR/Cas9-2A-RFP plasmid (Sigma-Aldrich, St. Louis, MO, USA) or CRISPR Universal Negative Control plasmid (Sigma-Aldrich, St. Louis, MO, USA). For the selection of RFP-positive-GnRHR knockout LNCaP cells, the transfected LNCaP cells were sorted using BD FACS Aria II flow cytometer (BD Biosciences, San Jose, CA, USA).

References

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