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Anti-cancer effect of GV1001 for prostate cancer: function as a ligand of GnRHR

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Abstract

GV1001, a 16-amino acid fragment of the human telomerase reverse transcriptase catalytic subunit (hTERT), has been developed as an injectable formulation of cancer vaccine. Here, we revealed for the first time that GV1001 is a novel ligand for gonadotropin-releasing hormone receptor (GnRHR). The docking prediction for GV1001 against GnRHR showed high binding affinity. Binding of GV1001 to GnRHR stimulated the Gαs-coupled cAMP signaling pathway and antagonized Gαq-coupled Ca2+ release by leuprolide acetate (LA), a GnRHR agonist. Repeated injection of GV1001 attenuated both serum testosterone level and seminal vesicle weight via desensitization of hypothalamic-pituitary-gonadal (HPG) axis. We then tested whether GV1001 has an inhibitory effect on tumor growth of LNCaP cells, androgen receptor–positive human prostate cancer (PCa) cells. GV1001 significantly inhibited tumor growth and induced apoptosis in LNCaP-implanted xenografts. Interestingly, mRNA expressions of matrix metalloproteinase 2 and matrix metalloproteinase 9 were suppressed by GV1001, but not by LA. Moreover, GV1001 significantly inhibited the proliferation and migration of PCa cells and induced apoptosis in a concentration-dependent manner. Our findings suggest that GV1001 functions as a biased GnRHR ligand to selectively stimulate the Gαs/cAMP pathway, with anti-proliferative and anti-migratory effects on human PCa.

Key Words
- GV1001
- GnRH
- prostate cancer
- hTERT
- cancer vaccine

Introduction

GV1001 is a peptide composed of 16 amino acids derived from the human telomerase reverse transcriptase catalytic subunit (hTERT). It has been developed as an injectable formulation of cancer vaccine for immunotherapy of many types of solid tumors because more than 90% of malignant tumors use telomerase to sustain immortality (Kim et al. 1994). GV1001 vaccination produces several CD4 clones that recognize naturally processed hTERT (Middleton et al. 2014). In the phase 2 trial of Bernhardt et al., immune responses were observed in 24 of 38 evaluable pancreatic cancer patients treated with GV1001 (Bernhardt et al. 2006). In addition, a greater median survival (216 days) was observed for responders compared with non-responders (88 days) (Bernhardt et al. 2006). GV1001 possesses potential therapeutic efficacy with proven safety in clinical trials not only in pancreatic cancer, but also in non-small-cell lung cancer (NSCLC) and melanoma (Bernhardt et al. 2006, Brunsvig et al. 2006, ...
These results demonstrate that GV1001 is immunogenic and safe for use in patients with diverse solid cancers. However, the mechanistic basis for the anti-cancer effect of GV1001 is not fully understood.

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of male cancer-related death in the United States (Siegel et al. 2011) and Europe (Ferlay et al. 2010). Many treatment options, including prostatectomy, radiation therapy and chemotherapy are clinically available, but hormonal therapy should be used in symptomatic metastatic PCa patients (Cornford et al. 2017). However, PCa can progress to castration-resistant prostate cancer (CRPC) within 12–18 months after ADT treatment (Lassi & Dawson 2010). When PCa progresses toward the CRPC stage, resistance to conventional chemotherapy or hormonal therapy can occur, leading to a mean survival time of only 9–30 months (Kirby et al. 2011). GnRHR agonists such as leuprolide acetate (LA) and goserelin have been frequently used as a first-line therapy for the treatment of PCa (McLeod 2003, Mottet et al. 2011). More recently, GnRHR antagonists such as abarelix and degarelix were approved for several types of hormone-related cancer with the benefits of rapid decreases in luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone levels without flare (Trachtenberg et al. 2002, Klotz et al. 2008). In the clinic, either agonists or antagonists of GnRHR inhibit the production of LH, causing suppression of testosterone and dihydrotestosterone and eventually reducing the blood prostate-specific antigen (PSA) level and the risk of death (Klotz et al. 2008, Tombal et al. 2010). These clinical benefits provide evidence of GnRHR analog application in patients with symptomatic metastatic diseases including bone metastases and neurologic symptoms due to impeding spinal cord compression and subvesical obstruction (Mottet et al. 2011). In a PCT patent (PCT/KR2014/005508), testosterone levels in male rats increased on 1st day after GV1001 injection (maximal 6.8-fold) and began to decrease from 2nd day (KAELE-GemVax CO, Ltd. 2014). Moreover, increased LH levels were observed on day 1 in the groups injected with GV1001. Based on the finding, we hypothesized that GV1001 may have function as a regulator of GnRHR.

In this study, we revealed for the first time that GV1001 functions as a biased ligand of GnRHR and functionally antagonizes Goq-mediated Ca2+ release induced by receptor stimulation. We then compared the pharmacodynamic effects of GV1001 on the downstream signaling of GnRHR with those of LA to understand its underlying mechanism. We also evaluated in vitro and in vivo anti-cancer efficacies of GV1001 in PCa.

Materials and methods

Materials

Antibodies recognizing GnRHR (Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP) 1, cleaved PARP1 (cl-PARP1), cleaved caspase3 (cl-casp3), Bcl-2 (Cell Signaling Technology) and ß-actin (Sigma-Aldrich) were obtained from Cell Signaling Technology. GV1001 peptide and fluorescein isothiocyanate (FITC)-conjugated anti-cancer efficacies of GV1001 in PCa. These clinical benefits provide evidence of GnRHR analog application in patients with symptomatic metastatic PCa patients (McLeod 2003, Mottet et al. 2011). More recently, GnRHR antagonists such as abarelix and degarelix were approved for several types of hormone-related cancer with the benefits of rapid decreases in luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone levels without flare (Trachtenberg et al. 2002, Klotz et al. 2008). In the clinic, either agonists or antagonists of GnRHR inhibit the production of LH, causing suppression of testosterone and dihydrotestosterone and eventually reducing the blood prostate-specific antigen (PSA) level and the risk of death (Klotz et al. 2008, Tombal et al. 2010). These clinical benefits provide evidence of GnRHR analog application in patients with symptomatic metastatic diseases including bone metastases and neurologic symptoms due to impeding spinal cord compression and subvesical obstruction (Mottet et al. 2011). In a PCT patent (PCT/KR2014/005508), testosterone levels in male rats increased on 1st day after GV1001 injection (maximal 6.8-fold) and began to decrease from 2nd day (KAELE-GemVax CO, Ltd. 2014). Moreover, increased LH levels were observed on day 1 in the groups injected with GV1001. Based on the finding, we hypothesized that GV1001 may have function as a regulator of GnRHR.

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Cell culture and establishment of stably GnRHR-overexpressing HEK293 cells

LNCaP (androgen receptor positive human PCa cell line), PC-3 (androgen receptor negative human PCa cell line) and N87 (human gastric cancer cell line) cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (100 U/mL, Hyclone). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (100 U/mL). All cultures were maintained in a humidified 5% CO2 environment at 37°C. To obtain GnRHR-overexpressing HEK293 (HEK293-GnRHR) cells, transfection of pcDNA3.1(+)–GnRHR vector was performed by using Lipofectamine 2000 as specified by the manufacturer’s instruction (Invitrogen). Genomic-resistant colonies were selected by adding G418 (800 μg/mL, Thermo Fisher Scientific) to the culture medium. HEK293-pcDNA3.1(+) vector was also used as mock-transfection group.

Western blot analysis

After washing with PBS, cells were lysed with lysis buffer containing 20 mM Tris–Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM ß-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 μg/mL leupeptin. The cell lysates were centrifuged at 13,000 × g for 15 min to remove insoluble material, the supernatants were fractionated using 10, 12 or 15% separating polyacrylamide gels, and electrophoretically transferred to nitrocellulose membranes. Horseradish peroxidase (HRP)-conjugated anti-cancer efficacies of GV1001 in PCa. These clinical benefits provide evidence of GnRHR analog application in patients with symptomatic metastatic diseases including bone metastases and neurologic symptoms due to impeding spinal cord compression and subvesical obstruction (Mottet et al. 2011). In a PCT patent (PCT/KR2014/005508), testosterone levels in male rats increased on 1st day after GV1001 injection (maximal 6.8-fold) and began to decrease from 2nd day (KAELE-GemVax CO, Ltd. 2014). Moreover, increased LH levels were observed on day 1 in the groups injected with GV1001. Based on the finding, we hypothesized that GV1001 may have function as a regulator of GnRHR.

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anti-IgG antibodies were used as the secondary antibodies (Cell Signaling Technology). The nitrocellulose papers were developed using an ECL chemiluminescence system (Millipore). For ECL chemiluminescence detection, LAS-3000 mini system (Fujifilm, Tokyo, Japan) was used.

**Streptavidin-biotin binding assay**

Total cell lysate (300 μg in 1 mL) was incubated with 4 nmol biotinylated GV1001 for 8 h at 4°C with gentle rotation to allow binding. 150 μL streptavidin-conjugated Dynabeads (Invitrogen) were blocked with 2% bovine serum albumin in PBS for 15 min, and then incubated with the biotinylated GV1001-preincubated cell lysates. Total cell lysates-Dynabeads mixture without biotinylated GV1001 were used as negative control. The streptavidin-biotin complex conjugated proteins were eluted by 10 min boiling in SDS loading buffer and separated on a polyacrylamide gel for GnRHR Western blotting.

**Reporter gene assay**

LNCaP, HEK293-pcDNA3.1(+) and HEK293-GnRHR cells (1 × 10^5 cells/well) were cultured in 48-well plates, and then transfected with luciferase reporter plasmid containing cAMP response element (CRE). The transfected cells were exposed to compounds for 24 h, and the promoter activity was measured using a dual-luciferase reporter assay system (Promega). The firefly and hRenilla luciferase activities in the cell lysates were measured using a luminometer (LB960, Berthold Technologies, Bad Wildbad, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity to phRL-SV (hRenilla) luciferase.

**siRNA transfection**

To knockdown the endogenous GnRHR, LNCaP cells were transfected with 75 nmol/L GnRHR siRNA (catalog no: SASI_Hs02_00302488, Sigma-Aldrich) or nonspecific control siRNA (catalog no: SIC001, Sigma-Aldrich) using Lipofectamine2000 (Invitrogen) for 24 h.

**Cell proliferation assay**

LNCaP and PC-3 cells were seeded in 96-well plate (3 × 10^3 cells/well) and cultured for 96 h in the presence or absence of GV1001 (0.01–10 μM) or LA (1–100 nM). GV1001 or LA was treated every 2 days. Viable cell numbers were counted using thiazolyl blue tetrazolium bromide (MTT, 2 mg/mL solution in PBS) and IncuCyte ZOOM live-cell analysis system (Essen BioScience, Ann Arbor, MI, USA). The kinetic of viable cell numbers were monitored using the IncuCyte label-free cell monolayer confluence algorithm, which provides the ability to acquire phase-contrast images and an integrated confluence metric as a surrogate for cell number.

**Transwell migration assay**

Cell migration was quantified by transwell migration assay. LNCaP and PC-3 cells were seeded in the upper chamber of the transwell plate (Costar, Corning Inc.) and the lower chamber was filled with serum-containing media. The cells were incubated at 37°C for 18 h and then fixed with formalin and methanol, and subsequently stained with hematoxylin for 10 min followed by 4 min eosin staining. With 20× magnification, migrated cells to the lower filter side were analyzed.

**Animal experiments**

Five-week-old male BALB/c-nu mice (Raon Bio Inc., Seoul, South Korea) were subcutaneously inoculated with 1 × 10^6 LNCaP cells in 0.1 mL PBS solution. Once tumors became palpable (>150 mm^3), mice were randomly divided into five groups and subcutaneously injected with vehicle (PBS), GV1001 (0.1, 1 and 10 mg/kg/day) or LA (0.1 mg/kg/day) for 13 days. For the xenograft assay using GnRHR-knockout LNCaP cells, 6-week-old male BALB/c-nu mice (Jung Ang Lab Animal Inc., Seoul, South Korea) were subcutaneously inoculated with 1 × 10^6 LNCaP-Control sgR/Cas9 cells or LNCaP-GnRHR sgR/Cas9 cells in 0.1 mL PBS solution. Once tumors became palpable (>150 mm^3), mice were randomly divided into two groups and subcutaneously injected with or without GV1001 (10 mg/kg/day) for 13 days. Mice were monitored twice per week and tumor length and width were detected by calipers and tumor volume was calculated using the formula (length × width^2) × 0.52. All the animal experiments were performed in accordance with NIH animal use guidelines and the present study was approved by the Seoul National University Institutional Animal Care and Use Committees.

**Caspase-3/7 activity assay**

Caspase-3/7 activity was determined with IncuCyte ZOOM live-cell analysis system according to the manufacturer’s instruction. LNCaP and PC-3 cells were seeded on 96-well plate (3 × 10^3 cells/well) and cultured for 96 h with or
without GV1001 (0.1–10μM), 100nM LA and 100nM CA. Diluted apoptosis reagent (IncuCyte, Cat No 4440, Essen BioScience, final concentration 5 μM) was simultaneously added in culture media. The cells were plated into the IncuCyte ZOOM live-cell analysis system and scanned every 4h in a humidified 5% CO₂ environment at 37°C.

Statistics

Statistical analysis was performed using one-way ANOVA and Tukey’s post hoc multiple comparisons to determine the differences. The statistical significance was accepted at *P<0.05 and **P<0.01.

Methods for GnRHR docking prediction and other studies were described in the Supplementary Materials and methods (see section on supplementary data given at the end of this article).

Results

Peptide structure superposition of GV1001 with GnRH analogs

We examined the peptide structure similarity between GV1001 and GnRH analogs, GnRH, LA (a representative GnRHR agonist) and CA (a representative GnRHR antagonist). GV1001 showed structural similarity with the three GnRH analogs in the 9-amino acid sequence Ile-Phe-Arg-Leu-Arg-Ser-Thr-Leu-Leu, which contains key residues for ligand selectivity (Fig. 1A and B) (Millar 2005). In addition, the peptide structure of GV1001 is more similar to CA than LA. Based on the peptide superposing results, we hypothesized that GV1001 might function as a GnRH analog.

Direct interaction between GnRHR and GV1001

To assess whether GV1001 acts on GnRHR, physical binding of FITC-labeled GV1001 with GnRHR was determined in GnRHR-overexpressing HEK293 cells. Western blot analysis confirmed that protein levels of GnRHR in HEK293-GnRHR cells were higher than those in mock-transfected HEK293-pcDNA3.1(+) cells (Fig. 1C). A fluorescence-labeled ligand-receptor-binding assay using HEK293-pcDNA3.1(+) cells and HEK293-GnRHR cells showed that binding of GV1001 to membrane fractions was higher in the HEK293-GnRHR cells compared with mock-transfected HEK293-pcDNA3.1(+) cells (Fig. 1D). A biotin-streptavidin-binding assay was also performed to confirm the specific binding between GnRHR and GV1001 (Fig. 1E). The synthesis process of biotinylated GV1001 was described in the Supplementary Materials and methods, and MALDI-TOF validation of the compound is illustrated in Supplementary Fig. 1. Non-biotinylated GV1001 was used as a control. Biotinylated GV1001 pulled down GnRHR efficiently in the HEK293-GnRHR cells but only marginally in the HEK293-pcDNA3.1(+) cells (Fig. 1E), suggesting direct binding of GV1001 to GnRHR.

hGnRH molecular modeling simulations

Homology modeling approaches were used to predict the structure of hGnRH which was used as a template on the basis of highest sequence identity. To check the authenticity of predicted 3D structure, model_6 was selected based on highest number of residues present in the allowed region of Ramachandran plot (Fig. 2A). GV1001 appears docked in a pocket surrounded by TM2, TM3, TM6 and TM7 and lies just below ECL1 and ECL3. The docking prediction for GV1001 against hGnRHR showed high binding affinity (ZDOCK score of Pose-427=10.42). Within the 5 Å region of the binding site, hydrophobic residues consist of the great network through (1) Phe13 (A-part of Fig. 1A) of GV1001 – Leu72 – Leu11 of GV1001, (2) Leu72 – Phe13 of GV1001 – Val138 and (3) Val138 – Ile14 of GV1001 – Val139 (Aftabuddin & Kundu 2007). In the region, two hydrogen bonds exist between Arg10 (B-part of Fig. 1A) of GV1001 and Ala246 (length: 2.4Å) and between Arg12 of GV1001 and Val250 (length: 3.0 Å). The two non-covalent bonding also can be stronger through intramolecular hydrogen bonding: (1) Leu11, Phe13, and Ile14 with Arg10 and (2) Arg12 and Ile14 with Arg12 in GV1001. The hydrogen bonding network of Arg10 and the hydrophobic network of Phe13 (A-part of Fig. 1A) can induce close binding of GV1001 with hGnRHR so that Leu11 and Arg12 of the ligand could be buried in the deep cavity of the binding site. In addition, some residue (from Ala5 to Ser9) exists in the shallow pocket relatively but intramolecular double hydrogen bonds in hydroxyl group and amide N-H of Thr8 and carbonyl C=O of Leu6 (backbone) could make the γ-turn structure more stable to elicit high affinity (Fig. 2B) (Kim et al. 2015).

From second homology model, ZDOCK docking generated an enormous number of poses and Pose-31 with the highest score was thus selected for further studies (ZDOCK score=13.10) (Fig. 2C). Within the 5 Å region of the binding site, Phe13 of GV1001 (A-part of Fig. 1A) is surrounded by hydrophobic residues: Leu97, Met100, Cys114, Leu117, Val197, Phe178, Cys196 and Phe309 (Fig. 2D). In addition, the pi-pi interaction of Phe13 with
Phe178 and Trp101 can be synergized with the pi-cation interaction between Phe313 and Lys121 surrounding three phenylalanine groups (Phe178, Phe309 and Phe313). Likewise, Pose-427 in Model_6, the hydrogen bonding interactions between GV1001 and backbone hGnRH can be potential through conformational change in (1) Arg10 (β-part of Fig. 1A) of GV1001 and Tyr283 (length: 3.22 Å), (2) Ile14 of GV1001 and Tyr283 (length: 3.10 Å), (3) Leu7 (E-part of Fig. 1A) of GV1001 and His306 (length: 3.18 Å) and (4) Arg12 of GV1001 and Thr198 (length: 2.27 Å). The four potent H-bonding also can be stronger through intramolecular hydrogen bonding interactions: (1) Leu11, Phe13 and Ile14 with Arg10 of GV1001, (2) Thr8 with Leu6 of GV1001 and (3) γ-turn structure from

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**Figure 1**

Binding of GV1001 to GnRHR. (A) Peptide structures of GV1001 and GnRH. Red-colored residues are overlapped with GnRH. (B) Peptide superposition analysis showing structural comparison between GV1001 and GnRH analogs, leuprolide acetate (LA) and cetrorelix acetate (CA). (C) Western blot analyses of GnRHR in HEK293-pcDNA3.1(+) and HEK293-GnRHR cells. Expression levels of the β-actin proteins were determined as loading controls. Densitometric analyses were performed by three independent experiments and data represent means ± s.d., *P < 0.05 vs pcDNA3.1(+) transfected control. (D) GV1001 binding to GnRHR. Streptavidin-biotin binding assays were performed to confirm the direct interaction between GV1001 and GnRHR. Total cell lysates (300 μg) from pcDNA3.1(+) and HEK293-GnRHR cells were incubated with or without 4 nmol biotinylated GV1001 for 8 h. The results were confirmed by multiple experiments. A full color version of this figure is available at [10.1530/ERC-18-0454](https://doi.org/10.1530/ERC-18-0454).
Ser9 to Leu11. Protonated Arg10 is able to have π–cation interaction with Phe309, Tyr283 and Tyr290 to enhance affinity of GV1001. Despite difference in the sequence and conformation of two homologies, best docking poses from two homology models commonly proposed the deep pocket binding part from Arg10 to Ile14 (part A and B of Fig. 1A) and the shallow pocket binding part from Ala5 to Ser9 (parts C, D and E of Fig. 1A).

**Selective activation of the Gαs-coupled cAMP pathway by GV1001 and functional antagonism against GnRHR agonist**

As a G protein-coupled receptor, GnRHR can be coupled with multiple G proteins (Ruf et al. 2003, Wu et al. 2009b). To investigate which downstream signaling pathway of GnRHR is activated by GV1001, we examined both Gαq-mediated intracellular calcium ([Ca²⁺]i) release and Gαs-stimulated cAMP activity after exposure of LNCaP cells to GV1001 and a known GnRHR agonist, LA. Incubation of LNCaP cells with LA (10 and 100 nM) potently increased the [Ca²⁺]i in LNCaP cells (Fig. 3A). We further tested [Ca²⁺]i content in HEK293-GnRHR cells. LA showed a more potent effect in HEK293-GnRHR cells than in HEK293-pcDNA3.1(+) cells (Fig. 3B). LA increased [Ca²⁺]i 19-fold in HEK293-GnRHR cells, but only a slight increase in [Ca²⁺]i was observed in GV1001-treated cells (Fig. 3B). Next, the reciprocal antagonism of GV1001 was assessed in HEK293-GnRHR cells. Pretreatment with CA (100 nM), a representative GnRHR antagonist, completely inhibited the LA-stimulated increase in [Ca²⁺]i (Fig. 3C). Interestingly, GV1001 (0.1, 1 and 10 μM) also showed significant inhibitory effects on the LA-stimulated [Ca²⁺]i increase, although the inhibition intensity was less than that for CA (Fig. 3C).

Because adenylyl cyclase-mediated cAMP signaling is activated by Gαs coupling with GnRHR, we also evaluated CRE reporter activity in HEK293-pcDNA3.1(+) or HEK293-GnRHR cells exposed to GV1001 or LA. In comparison with the [Ca²⁺]i assay results, 100 nM LA did not change CRE-derived reporter gene activity (Fig. 3D); however, cAMP-mediated promoter-binding activity was concentration dependently enhanced by GV1001 (Fig. 3E). We then assessed whether GV1001-induced CRE
reporter activity is dependent on GnRHR. Transfection of LNCaP cells with GnRHR siRNA efficiently inhibited protein expression of GnRHR (Fig. 3F). In control siRNA-transfected cells, LA pretreatment suppressed GV1001-induced CRE reporter activity, but showed no effect on forskolin (a direct adenyl cyclase activator)-stimulated CRE activation (Fig. 3G). However, no increase in CRE reporter activity was detected in GV1001-treated LNCaP cells transfected with GnRHR siRNA (Fig. 3G). Based on the docking simulation of GnRHR and GV1001, we synthesized mutant peptides to clarify key amino acids of GV1001 for Gαs-mediated cAMP signaling activation. Arg10, Arg12 or both of them which are predicted to be important for the binding of GV1001 and GnRHR were substituted to alanine. In LNCaP cells, CRE reporter activity was enhanced by 10 μM GV1001, but not by any
mutant peptides (0.1-10 μM) (Fig. 3H). We also evaluated CRE reporter activities in HEK293-pcDNA3.1 (+) or HEK293-GnRHR cells exposed to mutant peptides. There were no increases in CRE reporter activity in alanine-substituted peptide-treated HEK293-GnRHR cells (Fig. 3I). These observations imply that GV1001 as a GnRHR ligand selectively stimulates the Gas-coupled cAMP signaling pathway and functionally antagonizes Gq-mediated calcium signaling by canonical GnRHR agonist.

**Inhibition of prostate tumor growth by GV1001**

GnRHR agonists enhance secretion of LH and FSH by binding to GnRHR expressed on the pituitary gonadotropes and consequently stimulate secretion of steroid hormone from the gonads (Clayton 1988, Conn & Crowley 1994). However, repeated exposure of the GnRHR agonist leads to the downregulation or desensitization of GnRHR and eventually decreases production of testosterone (Clayton 1988, Eckstein & Haas 2014). The suppressive effect on the pituitary gonadal axis contributes to the anti-PCa effects of GnRH analogs (Conn & Crowley 1994, Chengalvala et al. 2003). To validate whether GV1001 functions as a GnRHR agonist, we determined serum testosterone level after repeated subcutaneous injection of GV1001 (10 mg/kg/day for 7 days). Serum testosterone level was 2.3-fold increased 4 h after 1st GV1001 injection compared to vehicle-injected control group. However, serum testosterone level at 7 days after repeated injection of GV1001 was significantly lower than vehicle-injected group (Fig. 4A). Moreover, seminal vesicle weights in GV1001-treated mice were significantly reduced compared to those in vehicle-treated group (Fig. 4B). The data indicate that continuous exposure of GV1001 may affect the function of pituitary gonadal axis and suppress the secretion of testosterone. Next, we estimated the effect of GV1001 on the tumor growth of LNCaP cells in xenograft assays. Subcutaneous injection of GV1001 (10 mg/kg, five times a week) significantly inhibited tumor growth in nude mice implanted with LNCaP cells 10 days after injection (Fig. 4C). As expected, we also observed reduced tumor growth in mice subcutaneously injected with LA (0.1 mg/kg, five times a week), but the percent inhibition of tumor volume was less than that achieved with 10 mg/kg GV1001 (Fig. 4C). TUNEL assay using tumor tissues revealed that GV1001 caused apoptotic cell death of cancer cells (Fig. 4D), and Ki-67 immunostaining confirmed that the cell proliferation marker was significantly decreased by GV1001 (Fig. 4E). We also quantified the mRNA levels of matrix metalloproteinase (MMP)2 and MMP9, representative metastasis markers, using real-time qPCR in tumor tissues. Interestingly, mRNA levels of MMP2 and MMP9 were significantly reduced by GV1001, but not by LA injection (Fig. 4F). To assess tumor selectivity of GV1001, we further examined the anti-cancer effect of GV1001 on an N87 gastric tumor xenograft model. It has been reported that GnRHR expression is very low in human gastric cancer tissues (Lu et al. 2015). When we determined the protein expression of GnRHR in N87 cells (Fig. 4G), extremely low level of GnRHR was detected in N87 cells in comparison to LNCaP or PC-3 cells. Intraperitoneal injection of 5-fluorouracil (5-FU, a representative chemotherapeutic agent against gastric cancer) resulted in a significant reduction of tumor growth in nude mice implanted with N87 cells (Fig. 4H). In contrast, GV1001 did not have any significant effect on N87 tumor growth (Fig. 4H). These results support a notion that GV1001 as a novel ligand on GnRHR selectively inhibits tumor growth of AR-positive PCa.

**Inhibition of proliferation and migration of PCa cells by GV1001**

Although GnRHR agonists preferentially act on pituitary gonadal axis (Clayton 1988, Conn & Crowley 1994), recent studies have also demonstrated that GnRHR modulation can directly affect in vitro cell proliferation in many cancer cells, such as endometrial, ovarian, breast and PCa cells (Miller et al. 1985, Dondi et al. 1994, Emons et al. 1997, Grundker et al. 2002, Volker et al. 2002). It has been reported that GV1001 penetrates the plasma membrane and is accumulated in the cytoplasm of Huh7 (human hepatocellular carcinoma cell line), HepG2 (human hepatocellular carcinoma), CHO (Chinese hamster ovary cell line) and bone marrow-derived dendritic cells (Lee et al. 2013). When we evaluated the cellular localization of FITC-labeled GV1001, it was mainly seen in the cytoplasm and plasma membrane (Fig. 5A). These results imply that GV1001 not only binds to cell-surface GnRHR, but also penetrates across the plasma membrane and localizes in the cytoplasm, supporting the possibility that GV1001 targets intracellular molecules. To determine the in vitro anti-proliferative effects of GV1001, cell proliferation of LNCaP cells was determined by MTT assay for 96 h. GV1001 exposure marginally, but significantly inhibited proliferation of LNCaP cells (Fig. 5B). Proliferation of LNCaP cells was also decreased by 100 nM LA (Fig. 5C), and the inhibition intensity was marginal as for GV1001. When we compared GV1001 (0.1-10 μM)-mediated anti-proliferative effects in control siRNA-transfected and
**Figure 4**

*In vivo* anti-cancer effect of GV1001. (A) Testosterone level in the serum of vehicle or GV1001 (10 mg/kg)-injected BALB/c mice was measured by ELISA. Data represent means ± s.d. (*n* = 10, *P* < 0.05, **P** < 0.01 vs vehicle-treated control group). (B) Whole mounts of seminal vesicles collected from BALB/c mice after 7 days treatment of either vehicle or GV1001 (10 mg/kg). Quantitative analysis of seminal vesicle weights (% body weight) was presented as mean ± s.d. (*n* = 10, *P* < 0.05 vs vehicle-treated control group). (C) Tumor growth of LNCaP cells-implanted xenografts. Left panel, representative images of tumor-bearing mice injected with vehicle, GV1001 (10 mg/kg) or LA (0.1 mg/kg) on 13 days after treatments; Right panel, time-dependent changes in tumor volume and body weight. Data represent means ± s.d. (*n* = 5, *P* < 0.05 vs vehicle-treated control group). (D) TUNEL assay of the xenograft tumor sections from the mice treated with vehicle, GV1001 (10 mg/kg) or LA (0.1 mg/kg). TUNEL-positive cells were stained with brown color. TUNEL-positive area was calculated as described in material and methods section. Data represent means ± s.d. (*n* = 5, *P* < 0.05, **P** < 0.01 vs vehicle-treated control group). (E) Ki-67 immunohistochemical staining of the xenograft tumor sections. Data represent means ± s.d. (*n* = 5, **P** < 0.01 vs vehicle-treated control group). (F) mRNA levels of MMP2 and MMP9. RT-qPCR was performed in xenograft tumor tissues. The expression levels were normalized by ß-actin mRNA. Data represent means ± s.d. (*n* = 5, *P* < 0.05, **P** < 0.01 vs vehicle-treated control group). (G) Expression levels of GnRHR in LNCaP, PC-3 cells and N87 cells. (H) Tumor growth of N87 cells-implanted xenografts. 30 mg/kg GV1001 showed no inhibitory effect on N87-tumor growth. 50 mg/kg 5-FU was intraperitoneally injected as positive control. Data represent means ± s.d. (*n* = 6, **P** < 0.01 vs vehicle-treated control group). A full color version of this figure is available at [https://doi.org/10.1530/ERC-18-0454](https://doi.org/10.1530/ERC-18-0454).
Figure 5
Effects of GV1001 on cell proliferation and migration in PCa cells. (A) Penetration of GV1001 in LNCaP cells. LNCaP cells were treated with 10 μM FITC-labeled GV1001. The peptide distribution was analyzed by confocal microscopy. (B and C) Effects of GV1001 (B) and LA (C) on cell proliferation of LNCaP cells. LNCaP cells were plated in 96-well plate and cell proliferation was determined by MTT assays after 96 h treatment with GV1001 (0.01–10 μM) or LA (1–100 nM). Data represent means ± s.d. (n = 6, *P < 0.05, **P < 0.01 vs vehicle-treated control). (D) GnRHR-mediated inhibition of LNCaP cell proliferation by GV1001. Control siRNA-transfected and GnRHR siRNA-transfected LNCaP cells were treated with GV1001 (0.1–10 μM) for 96 h and cell proliferation was determined by IncuCyte ZOOM live-cell analysis system. Data represent means ± s.d. (n = 6, *P < 0.05 vs vehicle-treated control). (E and F) Effects of GV1001 (E) and LA (F) on cell migration. Transwell migration assays were performed in LNCaP cells 18 h after treatment with GV1001 (1 and 10 μM) or LA (1, 10 and 100 nM). Representative pictures of migrated cells were shown (left). The relative cell numbers of migrated cells were counted (right). Data represent means ± s.d. (n = 12–15, *P < 0.05, **P < 0.01 vs vehicle-treated control). (G) Expression levels of GnRHR and AR in both AR-positive LNCaP cells and AR-negative PC-3 cells. Expression level of the β-actin protein was used as a loading control. The mRNA expression levels were normalized by GAPDH mRNA. Data represent means ± s.d. from three independent experiments. *P < 0.05 vs LNCaP cells. (H) Effect of GV1001 on cell proliferation of PC-3 cells. PC-3 cells were plated in 96-well plate and cell proliferation was determined by MTT assay after 96 h treatment with GV1001 (0.01–10 μM). Data represent means ± s.d. (n = 6, **P < 0.01 vs vehicle-treated control). (I) Effect of GV1001 on cell migration of PC-3 cells. Transwell migration assays were performed in PC-3 cells 18 h after treatment with GV1001 (1 and 10 μM). Representative pictures of migrated cells were shown (left). The relative cell numbers of migrated cells were counted (right). Data represent means ± s.d. (n = 12–15, *P < 0.05, **P < 0.01 vs vehicle-treated control). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0454.
GnRHR siRNA-transfected LNCaP cells, GV1001 did not cause cytostatic effect in GnRHR-knockdown LNCaP cells (Fig. 5D). Transwell migration assay also revealed that GV1001 potently suppressed migration of LNCaP cells (Fig. 5E), whereas LA had no effect (Fig. 5F). Because both AR-positive LNCaP and AR-negative PC-3 cells express GnRHR (Fig. 5G), we further assessed the inhibitory effects of GV1001 on the proliferation and migration of PC-3 cells. Treatment of PC-3 cells with 0.1-10 μM GV1001 inhibited both cell proliferation and migration (Fig. 5H and I). These data indicate that GV1001 acts on GnRHR in PCa cells as well as in that in the anterior pituitary, and this activity is required for the direct suppression of proliferation and migration of PCa.

**Induction of apoptosis by GV1001 in PCa cells**

To estimate whether GV1001-induced inhibition of cell proliferation is due to apoptosis, we determined caspase-3/7 activity using the IncuCyte real-time monitoring system. Caspase-3/7 activity was determined by fluorescence emission due to conversion of the specific enzyme substrates. Caspase-3/7 activity was maximum (12-fold enhanced) 72 h after treatment with 0.1, 1 and 10 μM GV1001 in LNCaP cells (Fig. 6A). We also compared caspase-3/7 activity after treatment with the same concentration (100 nm) of GV1001, CA or LA (Fig. 6B). Caspase-3/7 activity was significantly increased to a higher level (4.2-fold) in cells treated with GV1001 compared with CA (0.6-fold) or LA (2.4-fold). We further observed that protein levels of cleaved PARP1 (cl-PARP1, a representative apoptosis marker) and cleaved caspases-3 (active form of caspase-3) were enhanced 72 h after exposure of LNCaP cells to 3, 10 μM GV1001 (Fig. 6C). In contrast, the expression level of Bcl-2, a mitochondrial anti-apoptotic protein, was decreased in LNCaP cells exposed to 0.3-10 μM GV1001 (Fig. 6C). We also found that GV1001 increased the protein level of cl-PARP1 as well as caspase-3/7 activity in PC-3 cells (Fig. 6D and E). To test whether apoptosis induction by GV1001 is mediated through GnRHR, GnRHR siRNA was introduced. GV1001-induced caspase-3/7 activation was diminished in GnRHR siRNA-transfected LNCaP cells compared to control siRNA-transfected cells (Fig. 6F). We then tested whether GV1001 acts on GnRHR expressed in LNCaP cells in vivo. GnRHR-knockout LNCaP (LNCaP-GnRHR sgR/Cas9) cells and control (LNCaP-GnRHR sgR/Cas9) cells were established using CRISPR/Cas9 system (Fig. 6G). After formation of palpable tumors in xenografts implanted with both the cell types (LNCaP-Control sgR/Cas9 and LNCaP-GnRHR sgR/Cas9 cells), GV1001 (10 mg/kg) was subcutaneously injected for 13 days. As expected, GV1001 injection for 13 days significantly inhibited tumor growth of LNCaP-Control sgR/Cas9 xenografts (Fig. 6H). However, GV1001 marginally reduced tumor growth of GnRHR-knockout LNCaP (LNCaP-GnRHR sgR/Cas9) xenografts, but the inhibition intensity was not significant (P=0.382) (Fig. 6H). These results suggest that anti-cancer effects of GV1001 are dependent on GnRHR expressed in both the pituitary gonadotropes and the PCa cells.

**Discussion**

GV1001 has been developed as an injectable formulation of cancer vaccine (Kyte 2009). Furthermore, GV1001 can penetrate across the plasma membrane of cancer cells and induces cancer cell death in many types of solid tumor (Bernhardt et al. 2006, Brunsvig et al. 2006, 2011, Kyte 2009, Kyte et al. 2011, Lee et al. 2013, Kim et al. 2014). Through the superposition of GV1001 structure, we identified structural similarity between GV1001 and commonly used GnRH analogs. Reduction of gonadotropin secretion via desensitization of HPG axis has been demonstrated as a mode of action for the anti-cancer effects of GnRH analogs against AR-positive PCa (Jacobi & Wenderoth 1982). However, the underlying molecular mechanisms of GnRH analogs are not fully understood. Our current study demonstrates that GV1001 physically interacts with GnRHR and affects the downstream signaling pathways. In the conventional GnRHR downstream signaling pathway, GnRHR ligand activates phospholipase C-dependent calcium release via Gαq/11 coupling and stimulates LH release (Stanislaus et al. 1997, Krismanovic et al. 2003). In addition to coupling with Gαq/11, GnRHR also activates Gαi and Gαs proteins (Liu et al. 2002, Ruf et al. 2003, Wu et al. 2009b, Tsutsumi et al. 2010). Gαi activation in response to high levels of GnRH interrupts the increase in neurosecretion (Krlsmovic et al. 2003) and the phosphorylation of EGFR (Grundker et al. 2002), eventually inhibiting cell proliferation. Here, we found for the first time that GV1001 preferentially activated Gαs-dependent cAMP production after binding to GnRHR and competitively inhibited an increase in Gαq/11-mediated [Ca2+]i by LA. Thus, it seems plausible that GV1001 acts as a biased ligand or functional antagonist on GnRHR. Classic agonists and antagonists are known as activators or inactivators of the entire signaling pathway downstream of the receptor. However, biased ligands activate some specific signals and/or inactivate specific signals mediated by the same receptor.
Figure 6
GnRHR-dependent apoptosis induction by GV1001. (A) Concentration-dependent effect of GV1001 on caspase-3/7 activity in LNCaP cells. LNCaP cells were exposed to GV1001 (0.1, 1, and 10 μM) for 72 h and caspase-3/7 activity was determined by IncuCyte zoom system. Data represent means ± s.d. (n = 6, **P < 0.01 vs vehicle-treated control). (B) Comparison of apoptosis activity in GV1001-, CA- and LA-treated LNCaP cells. Caspase3/7 activity was determined in LNCaP cells exposed to 0.1 μM each compound for 72 h. Data represent means ± s.d. (n = 6, **P < 0.01 vs vehicle-treated control). (C) Determination of apoptosis marker proteins in LNCaP cells. LNCaP cells were incubated with or without GV1001 (0.1–10 μM) for 72 h and the total cell lysates were subjected to immunoblottings of PARP1, cleaved PARP1 (cl-PARP1), cleaved caspase-3 (cl-casp3) and Bcl-2. β-actin was used as loading controls. Data represent means ± s.d. from three independent experiments. *P < 0.05, **P < 0.01 vs vehicle-treated control. (D) Concentration-dependent effect of GV1001 on caspase-3/7 activity in PC-3 cells. Data represent means ± s.d. (n = 6, **P < 0.01 vs vehicle-treated control). (E) Determination of cl-PARP1 in PC-3 cells. Data represent means ± s.d. from three independent experiments. *P < 0.05, **P < 0.01 vs vehicle-treated control. (F) Effect of GnRHR siRNA on caspase-3/7 activity. Control siRNA or GnRHR siRNA-transfected LNCaP cells were exposed to 10 μM GV1001 for 72 h and caspase-3/7 activity was determined by IncuCyte ZOOM system. Data represent means ± s.d. (n = 3, **P < 0.01 vs vehicle-treated control; ***P < 0.01 vs control siRNA-transfected group). (G) Protein levels of GnRHR in LNCaP-Control sgR/Cas9 and LNCaP-GnRHR sgR/Cas9 cells. Data represent means ± s.d. (n = 4, *P < 0.05 vs LNCaP-Control sgR/Cas9 cells). (H) Tumor growth of LNCaP-Control sgR/Cas9- or LNCaP-GnRHR sgR/Cas9 implanted xenografts. Left panel, representative images of tumor-bearing mice injected with or without GV1001 (10 mg/kg) for 13 days; Right panel, time-dependent changes in tumor volume. Data represent means ± s.d. (n = 5, **P < 0.01 vs vehicle-treated control group). A full color version of this figure is available at https://doi.org/10.1530/ERC-18-0454.
Krsmanovic et al. (2003) have suggested that ligand-mediated switch in coupling of the GnRHR between the two pathways is concentration dependent. Here, we revealed that GV1001 selectively stimulates Gs-coupled cAMP pathway through its binding to GnRHR. It may result from the differences of receptor-binding affinity or binding pocket between GnRH analogs and GV1001. Further studies will be needed to clarify the underlining mechanism.

In xenograft analyses, both GV1001 and LA reduced LNCaP-derived tumor formation, and the frequency of Ki-67-positive cells in tumor tissues was also diminished in treatment groups for both compounds. Moreover, the TUNEL-positive area (apoptosis index) in the same tumor tissues was enhanced by GV1001 and LA treatments. A variety of studies have shown that GnRHR-dependent apoptosis is involved in anti-cancer effects of GnRH analogs as well as systemic endocrine effects on the HPG axis (Higashijima et al. 1996, Wang et al. 2002, Kraus et al. 2004, 2006, Clementi et al. 2009, Wu et al. 2009a, Kim et al. 2018). Indeed, Kim et al. (2018) demonstrated that 100 or 200μM GV1001 was effective to inhibit angiogenesis or to induce apoptosis in renal cell carcinoma cells. Anti-cancer effect of 10μg/kg GV1001 seems to be equivalent to 0.1mg/kg LA in LNCaP xenograft study (Fig. 4A). Considering concentration difference of GV1001 and LA for the activation of Gαs-mediated CRE reporter (by 10μM GV1001) or Gαq-mediated calcium increase (by 0.1μM LA), the effective dose of GV1001 and LA in xenograft study may be appropriate.

Because either AR-positive LNCaP cells or AR-negative PC-3 cells express GnRHR, we further examined the anti-proliferative effects of GV1001 on LNCaP and PC-3 cells. Cell proliferation of both PCa cell types was marginally, but significantly inhibited by GV1001, and the inhibition intensity was similar to that of LA. We further revealed that representative apoptosis indices such as caspase-3/7 activity and cleaved PARP were markedly increased by GV1001. Because the anti-proliferation and apoptosis-inducing effects of GV1001 in PCa cells were diminished by GnRHR-knockdown, anti-cancer effects of GV1001 may be mediated through its GnRHR binding. Future study would be needed to clarify if GV1001 is effective in GnRHR-null mice. A previous study demonstrated that GV1001 inhibited the growth of MCF-7 (breast cancer) and Jurkat (T cell leukemia) cells under hypoxic conditions, presumably through the inhibition of heat shock protein 70/90 and hypoxia-inducible factor-α (Kim et al. 2014). Since many cancer cells, including MCF-7 and Jurkat cells, consistently express GnRHR, the anti-proliferative effects of GV1001 on both cell types might partly depend on the direct action on GnRHR (Enomoto et al. 2004, Finch et al. 2004). Indeed, we revealed that in vivo anti-cancer effect of GV1001 was diminished in GnRHR-knockout LNCaP xenografts.

In general, androgen deprivation therapy (ADT) has been considered the most effective first-line therapy against AR-positive PCa (Akaza 2011). Unfortunately, the majority of cases of PCa that respond to ADT progress to the CRPC stage with increased invasion and migration and a higher metastasis rate within approximately 2 years (Lassi & Dawson 2010). We showed that the mRNA expression levels of MMP2 and MMP9 in tumor tissues from xenografts were decreased by GV1001, but not by LA. Transwell migration assay data also confirmed that GV1001 possessed potent anti-migratory effects on LNCaP and PC-3 cells, while LA did not show any effects. These data indicate that GV1001 has distinct anti-migratory activity even though both GV1001 and LA function as ligands of GnRHR. More recently, GnRHR antagonist has become an attractive option to overcome PCa growth because there are several clear advantages over GnRHR agonist (Rocco et al. 2005, Klotz et al. 2008, Schroder et al. 2010, Tombal et al. 2010, Albertsen et al. 2014, Cui et al. 2014, Klotz et al. 2014, Uehara et al. 2015). GnRHR antagonist achieves more rapid suppression of testosterone and PSA than GnRHR agonist because of its immediate onset of action, and the suppression is maintained for up to 1 year in patients with PCa (Klotz et al. 2008). PSA recurrence occurs almost exclusively in patients with metastatic PCa, and degarelix was found to be more effective than LA in patients with high PSA level (Tombal et al. 2010). Thus, the long-term PSA suppression by GnRHR antagonist might be related to a therapeutic benefit for metastatic PCa. In comparison with LA, GnRHR antagonist showed greater reduction in serum alkaline phosphatase (s-ALP) level, which is a prognostic marker for cancer metastasis (Schroder et al. 2010). In clinical studies, GnRHR antagonist showed a possible response in a patient with metastatic PCa even after GnRHR agonist failure (Rocco et al. 2005, Uehara et al. 2015). These reports suggest that GnRHR antagonists might offer better control of metastasis compared with GnRHR agonists. Considering the proven safety in clinical trials and novel function as a biased ligand for GnRHR, GV1001 could be applicable for metastatic PCa.
Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/ERC-18-0454.

Declaration of Interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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