Targeting GPR30 with G-1: a new therapeutic target for castration-resistant prostate cancer

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Abstract

Castration-resistant prostate cancer (CRPC) is an advanced-stage prostate cancer (PC) associated with high mortality. We reported that G-1, a selective agonist of G protein-coupled receptor 30 (GPR30), inhibited PC cell growth by inducing G2 cell cycle arrest and arrested PC-3 xenograft growth. However, the therapeutic actions of G-1 and their relationships with androgen in vivo are unclear. Using the LNCaP xenograft to model PC growth during the androgen-sensitive (AS) versus the castration-resistant (CR) phase, we found that G-1 inhibited growth of CR but not AS tumors with no observable toxicity to the host. Substantial necrosis (approximately 65%) accompanied by marked intratumoral infiltration of neutrophils was observed only in CR tumors. Global transcriptome profiling of human genes identified 99 differentially expressed genes with ‘interplay between innate and adaptive immune responses’ as the top pathway. Quantitative PCR confirmed upregulation of neutrophil-related chemokines and inflammation-mediated cytokines only in the G-1-treated CR tumors. Expression of murine neutrophil-related cytokines also was elevated in these tumors. GPR30 (GPER1) expression was significantly higher in CR tumors than in AS tumors. In cell-based experiments, androgen repressed GPR30 expression, a response reversible by anti-androgen or siRNA-induced androgen receptor silencing. Finally, in clinical specimens, 80% of CRPC metastases (n = 123) expressed a high level of GPR30, whereas only 54% of the primary PCs (n = 232) showed high GPR30 expression. Together, these results provide the first evidence, to our knowledge, that GPR30 is an androgen-repressed target and G-1 mediates the anti-tumor effect via

Key Words
- androgen deprivation therapy
- androgen-repressed gene
- metastases
- tumor-infiltrating neutrophils
neutrophil-infiltration-associated necrosis in CRPC. Additional studies are warranted to firmly establish GPR30 as a therapeutic target in CRPC.

Introduction

Androgen ablation therapies are mainstay treatments for advanced prostate cancer (PC; Tannock et al. 2004, Higano et al. 2009, de Bono et al. 2011). Unfortunately, almost all patients ultimately fail to respond to these therapies and develop castration-resistant PC (CRPC) that grows in the presence of castration levels of circulating testosterone (de Bono et al. 2004). Although chemotherapy (docetaxel or cabazitaxel; Tannock et al. 2011), immunotherapy (e.g. sipuleucel-T; Higano et al. 2009, Kantoff et al. 2010), or complete androgen blockade (e.g. abiraterone; de Bono et al. 2011) may extend the lives of some patients, these treatments all have documented side effects and a relatively short duration of response. Hence, the development of new CRPC therapies with durable efficacy and low toxicity is warranted.

Estrogens have a long history of efficacy for advanced PC (Oh 2002). Huggins & Hodges (2002) first reported the use of diethylstilbestrol for advanced PC in 1941. However, severe cardiovascular toxicity of oral estrogens limited their use in PC (Norman et al. 2008). The early efficacy of parenteral estrogen in recent studies (Schellhammer 2012, Langley et al. 2013) and especially the better toxicity profiles owing to hepatic bypass (Norman et al. 2008) reinvigorated interest in the use of estrogens as a therapy for PC. In addition to the suppression of testosterone effects by estrogens, estrogens are also directly cytotoxic to PC cells (Ho et al. 2011). The actions of parenteral estrogens are believed to be mediated by the classical estrogen receptors (ERs), ER1 and ER2. However, the exact effects of the two ERs and their isoforms on PC growth and metastases may vary according to cellular contexts (Claessens & Tilley 2014, Nelson et al. 2014). We have recently reported that G-1 (1(1-(4-(6-bromobenzo(1,3)dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta(c)quinolin-8-yl)-ethanone)), which selectively activates the third ER, G protein-coupled receptor 30 (GPR30 or GPER) (Bologa et al. 2006), inhibited the growth of multiple PC cell lines and PC-3 xenografts, and exerted few or no adverse effects on the animals (Chan et al. 2010). These results indicate that G-1, by targeting GPR30, might offer a new treatment option for PC.

GPR30 is structurally unrelated to the classical ERs (ESR1 and ESR1). It is a seven-transmembrane-domain receptor localized at the cell surface (Bologa et al. 2006, Funakoshi et al. 2006), endoplasmic reticulum (Thomas et al. 2005, Prossnitz et al. 2007, Otto et al. 2008), perinuclear compartment (Cheng et al. 2011), and nucleus (Madeo & Maggiolini 2010). The successful development of a highly selective non-steroidal agonist, G-1, for GPR30, provides a tool for studying the action of GPR30 independent of the actions mediated by ESR1 and ESR2 (Bologa et al. 2006, Blasko et al. 2009). Activation of GPR30 was found to play opposite roles in the regulation of the growth of various normal and neoplastic tissues, promoting growth of breast, endometrium, and ovarian tissues (Filardo et al. 2000, Vivacqua et al. 2006, Albanito et al. 2007, Pandey et al. 2009), but inhibiting growth of thymocytes, urothelial cells, vascular smooth muscle cells, and ER-positive breast cancer cells (Albanito et al. 2007). The dual action of GPR30 could be related in part to its differential activation of downstream mediators, including EGFR, PI3K, Erk1/2, cAMP, and intracellular Ca²⁺ (reviewed in Maggiolini & Picard (2010) and Prossnitz & Barton (2011)). We demonstrated that in PC cells, the activation of GPR30 by G-1 leads to growth inhibition via an ERK/p21-mediated cell cycle arrest at the G2 phase (Chan et al. 2010). In addition, we found that G-1 inhibited the growth of PC-3 xenografts that lack the androgen receptor (AR). Still unknown are the mode of action of G-1 in vivo and the potential link between its efficacy and androgen status in PC.

This study evaluated the efficacy of G-1 in inhibiting the growth of LNCaP xenografts during the androgen-sensitive (AS) or the castration-resistant (CR) phase. In this study, we report that G-1 inhibited the growth of the xenograft in castrated (low testosterone) animals but not in intact, androgen-supported animals (high testosterone). The G-1-induced growth inhibition in the CR xenograft was associated with massive necrosis, neutrophil infiltration, upregulation of a set of cell-mediated immune response genes, and enhanced expression of GPR30 (GPER1). Results obtained from cell-based experiments revealed that GPR30 is repressed by androgen,
whereas immunohistochemical results indicated a larger proportion of human CRPC metastases than primary PC express high GPR30 level. Collectively, these results provide support for targeting GPR30 with G-1 as a possible new approach for the treatment of CRPC.

Materials and methods

Human specimens

Human tissue microarrays were obtained from Massachusetts General Hospital (primary PC) and the University of Washington (metastatic CRPC). Samples were de-identified; only those with complete clinical information, follow-up data, and good tissue quality were included. The primary PC cohort comprised one specimen each from 232 patients with PC (i.e. 232 specimens) taken at prostatectomy (Leung et al. 2010). The metastatic CRPC cohort comprised patients who participated in the Rapid Autopsy Program during the period 1999–2006; it consisted of 123 CRPC specimens, including 75 bone (spine, ribs, pelvis, sternum, ischium, iliac, and sacrum), 29 lymph node, 14 liver, and five lung metastasis tissues from 24 patients. The use of the specimens was reviewed and approved by the Institutional Review Board committees of the respective universities.

Cell culture and siRNA experiments

Human PC cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and passaged for less than 3 months after resuscitation. Both LNCaP and PC-3 were retro-authenticated by ATCC with short tandem repeat profiling (March 13, 2013) and confirmed to be the original cell line. LNCaP cells were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS; sodium pyruvate, 1 mmol/l; l-glutamine, 2 mmol/l; and l-glucose, 1.25 g/l. PC-3 cells were maintained in F-12K medium (ATCC) supplemented with 10% FBS. Cells were cultured at 37°C in an atmosphere of 5% CO2. For androgen treatment (R1881 and dihydrotestosterone (DHT)), LNCaP (2.5 x 10^5) and PC-3 (2 x 10^5) cells were seeded in phenol-red-free RPMI-1640 (with supplements) and F-12K media, respectively, supplemented with 10% charcoal-stripped FBS. For drug treatment, drugs were added daily for 4 days, and the medium was changed every 2 days. For siRNA-AR (siAR) transfection, cells were replenished with 1.6 ml of fresh medium and 400 µl of siAR-DharmaFECT mixture (50 nM Stealth RNAi siAR, Invitrogen; DharmaFECT3 for LNCaP and DharmaFECT2 for PC-3 cells, Dharmacon, Lafayette, CO, USA) the following day. At day 3, cells were recovered with the respective medium containing 10% charcoal-stripped FBS, and drugs were added daily for 4 days. Transfection was repeated on day 2 of drug treatment. At the end of the experiments, cells were collected for RNA extraction. For the transfection-negative control, cells were treated with DharmaFECT and siRNA-non-targeting (siNT, Dharmacon).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP)-sequencing and ChIP were carried out as described previously using an antibody to AR (ab74272, Abcam, Cambridge, MA, USA; Decker et al. 2012). The site-specific qPCR primers for the AR-binding site at the GPER1 (GPR30) locus were as follows: forward, 5'-CTGGGACAACGTGAGCATAG-3' and, reverse, 5'-CCAACACCATTTACCAAGCCA-3'. The primers for prostate-specific antigen (PSA (KLK3)) enhancer and control regions have been described previously (Zheng et al. 2013).

Microarray experiment and analysis

RNA was extracted from LNCaP xenografts with TRIzol Reagent (Invitrogen); RNA extracts with integrity numbers of less than 8 (four animals in each group), as measured by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA), were used for microarray analysis. The detailed microarray study is available in the Supplementary Methods, see section on supplementary data given at the end of this article. The data are accessible through the NCBI Gene Expression Omnibus Series accession number: GSE54974.

Xenograft study

In the first set of experiments, GPR30 mRNA expression was compared in tumors grown before and after the castration of mice. Male athymic nude mice (4–6 weeks old, 20–25 g, Taconic, Hudson, NY, USA) each received a subcutaneous implant of a 2 cm-long silastic capsule containing ~15 mg testosterone (Sigma), while the animals were under general anesthesia using isoflurane. After 2 days, LNCaP cells (5 x 10^6 cells) in 150 µl of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were injected subcutaneously into the flanks of mice, and the tumors that developed were measured twice weekly (Chan et al. 2010). When the tumors reached 150–300 mm3, mice were divided into two groups: intact and castrated animals. Tumors growing in the intact mice are referred
as AS tumors. For the castrated group, the silastic capsules were removed and mice were surgically castrated under general anesthesia using isoflurane. Tumors regressed and then regrew after castration (approximately 3 weeks post-castration); these tumors are referred as CR. AS or CR tumors at approximately 1000 mm$^3$ were collected to determine the expression of GPR30 mRNA.

In the second set of experiments, the therapeutic efficacy of G-1 on AS and CR tumors was evaluated and compared. LNCaP xenografts were developed as described in the first set of experiments. Both AS and CR tumors were enrolled when tumors reach approximately 300–400 mm$^3$ in size. Mice received subcutaneous injections of a vehicle (95% PBS, 2.5% DMSO, and 2.5% ethanol; v/v) or G-1 (4 mg/kg) daily for 16 days. Tumors and body weight were measured twice weekly. Mice were killed and weighed after removal of the xenografts. The protocol for animal use was approved by the Institutional Animal Care Committee at the University of Cincinnati.

Serum enzyme assays

Serum obtained from mice was assayed for creatine kinase (CK), lactate dehydrogenase (LDH), alanine transaminase (ALT), and aspartate transaminase (AST) using IDTox enzyme assay kits (ID Labs, London, ON, Canada) following the manufacturer’s protocols.

Quantitative real-time PCR

Total RNA was treated with RNase-free DNase (Qiagen) and reverse-transcribed (Chan et al. 2010). Real-time PCR was carried out as described previously (Chan et al. 2010). Species-specific primer sequences are presented in Supplementary Table S1, see section on supplementary data given at the end of this article. PCRs with SYBR GreenER PCR Master-Mix (Invitrogen) were monitored using the 7900HT Fast Real-time PCR System (Applied Biosystems). Individual mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Histopathology and immunohistochemistry analyses

Formalin-fixed xenograft samples were processed for hematoxylin and eosin (H&E) staining and subjected to histological examination for necrosis and inflammation; the thickness of the tumor capsule was determined by the surgical pathologist (J W). Analysis of paraffin-embedded human PC and LNCaP xenograft sections by immunohistochemistry (IHC) was performed as described previously (Leav et al. 2001). Antibodies and quantification of necrosis and markers are described in the Supplementary Methods and Table S2, see section on supplementary data given at the end of this article.

For clinical specimens, GPR30 expression was graded independently by two investigators (H-M L and J W) in a blinded manner. Signal intensity (0–3) and percentage of signal coverage (0–100) of each section were scored, and the product of the intensity and coverage was represented as an H-score (0–300) (Huang et al. 2005). For the metastatic CRPC cohort, H-scores were an average of duplicated cores in a specified metastatic site of each patient. In all cases of bone metastases, two to three sites were acquired per patient and an average H-score was calculated. The distribution of the H-score showed bi-modal or multi-modal properties in the clinical data: 45% of the specimens showed H-scores of less than 100, approximately 32% of the specimens showed H-scores of 100–199 and 23% of the specimens amassed H-scores of 200–300. In this study, we used a dichotomous variable of H-score group (i.e. H-score of 100 or more versus less than 100) in the analysis to reduce possible bias due to the distribution of the original H-score and to improve the statistical power. In order to assess the sensitivity of using different definitions to the H-score variables, the same analysis was repeated after replacing the dichotomous variable with a three-level category variable (i.e. H-score 0–99 versus 100–199 versus 200–300) as well as the original H-score. Those results were found to be consistent with the results of the main analysis using the dichotomous variable presented in this study.

Statistical analyses

Numerical dependent variables were analyzed by one-way ANOVA and the post hoc Bonferroni tests to compare means if more than two groups were involved. t-tests were used if means of two groups were compared. Categorical dependent variables were compared among groups using $\chi^2$ tests. All differences were considered significant when $P<0.05$.

Results

G-1 inhibits growth and induces necrosis in CR tumors with no apparent toxicity to the host

We compared the inhibitory effect of G-1 on AS or CR tumors growing in intact or castrated (low testosterone) mice respectively. Administration of G-1 significantly
inhibited the growth of CR tumors after 16 days of treatment ($P<0.05$, Fig. 1). Similar results were obtained in CR tumors including C4-2 and PC-3 (Supplementary Figure S3, see section on supplementary data given at the end of this article). Massive necrosis and inflammation were observed only in the G-1-treated LNCaP CR tumors (in seven out of eight mice). Inflammation was attended by considerable neutrophil infiltration of the necrotic area as well as of the healthy area of these tumors (Fig. 2A). This intratumoral neutrophil infiltration was not observed in either vehicle-treated CR tumors or vehicle/G-1-treated AS tumors that displayed only ischemic necrotic foci with no inflammation/neutrophils (Fig. 2A). We did not examine T cells in this study because nude mice are deficient in these cells (Pelleitier & Montplaisir 1975).

**Figure 1**
G-1 inhibited growth and induced necrosis in the castration-resistant tumors. G-1 inhibited growth of the CR tumor (bottom panel) but not the androgen-sensitive tumors (top panel). When LNCaP xenografts grew to 150 mm$^3$, mice were divided into two groups: intact and castrated. Intact animals received subcutaneous injections of a vehicle (2.5% DMSO and 5% ethanol) or G-1 (4 mg/kg) daily for 16 days. For the castrated group, mice were castrated and, when the tumor re-emerged, they were treated with a vehicle or G-1 daily for 16 days. Error bars represent mean±S.E.M., n=6–8/group, *$P<0.05$.

B cells and macrophages were found exclusively in the intratumoral stroma and the tumor capsule, respectively, in all treatment groups (Supplementary Figure S1). Notably, G-1-induced necrosis occupied an average of 65% of the tumor volume ($P=0.0003$, Fig. 2B). Furthermore, G-1 significantly reduced the intratumoral microvessel density in CR tumors but not in AS tumors (Fig. 2C, left panel). No significant alteration in microvessel density was observed in the tumor capsule with G-1 treatment (Fig. 2C, right panel). In the viable area of the tumors, cell proliferation (Ki67 staining) remained relatively constant in the four treatment groups except for an increase of 10–20% when compared with vehicle-treated counterparts in Ki67-staining cells in G-1-treated CR tumors (Fig. 2D, left panel). G-1 induced a slight but significant increase in apoptosis (cleaved caspase-3 staining) in the CR tumors (Fig. 2D, right panel).

Our previous work had demonstrated that G-1 did not have general toxicity (on the basis of body weight and tissue histology) in the animals (Chan et al. 2010). In this study, we further report that G-1 did not induce any changes in body weight or cause functional damage to the heart or the liver in mice after 16 days of treatment with G-1, as indicated by the levels of injury biomarkers in the serum (CK and LDH for heart injuries; AST and ALT for liver injuries, Fig. 2E).

**G-1 induced specific changes in gene expression exclusively in CR tumors**

Global transcriptome profiling was performed on vehicle/G-1-treated AS and CR tumors (four groups of tumors, $n=4$). Overall, the profiling results identified 2446 differentially expressed genes among the four treatment groups (false discovery rate (FDR)<0.1, $P<0.01$, $n=4$ per group). Unbiased hierarchical clustering analysis showed no significant differences in gene expression between the vehicle-treated and the G-1-treated AS tumors (Fig. 3, left side of heat map). However, this analysis identified two clusters of genes (a total of 1082) that were altered by G-1 exclusively in the CR tumors (Fig. 3, right side of heat map). Subsequent gene shaving using two additional criteria – $P<0.01$ and a difference of at least 1.5-fold between G-1-treated and vehicle-treated CR tumors – yielded a final set of 99 genes (Fig. 3A, gray panel). Ingenuity Pathway Analysis (IPA) of the 99 genes showed enrichment of the top biological pathway ‘antigen presentation, cell-to-cell signaling and interaction, and inflammatory response’, followed by ‘genetic disorder, neurological disease, and skeletal and muscular disorders’. Furthermore, the top canonical
pathway identified in this specific set of G-1-associated genes is ‘communication between innate and adaptive immune cells’ (Supplementary Table S3, see section on supplementary data given at the end of this article).

To focus on identifying molecular mediators of G-1-induced inflammation/neutrophil infiltration, we selected a set of genes from the 99-gene panel for confirmation based on a literature search showing their relatedness to cell-mediated immune responses. Quantitative real-time PCR analyses \((n=6\) per group) validated the upregulation of the expression of these genes in G-1-treated CR tumors but not in G-1-treated AS tumors when compared with their respective vehicle-treated controls. These include four chemokine genes \(CP, IL8\ (CXCL8), CCL2,\) and \(CXCL12;\) three interferon-induced antiviral genes \(IFIT2, IFIT3, IFIT4;\) and \(SOD2,\) an important oxidative stress response gene (Fig. 3B). As human interleukin 8 (IL8) is a strong chemo-attractant for both human and mouse neutrophils (Geiser et al. 1993, Schaider et al. 2003), we analyzed murine neutrophil-related cytokine genes using quantitative real-time PCR. Expression of murine genes involved in neutrophil movement, accumulation, adhesion, activation, and phagocytic respiratory burst, including \(Il1b, Il6, Il18, Tnf\alpha\ (Tnf), Cxcl12, Cxcl1, Cxcl3, S100a8, S100a9,\) and \(Cd14\) (Cacalano et al. 1994, Leung et al. 2001, Ryckman et al. 2003, Harokopakis &

![Figure 2](http://erc.endocrinology-journals.org)
Hajishengallis 2005, Eash et al. 2010), was elevated by 1.8- to 50.9-fold in G-1-treated vs vehicle-treated CR tumors (Fig. 3B). Interestingly, the expression of human ILIB was not altered in CR tumors with G-1 treatment.

**Androgen represses GPR30 expression via AR, and castration increases GPR30 expression**

In an attempt to explain why G-1 inhibited growth only in an androgen-deprived environment, we determined the effect of androgen on GPR30 expression. Androgen is the principal hormone regulating prostate function. Treatment of LNCaP cell cultures with R1881 (a synthetic androgen) or DHT (the physiologically active androgen) reduced the expression of GPR30 mRNA; the effects of these androgens were abolished by cotreatment with bicalutamide, an AR antagonist, or by transduction of a siRNA against AR (Fig. 4 A and B, upper panels). These results indicate that androgen represses GPR30 expression via mechanisms involving the AR. Furthermore, ChIP-seqencing analyses of LNCaP cells revealed a strong AR-binding site approximately 3.5 kb downstream of the 3' end of the GPR30 (GPER1) gene after androgen stimulation (Supplementary Figure S2, see section on supplementary data given at the end of this article, upper panel). This AR-binding site on GPR30 was further validated by an independent site-specific ChIP-qPCR analysis (Supplementary Figure S2, lower panel).

In the LNCaP xenograft model, expression of GPR30 mRNA was significantly higher (approximately eightfold) in CR tumors grown in castrated mice than in AS tumors grown in intact mice (Fig. 4C). Expression of AR mRNA in CR tumors was increased by 1.8- to 4.6-fold when compared with that in AS tumors (data not shown). These results are in concordance with those from cell-based studies, indicating that GPR30 expression is repressed by androgen via AR-mediated signaling.

**GPR30 expression is higher in metastatic CRPC than in primary PC**

We reasoned that GPR30 in CRPC metastases needs to be expressed at significant levels before we can consider it as a new therapeutic target for CRPC. Hence, we used IHC to assess the level of GPR30 expression in specimens obtained from two cohorts of patients. The first cohort included only primary cancers from specimens obtained at prostatectomy (n = 232) and the second comprised CRPC metastases (n = 123). We found that 80% of the metastatic CRPC specimens expressed high levels of GPR30, with an
H-score of 100 or more when compared with 54% of primary PC specimens with an H-score of 100 or more (P<0.001) (Fig. 5). GPR30 staining in PC was not correlated with age, the Gleason score of primary cancer, final PSA level, type of androgen deprivation therapy (ADT), or duration of ADT (Supplementary Table S4, see section on supplementary data given at the end of this article). Interestingly, no difference in the H-scores of GPR30 was observed among the 75 bone metastases obtained from different locations (H-score approximately 162–165; pelvis/sternum/ischium/iliac/sacrum versus rib/limb versus spine; Supplementary Table S5).

Discussion

In this study, we determined that G-1, a GPR30 agonist, inhibited the growth of CR tumors but not during their preceding AS growth phase, with no detectable toxicity to the host. The G-1-induced growth inhibitory response was manifested as massive necrosis attended by marked neutrophil infiltration in the affected tumors, associated with the activation of gene pathways involved in innate antitumor immunity. We also demonstrated that androgen suppressed GPR30 expression in an AR-dependent manner and that castration markedly upregulated its expression. Clinically, we observed an elevated prevalence of high levels of GPR30 in CRPC metastases when compared with that in primary PC. Taken together, these findings provide evidence for the effective preclinical targeting of GPR30 with G-1 for CRPC.

In this study, we aimed to examine the activation of GPR30 by G-1 in both an androgen-supported (intact) and an androgen-deprived (castrated) environment in vivo. We had previously demonstrated that G-1 inhibited growth in cell culture experiments and a hormone-independent PC-3
xenograft in castrated hosts (Chan et al. 2010). This study further demonstrated the efficacy of G-1 in the LNCaP xenograft model, which recapitulates the natural history of PC progression from AS to CR. We found that, in the LNCaP xenograft model, G-1 inhibited the growth of CR tumors but not AS tumors, indicating that the androgen deprivation may favor the anti-tumor action of G-1.

Histological examinations have indicated that G-1 induced massive tumor necrosis in the castrated mice and invasion of the viable region of G1-treated tumors by numerous tumor-infiltrating neutrophils (TINs). At the molecular level, upregulation of chemokine and inflammatory response genes, including CP, IL8, CCL2, CXCL12, and IFITs, were uncovered by transcriptome profiling and confirmed by qPCR. Thus, one hypothesis is that chemokines secreted by viable CRPC cells and/or additional tumor tissue remodeling factors stimulated by G-1 may direct the migration of neutrophils (illustrated in Fig. 6). Neutrophils have been implicated in tumor progression and antitumor response. Mild infiltration of neutrophils stimulates proliferation and metastasis in cancer (Gregory & Houghton 2011). However, high levels of TINs induce a destructive oncolytic response (Fu et al. 2011) and are associated with cytotoxicity and tumor regression (Di Carlo et al. 2001). Neutrophils produce cytotoxic mediators, including reactive oxygen species, proteases, membrane-perforating agents, and soluble cell-kill mediators (Di Carlo et al. 2001). Moderate or extensive...
levels of TINs are associated with reduced mortality in gastric cancer (Caruso et al. 2002), indicating that neutrophils are active in immunosurveillance against cancer. Key TIN-associated cell-kill mediators, including IL1β and tumour necrosis factor alpha (TNFα) (Di Carlo et al. 2001), were detected in the G-1-induced tumor necrosis. Previous results have indicated that transgenic expression of IL8 and TNFα in tumor cells elicited prominent neutrophil-mediated antitumor activity (Hirose et al. 1995, Musiani et al. 1996). In addition, ceruloplasmin (CP) produced by the CR tumor cells attracts neutrophils and enhances phagocytosis of neutrophils (Saenko et al. 1994). In contrast to the systemic upregulation of cytokines, which may pose a health hazard to immunocompromised patients with cancer, local and specific recruitment of neutrophils may provide a new approach to the targeted treatment of cancer (Hirose et al. 1995, Fu et al. 2011).

GPR30 expression has been reported to be upregulated by various growth factors, HIF1α, and progesterin (Ahola et al. 2002, Albanito et al. 2008, Recchia et al. 2011, De Marco et al. 2013). However, only one report described a decrease in GPR30 expression after estrogen treatment in the human internal mammary artery (Haas et al. 2007). This estrogen-induced suppression of GPR30 was not detected in neurons (Jacobi et al. 2007), indicating that the regulation of GPR30 expression is cell-context-specific. In this study, we demonstrated for the first time that androgen, the principal hormone in the prostate, inhibited GPR30 expression that was dependent on AR.

Interest has started to focus on the crosstalk between AR and ER signaling in PC (Yang et al. 2012, Claessens & Tilley 2014, Nelson et al. 2014). The goal of current treatments of CRPC is to maximally suppress androgen signaling, which may in turn remove the androgen suppression of GPR30 expression, resulting in a high level of GPR30 in late-stage CRPC. In this study, we provided convincing evidence that, in both a preclinical model and in human specimens, reduced androgen levels in CRPC enhanced GPR30 expression when compared with hormone-naive PC. The wide expression and high levels of GPR30 may highlight an unprecedented opportunity to target this protein in clinical metastases of CRPC.

Existing therapies for CRPC offer limited gains in survival and trigger adverse effects; thus, attention has begun to focus on the sequence of application of these treatments (Higano & Crawford 2011). The current LNCaP model represents a subtype of CRPC in which G-1 induced intra-tumoral neutrophil infiltration associated with tumor necrosis. Similarly, we reasoned that a subset of patients harboring CRPC may benefit from G-1 therapy if it is delivered before the patients receive chemotherapy, which can compromise neutrophil production. In light of the most recent CRPC that failed second-generation ADT (i.e. abiraterone acetate and MDV3100), whether or not the expression of GPR30 or the population of patients expressing high levels of GPR30 is increased upon resistance is a clinically interesting question with respect to the further exploration of GPR30 as a novel targeted therapy for late-stage CRPC. Importantly, in all the animal studies reported to date, G-1 did not induce adverse effects (Blasko et al. 2009, Dennis et al. 2009, Chan et al. 2010, Gao et al. 2011). G-1 toxicity to the functions of vital organs including heart and liver has been further proven to be undetectable in this study. One major concern regarding estrogen-related treatment in PC is the increase in the risk of venous thromboembolism (reviewed in Cox & Crawford (1995)). Although G-1 is a specific GPR30 agonist that has been shown not to bind ERα at a concentration up to 10 μM (Bologa et al. 2006), definitive evidence for the absence of estrogen-mediated coagulopathy in vivo is required.

Our findings, taken together, indicated that G-1 effectively inhibited preclinical CRPC growth with a low risk of toxicity; underscoring that G-1 or other GPR30-specific agonists might serve as novel anticancer agents for CRPC that expresses GPR30. The upregulation of GPR30 expression after androgen ablation and the recruitment of neutrophils to the CR tumors are both indicative of a potentially important therapeutic window for G-1/GPR30-targeted therapy preferably under the conditions of a low or ultra-low androgen levels in CRPC before chemotherapy.

**Supplementary data**
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0402.

**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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