G-Protein-coupled Estrogen Receptor 1 Agonist G-1 Perturbs Sunitinib Resistance-related Phosphoproteomic Signatures in Renal Cell Carcinoma

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Abstract. Background: Metastatic renal cell carcinoma (RCC) often develops resistance to first-line targeted therapy such as sunitinib. G-Protein-coupled estrogen receptor 1 (GPER1) agonist G-1 was recently reported to regulate RCC physiology but the role of G-1 in RCC tumorigenesis and sunitinib resistance remains largely unknown. Materials and Methods: Parental and sunitinib-resistant 786-O cells were treated with GPER1 agonist G-1, and quantitative phosphoproteomics was performed. Bioinformatic analyses and validations, including immunoblotting, cell migration, and cell cycle distribution, were performed. Results: G-1 repressed cell proliferation and migration in both parental and sunitinib-resistant 786-O cells. Phosphoproteomic signatures, including phosphoinositide 3-kinase and protein kinase B (PI3K-AKT) as well as other pathways, were up-regulated in sunitinib-resistant cells but application of G-1 reversed this effect. Among phosphoprotein candidates, activating transcription factor 2 (ATF2) Thr69/71 phosphorylation was antagonistically regulated by sunitinib resistance and G-1. Conclusion: Our results open up the possibility for managing RCC and sunitinib resistance by GPER1 agonist G-1 and its regulated pathways.

Renal cell carcinoma (RCC) is the leading cause of kidney malignancy, contributing to an estimated 4-5% of new cases in 2020 (1). In addition to radical or partial nephrectomy, sunitinib is the first-line standard therapy for low-risk recurrent or metastatic RCC (2, 3). Primary or secondary resistance, however, usually develops after sunitinib administration (4). Although the combination of other target therapeutics, such as immune checkpoint inhibitors, seems to be a promising treatment option (5), there is still a need to investigate alternative strategies for managing RCC, especially in cases of sunitinib resistance.

The incidence of RCC has a male-to-female ratio of 2:1 in young patients but becomes approximately 1:1 in patients over 70 years old (6), implying the inhibitory role of estrogen in RCC carcinogenesis. Estrogen can repress RCC growth by non-genomic signaling pathways via classical, nuclear estrogen receptors (7-9). In addition to nuclear estrogen receptors, G-protein-coupled estrogen receptor 1 (GPER1) was identified as a non-classical estrogen receptor, and its expression correlated with tumorigenesis and poor prognosis in several cancer types, including breast and prostate cancer (10). Treatment of in vitro cancer cell lines by the GPER1 agonist, G-1, led to variable results. For example, G-1 inhibited cell growth in prostate cancer (11) but promoted cell survival and migration in RCC cell lines (12). Recently, we demonstrated that estrogen inhibited RCC cell growth and alleviated sunitinib resistance in vitro through estrogen-triggered modulation of phosphorylation (9, 13). However, it remains unclear whether G-1 regulates phosphorylation dynamics in RCC tumorigenesis as well as and sunitinib resistance.

Quantitative phosphoproteomics has been used to study phosphorylation signaling in cancer development and drug resistance. Smith et al. found the novel phosphoproteomic
signature in GPER1 agonist G-1-treated breast cancer cells (14). In addition, by combining immunoprecipitation with quantitative phosphoproteomics, a sunitinib-regulated tyrosine phosphorylation network was revealed (15). In the present study, we utilized a quantitative phosphoproteomics approach to survey G-1-regulated phosphorylation changes and their effect on sunitinib resistance with respect to cell proliferation, cell cycle, and cell migration. The identification of phosphopeptides that are regulated with G-1 may provide novel therapeutic targets for sunitinib-resistant RCC.

Materials and Methods

Chemicals. All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Generation of sunitinib-resistant RCC cells. The human RCC cell line 786-O was purchased from the Biological Collection and Research Center (Hsinchu, Taiwan) and maintained in RPMI 1640 medium (without phenol red) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA). Sunitinib-resistant 786-O cells were generated in-house by gradually increasing exposure up to 10 μM sunitinib in vitro (16). Sunitinib resistance (SunR) was confirmed by cell growth and sunitinib accumulation in lysosomes (17).

Treatment with G-1 and determination of cell viability. Both parental (PAR) and SunR 786-O cells were seeded at 4×10^3 cells/well in 100 μl culture medium in 96-well plates and cultured for 24 h. The cells were then treated with sunitinib (2.5 to 10 μM), estrogen (1 to 5 μM), GPER1 agonist G-1 (1 to 5 μM), or GPER1 antagonist G-15 (1 to 5 μM) for an additional 48 h. Cell viability and number were measured by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay as previously described (18).

Peptide dimethyl labeling and phosphopeptide enrichment. For quantitative phosphoproteomic analysis, both PAR and SunR 786-O cells were treated either with vehicle only (i.e. 0.1% dimethyl sulfoxide) or with 2 μM G-1 for 48 h. Cellular proteins were then harvested in a lysis buffer containing 4% sodium dodecyl sulfate, 100 mM triethylammonium bicarbonate, as well as protease and phosphatase inhibitor cocktails, and sonicated using a Bioruptor Plus Sonicator (Diagenode, Denville, NJ, USA). After acetone precipitation, protein pellets were resuspended in 100 mM triethylammonium bicarbonate, and 8 M urea, followed by trypsin digestion and dimethyl labeling as previously described (9). Peptides from G-1-treated PAR 786-O cells, vehicle-treated SunR cells, and G-1-treated SunR cells were labeled with stable isotopic formyldehyde (3^13C3O, or heavy labeled), for the vehicle-treated PAR 786-O cells, the peptides were labeled with formyldehyde (CH2O, or light labeled). After dimethyl labeling, equal amounts (500 μg) of the heavy- and light-labeled peptides were combined and subjected to sequential phosphopeptide enrichment by Ga- and Fe-immobilized metal affinity chromatography as previously described (19). The eluted phosphopeptides were further fractionated into six fractions using the high-pH reverse-phase StageTip method (20).

Nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) and statistical analysis of phosphosites.

The high-pH reverse phase-fractionated phosphopeptides were analyzed using an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled on-line to a Dionex Ultimate 3000 RSLC nano system (Thermo Fisher Scientific). Phosphopeptides were separated by in-house-prepared capillary column (100 μm × 15 cm tip column) packed with 3-μm ReproSil-Pur 120 C18-AQ reverse-phase beads (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany), and data-dependent acquisition in LTQ-Orbitrap XL were performed as described previously (9). In the present study, three independent biological batches with duplicate nanoLC-MS/MS analyses were performed. Phosphopeptide identification and dimethyl quantification were performed using MaxQuant software (version 1.5.3.8) (21) against a reviewed SWISS-PROT sequence database (version 2016_05, with 20, 207 human canonical protein sequences) as previously described (9). The false discovery rate (FDR) at the peptide and protein levels was fixed at 1% in MaxQuant. The localization probability of each identified phosphorylation site (phosphosite) was also determined by MaxQuant. All nanoLC-MS/MS raw files as well as MaxQuant-generated result data were deposited onto the ProteomeXchange (22) Consortium via the Proteomics Identifications (PRIDE) partner repository with the dataset identifier PXD021254.

All phosphosites analyses were performed in Perseus platform (version 1.6.2.3) (23). Only high-confidence phosphosites (localization probability >0.75 or class I phosphosites) (24) were analyzed. For the SunR group, light-labeled intensity (PAR) and heavy-labeled intensity (SunR) were filtered to remove missing values. The phosphosite intensities were normalized by the internal reference scale (IRS) method (25), log2-transformed, and subjected to permutation FDR testing with s0 setting of 0.1 to identify dysregulated phosphosites (p<0.01) (23). The G-1-treated group was analyzed using the same approach. For comparing the G-1-treated and untreated SunR groups, differences in phosphosite levels were calculated as the “ratio of ratios”, according to a previous study (26). We first filtered for phosphosites with quantification information in the SunR (vs. PAR) and G-1-treated SunR (vs. PAR) groups. After IRS normalization across the two groups, the expression ratio for each group was calculated as SunR/PAR or (G-1-treated SunR)/PAR. Finally, the obtained ratio of G-1-treated SunR was divided by that of SunR. The resulting expression ratios were subjected to permutation FDR testing.

Bioinformatics of differentially regulated phosphosites. Gene Ontology network analysis was performed on the dysregulated phosphosites using Cytoscape (v3.8.0) (27) plug-in ClueGO (v2.5.7) (28, 29). Briefly, the regulated phosphosites were clustered (cut-off p-value<0.01) using ontologies/pathways information from the Gene Ontology biological pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) (30, 31), Comprehensive Resource of Mammalian Protein Complexes (32), and WikiPathway (33) databases. Enriched clusters were annotated by AutoAnnotate (v1.3.3) (34), a Cytoscape application for summarizing networks with semantic annotations. In addition, the Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation module (35) with default settings was used to analyze the Gene Ontology information of the regulated phosphosites. The kinase–substrate relationship of the regulated phosphosites were analyzed using the post-translational modification–signature enrichment analysis (PTM-SEA) algorithm (36) with default settings and a cut-off FDR of 0.01. Finally, the network visualization of the regulated phosphosites was performed using the Cytoscape application PhosphoPath (v3.2) (37).
Qualitative immunoblotting. Ten micrograms of cellular lysates were used for western blotting as previously described (9). The primary antibodies used in the present study and their dilution factors were as follows: anti-phospho (p)-activating transcription factor 2 (ATF2) (Thr69/71) (Cat. 9225) at 1:1000, anti-ATF2 (Cat. 9226) at 1:1500; anti-cyclin-dependent kinase 1 (CDK1) (Cat. 9116) at 1:1000 from Cell Signaling Technology (Danvers, MA, USA); anti-cyclin B (Cat. sc-245) at 1:1000 and anti-cyclin D (M20) (Cat. sc-718) from Santa Cruz Biotechnology (Dallas, TX, USA); and anti-glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. 60004-1-lg) at 1:40000 from Proteintech (Rosemont, IL, USA).

Quantitative phosphoproteomics revealed sunitinib resistance- and G-1-regulated phosphosites. Although G-1 is believed to be a specific GPER1 agonist (41), G-1 may affect both GPER1-dependent and -independent signaling pathways. To further dissect the possible phosphorylation signaling pathways regulated by G-1 treatment, chronic sunitinib treatment, and G-1 treatment in SunR cells, quantitative phosphoproteomics revealed 2893 and 3098 quantifiable phosphosites in the SunR and G-1 treatment groups, respectively. After IRS normalization (25), followed by permutation FDR testing (p<0.01), we identified 615 up-regulated and 886 down-regulated phosphosites in SunR 786-O cells (Figure 2A) as well as 629 up-regulated and 690 down-regulated phosphosites in G-1-treated PAR 786-O cells (vs. control) (Figure 2B).

We further analyzed the dysregulated phosphosites using ClueGO (28, 29) to explore the functions of phosphosites in SunR and G-1-treated PAR 786-O cells. For SunR 786-O cells, two clusters were up-regulated in response to chronic sunitinib treatment, including the adherens junction organization as well as vascular endothelial growth factor receptor (VEGFR) pathway and focal adhesion (Figure 2C). This agrees with previous observations, where sunitinib-resistance up-regulated VEGF-related pathways (4, 17). In contrast, phosphosites involved in transcription factor binding and mRNA splicing were down-regulated, implying global repression of gene transcription in SunR cells (Figure 2A). Microtubule organization, negative regulation of organelle organization, as well as nuclear division and segregation were down-regulated in SunR cells (Figure 2A).

On the other hand, G-1 up-regulated phosphosites involved in protein sumoylation and ubiquitinylation, nucleocytoplasmic transport, DNA integrity checkpoints, and actin filament bundles (Figure 2B). Phosphosites involved in the receptor-signaling pathway, adhesion/tight junctions, and spliceosome were down-regulated in G-1-treated PAR cells (Figure 2B).

G-1 antagonized sunitinib resistance-related phosphorylation pathways. To explore the potential effect of G-1 on the sunitinib resistance-regulated phosphosites, the phosphosite intensities in G-1-treated SunR cells were compared to those of the untreated SunR cells. We identified 24 up-regulated (Log₂ ratio >0.5) and 10 down-regulated (Log₂ ratio < -0.5)
Enrichment for protein functions and pathway information of the differentially expressed phosphosites was determined by Fisher’s exact test \((p<0.01)\) using gene annotation databases (Table I). For example, arrest defective 1 and \(N\)-acetyltransferase human (ARD1–NATH) complex, Ku-antigen and \(N\)-methyl-D-aspartic acid receptor-regulated protein 1 (Ku-antigen–NARG1) complex, as well as activating transcription factor 2, hexokinase-1 and voltage-dependent anion channel-1 (ATF2–HK1–VDAC1) complex were enriched. In addition, mitogen-activated protein kinase (MAPK) signaling pathway and nucleotide-binding domain (NOD)-like receptor signaling pathway were enriched. Finally, proteins related to DNA-damage checkpoint and repair were enriched.

To address the potential kinase motif(s) for the regulated phosphosites, the differentially expressed phosphosites were subjected to PTM-SEA analysis (36). Kinase motifs related to the cyclin dependent kinase (CDK) family were down-regulated in both SunR and PAR 786-O cells (Figure 3B). These data indicate that sunitinib resistance reduced cell-cycle/division activity, and G-1 further reduced CDK activities, such as of CDK1, CDK2, CDK4, and CDK6. On the other hand, several kinases and pathways, including C-C chemokine receptor type 7 (CCR7), phosphoinositide 3-kinase

Figure 1. G-Protein-coupled estrogen receptor 1 (GPER1) agonist G-1 suppressed cell growth in both parental (PAR) and sunitinib-resistant (SunR) 786-O cells. SunR 786-O cells were first generated by chronic exposure to 10 \(\mu\)M sunitinib for over 6 months. Both PAR and SunR 786-O cells were incubated with sunitinib (2.5 or 10 \(\mu\)M) (A), estrogen (1 or 5 \(\mu\)M) (B), GPER1 agonist G-1 (1 or 5 \(\mu\)M) (C), or GPER1 antagonist G-15 (1 or 5 \(\mu\)M) (D) for 48 h, and then viable cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. All treatments were repeated in triplicate. Significantly different at \(p<0.05\) from *vehicle control, and #indicated group.
and protein kinase B (PI3K-AKT), epidermal growth factor receptor (EGFR), tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2), tyrosine-protein kinase KIT (KIT) receptor, and interleukin 33 (IL33) signaling pathways, as well as glycogen synthase kinase 3 beta (GSK3B) and MAPK1 kinases were notably up-regulated in SunR 786-O cells. EGFR and PIK3-AKT pathways are involved in sunitinib resistance (42, 43). CCR7, GSKB, and KIT pathways have been correlated with prognostic and therapeutic resistance in RCC cells (44-46). G-1 treatment also alleviated these sunitinib resistance-activated kinases/pathways. Finally, motifs related to mammalian target of rapamycin (mTOR) and polo-like kinase 1 (PLK1) were up-regulated in G-1-treated SunR 786-O cells compared to that in untreated cells, indicating that G-1 may activate the mTOR and PLK1 pathways in SunR 786-O cells.

We also performed hierarchical clustering of the G-1-regulated phosphosites in SunR 786-O cells (Figure 3C). In general, SunR 786-O and G-1-treated SunR 786-O cells showed opposite expression profiles. For example, phosphosites in clusters 1 and 2 were up-regulated in SunR 786-O cells but down-regulated in G-1-treated SunR 786-O cells. In clusters 3-5, phosphosite expression was down-regulated in SunR 786-O but up-regulated in G-1-treated SunR 786-O cells (Figure 3C). These data suggest that G-1 can antagonize changes in phosphorylation during chronic sunitinib treatment. In addition, PhosphoPath analysis (37) showed that 20 out of 34 regulated phosphosites showed functional connections centered on ATF2, filamin A (FLNA), p21 activated kinase 1 (PAK1), and SH3 domain-containing kinase-binding protein 1 (SH3KBP1) (Figure 3D). Finally, we used the DAVID functional annotation module with UniProtKB keywords (UP_KEYWORDS), KEGG pathway (KEGG_PATHWAY), and Gene Ontology (GOTERM_MF_DIRECT) to identify enrichment in phosphosite clusters (35). Functions in the MAPK signaling

Figure 2. Quantitative phosphoproteomics revealed sunitinib resistance- and G-protein-coupled estrogen receptor 1 agonist G-1-regulated phosphosites in sunitinib-resistant (SunR) (A) and G-1-treated (B) 786-O cells compared with the parental cells (PAR). Upper panel: Volcano plots of differentially regulated phosphosites (false discovery rate<0.01 and s0=0.1). Lower panel: The ClueGO enriched protein functions of the regulated phosphosites. Red and green colors denote up-regulated and down-regulated phosphosites, respectively.
Figure 3. G-Protein-coupled estrogen receptor 1 agonist G-1-regulated phosphoproteomic signatures in sunitinib-resistant (SunR) 786-O cells. A: Volcano plot of G-1-regulated phosphosites (p<0.01, Log2 ratio >0.5 or<−0.5) in G1-treated SunR 786-O cells compared with untreated SunR cells. B: Heatmap of phosphoproteomic signatures using the normalized enrichment scores by post-translational modification–signature enrichment analysis. The significantly enriched or depleted signatures at false discovery rate <0.05 are marked by asterisks. C: Hierarchical clustering of G-1-regulated phosphosites in SunR cells. The comparative expression profiles of the selected phosphosites are also shown. D: The network of G-1-regulated phosphosites in SunR cells depicted by PhosphoPath analysis. The relative expression of specific phosphosites is indicated by small colored boxes from left to right in: SunR, G-1-treated parental, G-1-treated SunR vs. untreated SunR (SunR&G-1 vs. SunR), and G-1-treated SunR (SunR&G-1) 786-O cells, with red and green colors denoting up-regulation and down-regulation, respectively.
Table I. Protein functions of phosphosites significantly regulated by G-protein-coupled estrogen receptor 1 agonist G-1 in sunitinib-resistant 786-O cells.

| Source            | Enriched protein functions                                               |
|-------------------|------------------------------------------------------------------------|
| CORUM             | ARD1–NATH complex                                                       |
| CORUM             | Ku antigen–NARG1 complex                                                |
| CORUM             | ATF2–HK1–VDAC1 complex                                                  |
| CORUM             | TP53BP1-containing complex                                              |
| CORUM             | PTIP–DNA damage-response complex                                        |
| CORUM             | MDC1–TP53BP1–SMC1 complex                                               |
| CORUM             | MDC1–H2AX–TP53BP1 complex                                               |
| CORUM             | Prohibitin 2 complex, mitochondrial                                     |
| KEGG brite        | MAPK signaling pathway                                                  |
| KEGG brite        | NOD-like receptor signaling pathway                                     |
| KEGG brite        | Cortisol synthesis and secretion                                        |
| KEGG brite        | Insulin secretion                                                       |
| KEGG brite        | Cocaine addiction                                                       |
| KEGG brite        | Viral carcinogenesis                                                     |
| KEGG brite        | Aldosterone synthesis and secretion                                     |
| KEGG brite        | Thyroid hormone synthesis                                               |
| KEGG brite        | Amphetamine addiction                                                   |
| Reactome          | Recruitment and ATM-mediated phosphorylation of repair and signaling proteins at DNA double strand breaks |
| Reactome          | SUMOylation of transcription factors                                    |
| Reactome          | Processing of ends of DNA double-strand breaks                          |
| Reactome          | Activation of the AP-1 family of transcription factors                  |

**Pathway, acetylation, DNA-binding, and alternative splicing, were enriched in clusters 1 and 2 (Table II). On the other hand, functions in the SH3 domain, cell–cell adhesion, transcription regulation, and methylation were enriched in clusters 3-5.**

**Integral network analysis highlighted the crosstalk of phosphorylation dynamics between G-1-treated PAR 786-O cells, SunR 786-O cells, and G-1-treated SunR 786-O cells.** We dissected the possible crosstalk between G-1 and sunitinib in RCC cells by performing integral network analysis. We used the phosphosites with significant changes in at least one of the following conditions: G-1-treated PAR vs. PAR 786-O cells, SunR 786-O vs. PAR 786-O, and G-1-treated SunR vs. untreated SunR 786-O cells. A total of 2,286 phosphosites were analyzed by the PhosphoPath analysis. In agreement with PTM-SEA analysis (Figure 3B), PhosphoPath analysis indicated enrichment of the VEGFA-VEGFR2 signaling pathway (p<0.0001), focal adhesion-PIK3-AKT-mTOR signaling pathway (p<0.0001), and cell cycle checkpoint (p<0.0001) (Figure 4).

**G-1 inhibited ATF2 phosphorylation and cell-cycle progression.** According to our phosphoproteomics data, ATF2 phosphorylation at Thr69 and Thr71 was differentially regulated in G-1-treated and untreated SunR 786-O cells (Figure 3C and 3D). In addition, ATF2 was also found in the VEGFA-VEGFR2 signaling pathway (Figure 4A) and focal adhesion-PIK3-AKT-mTOR signaling pathway (Figure 4B), suggesting that ATF2 may be affected by G-1-mediated and sunitinib resistance-related signaling pathways. In agreement with the phosphoproteomics data, we confirmed that Thr69/71 phosphorylation in ATF2 was up-regulated in SunR 786-O cells, and that G-1 treatment inhibited Thr69/71 phosphorylation in both PAR and SunR 786-O cells (Figure 5A). In contrast, ATF2 expression did not significantly change by treatment, demonstrating that G-1 and sunitinib resistance affect ATF2 activity by protein phosphorylation.

Recently, Hasegawa et al. reported that CDK1 and cyclin B complex promote cell-cycle progression into M phase by phosphorylating Thr69/71 in ATF2 (47). In addition, our phosphoproteomics data suggested that G-1 regulates the
cell-cycle checkpoint (Figure 4C). Therefore, we performed flow cytometry to examine the cell-cycle progression in cells. Firstly, G-1 treatment promoted S-phase entry in PAR 786-O cells (Figure 5B). The entry of S-phase after G-1 treatment may be explained by the increased expression of G1/S-phase cyclin D1 in response to G-1 treatment in PAR 786-O cells (Figure 5A). On the other hand, G-1 treatment blocked the G2/M phase transition in PAR 786-O cells (Figure 5B). This G2/M arrest coincided with reduced CDK1 and cyclin B expression by G-1 treatment, as well as the down-regulation of ATF2 Thr69/71 phosphorylation (Figure 5A). In contrast, G-1 treatment did not lead to any significant changes in cell-cycle progression in SunR 786-O cells (Figure 5B), although ATF2 phosphorylation as well as cell-cycle regulators, such as CDK1, cyclin B and cyclin D were inhibited by G-1 treatment in SunR cells.

**Effect of G-1 on RCC cell migration.** According to our phosphoproteomics data, both G-1 treatment and sunitinib resistance may affect cell migration and cell adhesion. We therefore monitored cell migration using Oris™ cell migration assays (48). Sunitinib resistance reduced cell migration by ~50% in 786-O cells, and G-1 inhibited cell migration by nearly 50% in both PAR and SunR 786-O cells (Figure 5C and D). Taken together, these data show G-1 can repress cell migration in both PAR and SunR 786-O cells.

**Discussion**

It has been shown that estrogen represses RCC cell growth via classical estrogen receptors (8, 9), and perturbs sunitinib resistance in *in vitro* RCC cell culture (13). The role of non-classical estrogen receptor GPER1 in RCC physiology, however, is still unclear. In the present study, we showed that GPER1 agonist G-1 repressed cell growth in both PAR and SunR 786-O RCC cells. Using quantitative phosphoproteomics, we further demonstrated that G-1 perturbs phosphoproteomic signatures involved in VEGFR pathway, cell-cycle progression, as well cell migration. Although the exact role of GPER1 remains to be further addressed, our observations imply the importance of the GPER1 pathway in regulating RCC growth. Because estrogen may induce genomic changes and cause delirious side-effects via classical estrogen receptors (49), we anticipate that the G-1-regulated extranuclear-initiated (‘nongenomic’) pathway, either GPER1-dependent or -independent, provides an alternative approach for managing RCC.

Our phosphoproteomics data also highlighted the potential roles of ATF2 in sunitinib resistance and G-1-regulated signaling pathways. Elevated ATF2 expression contributes to resistance to sorafenib, another first-line tyrosine kinase inhibitor, in liver cancer (50). Silencing of ATF2 inhibited the growth of pancreatic cancer cells and enhanced
sensitivity to chemotherapy (51). In addition, up-regulation of ATF2 expression is also correlated with poor prognosis of RCC (52). Together, these observations demonstrated that ATF2 might participate in RCC carcinogenesis and tyrosine kinase inhibitor resistance, although regulation of ATF2 phosphorylation adds another level of regulation to this system. In the present study, we observed the up-regulation of Thr69/71 phosphorylation of ATF2 in sunitinib resistance, and treatment with G-1 reversed this. ATF2 Thr69/71 phosphorylation is required for its association with activator protein 1 (AP-1), nuclear localization, and transcriptional activation of downstream genes related to DNA repair and apoptotic signaling (53), which may promote resistance to therapeutic agents. The down-regulation of Thr69/71 phosphorylation suggests a role of ATF2 in the mechanism of resistance. Further studies are needed to elucidate the role of ATF2 in resistance to sunitinib treatment and its potential as a therapeutic target.
phosphorylation, as shown by G-1 treatment, may restrict ATF2 to the cytoplasm, and possibly be correlated with mitochondrial death (54). According to our results, ATF2 phosphorylation participated in the focal adhesion pathway, which affects cell migration. Therefore, the exact role of ATF2 phosphorylation in the GPER1 pathway warrants future studies.

Sunitinib resistance is correlated with cell survival and migration. However, there is controversy over how sunitinib resistance contributes to cell migration; in some studies, sunitinib resistance increased the cell migration rate (55, 56), while others have reported its reduction (57). In the present study, we demonstrated that sunitinib resistance in 786-O cells reduced cell migration. These results may be dependent on the sunitinib concentration, as low-dose sunitinib (1 μM) seemed to promote cell migration, while high concentrations, such as 10 μM, inhibited cell migration. As the half-maximal inhibitory concentration of sunitinib is ~10 μM in the 786-O cell lines (Figure 1A), we anticipate that high-dose sunitinib would provide a better model mimicking acquired resistance, as shown in previous studies (16).

Although G-1 inhibits cell growth and antagonizes sunitinib-resistant pathways, our phosphoproteomics analysis demonstrated that mTOR kinase was up-regulated in SunR 786-O cells, but inhibited in G-1-treated SunR 786-O cells. When compared to SunR cells, however, G-1 treatment of SunR cells further up-regulated mTOR kinase activity. Similar results were also observed for PLK activity. RCC is known to activate the mTOR pathway during the development of resistance to therapies (4). In a recent study, activation of the PLK pathway was shown to promote proliferation and suppress apoptosis in RCC (58). Taken together, the significant up-regulation of mTOR kinase and PLK kinase in G-1-treated SunR 786-O cells raises concerns about the potential side-effects of G-1 on sunitinib-resistant cells.

Some limitations are present in this study. Although G-1 has been recognized as a specific GPER1 agonist, it may regulate RCC growth and phosphorylation dynamics in a GPER1-independent manner. Therefore, further studies are required to dissect the exact role of G-1 by using RCC cells with different GPER1 expression levels, such as GPER1 knock-down or overexpression.

**Conclusion**

In conclusion, we demonstrated that the GPER1 agonist G-1 can regulate phosphorylation pathways related to RCC cell growth and migration, and antagonize the phosphorylation pathways activated by sunitinib resistance. Our results suggest a promising strategy for managing RCC and sunitinib resistance by GPER1 agonist G-1 and its regulated pathways.
Conflicts of Interest

The Authors declare that they have no competing interests in regard to this study.

Authors’ Contributions

SKC, YCW, and WCK designed the experiments. TYL and H JW performed experiments. CJH, and WCK analyzed the data. SKC, CJH, and WCK wrote the article.

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Figure 5. G-Protein-coupled estrogen receptor 1 agonist G-1 down-regulated cell-cycle progression and cell migration. A: Parental (PAR) and sunitinib-resistant (SunR) 786-O cells were treated with dimethyl sulfoxide (DMSO) or 2 μM G-1 for 48 h, and subjected to the designated analyses. A: Immunoblotting of cell-cycle-related proteins phospho-activating transcription factor 2 (P-ATF2) at Thr69 and 71 (T69/71), ATF2, cyclin-dependent kinase 1 (CDK1), cyclin B, cyclin D, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). B: Quantitation of cells in each cell-cycle phase (average±SD) from flow cytometry. C: Typical photograph of migrated cells stained by calcein-AM. D: Quantification (average±SD) of migrated cells shown in (C). All treatments were repeated in triplicate. Significantly different at p<0.05 from *vehicle control, and #indicated group.
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