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Original Article

G-PROTEIN-COUPLED ESTROGEN RECEPTOR (GPER)-SPECIFIC AGONIST G-1 REDUCES CELL PROLIFERATION IN GASTROINTESTINAL CANCER CELLS INDEPENDENTLY OF GPER1 RECEPTOR PRESENCE

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ABSTRACT

Cancer is considered the second leading cause of death worldwide, following cardiovascular diseases. It is believed that the effect of estrogen is not only limited to tumors of hormonally regulated tissues such as the breast, endometrium, and ovary, but also plays a role in the development of gastric and colorectal cancer. Therefore, this study aimed to investigate the apoptotic and antiproliferative effects of G-Protein-Coupled Estrogen Receptor (GPER)-Specific Agonist G-1 on gastrointestinal adenocarcinoma cells. G-1 was applied to human gastric cancer (AGS) and human colon cancer (HT-29) cells at different concentrations. Real-time cellular analysis (RTCA) and MTT assay were used to evaluate cell proliferation. Flow cytometry was employed for the detection of early and late apoptosis using the Annexin-V FITC Kit, Hoechst staining, and analysis of Caspase-3 activation. siRNA was used to knockdown GPER1 expression at the transcriptional level. G-1 exhibited concentration- and time-dependent antiproliferative effects on AGS and HT-29 cells. At the highest concentration, G-1 induced apoptosis in both cell lines. G-1 showed similar antiproliferative effects on cells with and without siRNA treatment. The data suggest that the antiproliferative effects of G-1 appeared to be independent of GPER1 activation G-1 appears to be an attractive tool for suggesting novel targets in the treatment of gastric and colorectal adenocarcinomas.

KEYWORDS: AGS cells, colon cancer, estrogen receptors, gastric cancer, HT-29 cells

1. Introduction

Cancer is considered the second leading cause of death worldwide, following cardiovascular diseases [1]. According to Globocan 2022 data, it ranks 3rd and 5th in terms of colorectal (9.6%) and stomach cancer (4.9%) incidence worldwide [2]. As one of the most common cancers in the world, colorectal cancer (CRC) has a high mortality rate [3]. The global lifetime risk of developing gastric-CRC is approximately 1 in 12 people, and 1 in 16 people diagnosed with this cancer will die from CRC [4].

Epidemiological studies have shown that gastric cancer and CRC occur less frequently in women compared to men of the same age. These data suggest a protective role of estrogen in CRC types [5]. However, this protection is lost when a woman reaches menopause. Cellular and animal studies have also demonstrated the role of estrogen in reducing the formation of colon cancer [5]. The risk of developing colon cancer in women who have never given birth is higher compared to women who have given birth, and hormone replacement therapy is also thought to reduce the development of colon cancer [6-8]. Additionally, the high likelihood of breast cancer and colon cancer co-occurrence is noteworthy [9, 10].

Understanding the progression of cancer, a significant cause of mortality, plays a crucial role in clinical practice and prolonging healthy lifespans. Cancer progression consists of multistage carcinogenesis and metastasis processes involving complex pathways encompassing

genetic and environmental factors [11]. Among these, sex hormones and receptors are important factors in promoting cancer progression. The impact of sex hormones and receptors on cancer progression has been studied for a long time. Until recently, it was known that the effects of estrogens were mediated only by classical estrogen receptors (ERα and ERβ). However, inhibition of ERα and ERβ or deletion of these receptors (knock-out, KO) or knockdown of the corresponding mRNA (silence) did not eliminate estrogen responses. Studies conducted in recent years have demonstrated the presence of the G-protein-coupled membrane estrogen receptor GPER1 and have shown that GPER-1–selective agents are associated with a wide range of estrogen-regulated physiological and pathological processes, including those involving the central nervous, immune, renal, reproductive, and cardiovascular systems. Despite having lower saturation, GPER1 has a single high-affinity binding site for estrogen and lower binding affinity for 17β-estradiol [12]. Binding and dissociation of the receptor and ligand are completed within a few minutes [13]. GPER1 mediates rapid estrogen-dependent signaling events independent of classical estrogen nuclear receptors [12]. GPER1 activates multiple downstream signaling pathways, increasing cyclic AMP (cAMP) levels that support the activation of adenylate cyclase (AC) and intracellular calcium ($Ca^{2+}{}_i$) mobilization and phosphatidylinositol 3,4,5-trisphosphate (IP3) synthesis within the nucleus [14, 15]. GPER1 was first cloned in 1996 and initially named GPR30 [16]. Haas et al. reported the expression of GPR30 in human arterial and venous smooth muscle cells in 2007 and suggested that its effects could vary depending on cellular structures [17-19]. Localization studies have shown that GPER1 is present in the plasma membrane, nucleus, endoplasmic reticulum, mitochondria, and Golgi apparatus and synthesized in various tissues of the human body, including the brain, heart, lungs, liver, kidneys, ovaries, uterus, breasts, testes, stomach, and colon [17, 18]. GPER1 can be activated by estrogen at physiological concentrations, thereby modulating the functions of multiple cellular pathways, including phosphatidylinositol 3-kinase (PI3K), protein kinase (PK), and $Ca^{2+}{}_i$ homeostasis [20]. In recent years, researchers have shown that estrogen and its receptors play significant roles in various gastrointestinal (GI) diseases, including gastroesophageal reflux (GER), esophageal cancer, peptic ulcers, gastric cancer, inflammatory bowel disease, irritable bowel syndrome (IBS), and CRC [21]. The epigenetic downregulation of GPER1 suggests that it functions as a tumor suppressor in CRC, and its activation may hold potential therapeutic value for CRC treatment [22].

G-1 (IUPAC name: (±)-1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone) is a synthetic, non-steroidal small molecule that selectively activates GPER1. It was first developed by Bologa et al. (2006) as a selective agonist that does not interact with classical nuclear estrogen receptors ERα or ERβ, providing a critical tool to distinguish GPER1-dependent signaling from ERα/ERβ-mediated pathways [23]. Over the past two decades, evidence has shifted the understanding of estrogen signaling from a solely nuclear receptor–dependent mechanism to a broader model that includes rapid, membrane-initiated pathways [24]. The identification of GPER1 as a mediator of these rapid responses has generated substantial interest in its physiological and pathological roles, particularly in cancer biology.

It is believed that the effects of estrogen are not limited to hormonally regulated tissues such as the breast, endometrium, and ovary, but also extend to the development of gastric and CRCs. Estrogens mediate their actions through nuclear receptors ERα and ERβ as well as the membrane-bound GPER1. Although the antitumor properties of G-1 have been demonstrated in several cancer types, including lung, breast, and prostate cancers, through GPER1-mediated signaling pathways that induce cell-cycle arrest and apoptosis, its role in gastrointestinal cancers remains poorly characterized [25, 26]. Limited data exist regarding the impact of GPER1 activation on gastric and CRC cell survival, proliferation, and apoptosis. Therefore, further investigation of this pathway represents an unmet need in the field.

G-1 was selected for this study because it is the most widely validated and highly selective GPER1 agonist, enabling specific activation of GPER1 without confounding ERα/ERβ signaling. This specificity makes G-1 an ideal tool for elucidating the potential contribution of GPER1 to gastrointestinal cancer biology, particularly in the human gastric cancer (AGS) and human colon cancer (HT-29) cell lines.

2. Materials and Methods

2.1. Chemicals and reagents

GPER1 agonist G-1 (Cayman Chemical, CAS No: 881639-98-1) was dissolved in 100% DMSO (SERVA, Germany) to prepare the stock solution. Cell culture media, including F-12K and McCoy's 5A (Gibco/BRL, Gaithersburg, MD, USA), were supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL), 1% penicillin-streptomycin (Gibco/BRL), phosphate-buffered saline (PBS, Gibco, UK), trypsin-EDTA (Capricorn, USA) and 1% L-glutamine (Hyclone, Logan, Utah). Hoechst dye, MTT reagent, and Annexin V-PI staining kit were obtained from Sigma-Aldrich. The Caspase-3/CPP32 Colorimetric Assay Kit and the ApoDIRECT In Situ DNA Fragmentation Assay Kit were purchased from BioVision. The ON-TARGETplus siRNA pool targeting GPER1 (Dharmacon, Cat. No: L-005563-00) was used for gene silencing, and a non-targeting siRNA pool (Dharmacon, Cat. No: D-0018-10-05) served as the negative control.

2.2. Cell culturing

Human gastric cancer AGS and human colon cancer HT-29 cell lines were obtained from the American Type Tissue Culture Collection (ATCC). The cells were cultured in F-12K and McCoy's 5A media (Gibco/BRL, Gaithersburg, MD, USA) supplemented with 1% penicillin-streptomycin (Gibco/BRL, Gaithersburg, MD, USA) 1% L-glutamine (Hyclone, Logan, Utah) and 10% Fetal Bovine Serum (FBS) (Gibco/BRL, Gaithersburg, MD, USA) at 37°C with 5% CO_2. Routine checks of the cells were performed by using an inverted microscope. The cells were passaged at approximately 70-80% confluency. A portion of these expanded cells was stored frozen at -80°C for use in subsequent experiments. G-1 (CAS No: 881639-98-1; Cayman Chemical) was initially dissolved in 100% dimethyl sulfoxide (DMSO) (SERVA, Germany) to prepare a 10^{-2} M

stock solution. Serial dilutions were then performed using cell culture medium to obtain final working concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M). All working concentrations used in this study were prepared following this same dilution scheme, resulting in final DMSO concentrations in the treatment medium ranging from 0.1% to 0.00001% (v/v). Hoechst dye (Sigma-Aldrich, Germany) was diluted with water to prepare a 10 mg/mL stock solution, which was further diluted 1:1000 with cell culture medium prior to use.

2.3. Cytotoxicity analysis

Cell viability and cytotoxicity analysis were assessed via the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is a rapid and reliable method based on the conversion of the tetrazolium salt to a formazan product and the measurement of the resulting product's quantity by colorimetric assay using a spectrophotometer. For this purpose, AGS and HT-29 cells were seeded into 96-well plates at a density of 10,000 cells per well (n=8). The G-1 agonist was prepared at increasing concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) was applied to the wells of the 96-well plate. After incubation for 48 and 72 hours, 0.5 mg/mL MTT solution (Sigma-Aldrich, Germany) was added to each well, and the plate was incubated for 4 hours at 37°C. At the end of this period, 100 µL of DMSO solvent was added to each well, and the absorbance measured at wavelengths of 490 nm and 630 nm as reference range using a spectrophotometer (ELx800 Absorbance Reader; BioTek Instruments, Inc., Winooski, VT, USA) [27].

A real-time cell analysis system (RTCA, xCELLigence, Agilent) was also used, to evaluate cell viability and proliferation without labeling, through monitoring changes in live-cell impedance (cell index) [28]. Initially, cell titration experiments were conducted for each cell line in the RTCA device. The optimum seeding density (cells/well) was 125,000 and 31,250 for AGS and HT-29 cells, respectively (n=8). After seeding the cells into the e-plate, the G-1 agonist at increasing concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) was applied to the cells 24 hours later for cytotoxicity experiments. The cell index was monitored every 15 minutes for 72 hours. After completing the experiment, proliferation curves were generated using the built-in software of the instrument, and IC50 values were calculated [28].

After incubating the cells with different concentrations of G-1 for 48 hours, they were collected, and 100 µL of Hoechst dye was pipetted and mixed (n=6). After incubating for 15 minutes at 37°C in an incubator, the cells were centrifuged to form a cell pellet again. The dye was removed, and the cells were washed with preheated PBS at 37°C and examined under a fluorescence microscope [29].

2.4. Apoptosis analysis

For the demonstration of the apoptotic effects of G-1 in our study, Annexin V-PI staining (Sigma-Aldrich) was performed, and Caspase-3 activation and DNA fragmentation were analyzed.

2.4.1. Annexin V-PI staining

For apoptosis analysis, AGS ($2.5x10^5$) and HT-29 (10^5) cells were seeded into 24-well plates (n=3). After a 24-hour incubation period, cells were treated with different concentrations of the GPER1 agonist G-1 (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, $5x10^{-6}$, 10^{-5} and $5x10^{-5}$ M). After 48 hours of treatment, cells were washed with PBS, trypsinized, and collected in Eppendorf tubes containing PBS. The resulting cell pellet was resuspended in 100 µL of binding buffer for further analysis. After incubating with Annexin V and PI dyes for 15 minutes in the dark, the samples were analyzed using a flow cytometer (BD Biosciences, San Jose, CA) [30].

2.4.2. DNA fragmentation assay

For the analysis of DNA fragmentation in cells treated with G-1, the ApoDIRECT In Situ DNA Fragmentation Assay (BioVision) was utilized (n=6). This kit, which includes positive and negative controls, enables the detection of DNA fragmentation both under a fluorescence microscope and using a flow cytometer. Following the same procedure as described for caspase-3 activation analysis, cells were harvested by trypsinization 48 hours after treatment with the compound. The harvested cells were then prepared based on the manufacturer' guidelines and examined under a fluorescence microscope [29].

2.4.3. Caspase-3 activation analysis

For the analysis of caspase-3 activation, the Caspase-3/CPP32 Colorimetric Assay Kit (BioVision) was used. AGS and HT-29 cells were seeded into 6-well plates at a density of $1x10^6$ cells per well (n=6). After incubating the cells for 24 hours to allow for attachment, the culture media in the wells were removed, and compound-DMEM mixtures containing 10^{-6} and 10^{-5} M of the G-1 were added. Only DMEM was added to the wells containing control cells. After 48 hours of incubation, 1 mL of culture medium was removed from the wells and discarded. The remaining medium was aspirated, and the cells were scraped into an Eppendorf tube. The absorbance of each well at 400-405 nm was read using an ELISA plate reader (Thermo, Vantaa, Finland) [31].

2.5. Suppression of GPER1 expression

Post-transcriptional control mechanisms of gene expression regulate the localization and stability of mRNAs in the cytoplasm or their translation into proteins. One of these mechanisms involves short interfering RNAs (siRNAs) that induce the degradation of target mRNAs. In our study, suppression of gene expression was achieved by transfecting the ON-TARGET plus siRNA pool targeting the GPER1 mRNA (Dharmacon, Cat. No: L-005563-00). A non-targeting siRNA pool was used as a negative control (Dharmacon, Cat. No: D-0018-10-05).

For transfection, $4x10^5$ cells were seeded into each well of a 24-well plate containing 1000 µL of antibiotic-free growth medium (n=3). When the cells reached 85-90% confluence, siRNA transfection was performed using Dharmafect transfection reagent (2 µL) in each well. 5 µM siRNA was diluted in serum free medium and mixed with diluted transfection reagent. After incubating at room temperature for 20 minutes, 100 µL of the mixture was added to the wells containing antibiotic free cell medium. The cells were then incubated at 37°C in a 5% CO_2 incubator for 48 hours for mRNA analysis [32]. Real-time PCR (RT-PCR) amplification of the cDNA was performed using a PikoReal 24 RT-PCR (Thermo Fisher Scientific) and analyzed with PikoReal software 2.2 (Thermo Fisher Scientific).

2.6. Analysis of GPER1 mRNA expression

GPER1 mRNA expression was determined using qRT-PCR with gene-specific primer-probes. For this purpose, total RNA isolation was performed from the experimental groups, and after constructing complementary DNA (cDNA), GPER1 expression was determined using qRT-PCR (n=6). *GADPH* was used as the housekeeping gene to normalize GPER1 expression [32].

2.7. Statistical analysis

All data were expressed as mean ± standard deviation. For statistical analysis, one-way analysis of variance (ANOVA) and Dunnett post hoc test were applied using GraphPad Prism 10.0. Data with $P<0.05$ were considered significant. Analysis of the findings obtained from cytotoxicity experiments, determination of IC50 values, and statistical analysis of changes occurring at the IC50 concentration by comparing different data sets were performed using the software program of the RTCA system.

3. Results

3.1. The cytotoxic effects of GPER1 agonist G-1

According to the MTT results, GPER1 agonist G-1 exhibited concentration- and time-dependent cytotoxic effects on AGS cells. G-1 significantly reduced cell viability at concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M at 48 hours. At 72 hours, significant reductions in cell viability compared to control were determined with doses of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M (Fig. 1A).

The concentration and time-dependent cytotoxic effects of G-1 were also observed in HT-29 cells. According to the MTT results, cell viability significantly decreased in HT-29 cells treated with G-1 (10^{-7}, 10^{-6} and 10^{-5} M) at 48 hours. At 72 hours, significant reductions in cell viability were determined with doses of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M compared to control (Fig. 1B).

In proliferation experiments conducted using RTCA, it was observed that the G-1 did not affect proliferation at low concentrations in AGS and HT-29 cells, while at intermediate concentrations, it reduced the growth and proliferation of cancer cells. The highest concentration, $5x10^{-5}$ M, produced a significant antiproliferative effect (Fig. 2A, 2B). The results of the RTCA proliferation experiments were evaluated using the device's built-in software, and the IC_{50} concentration of G-1 was calculated for each cell line. Accordingly, the IC_{50} of G-1 was determined to be $1.27x10^{-5}$ M for the AGS cell line, and $1.76x10^{-5}$ M for the HT-29 cell line.

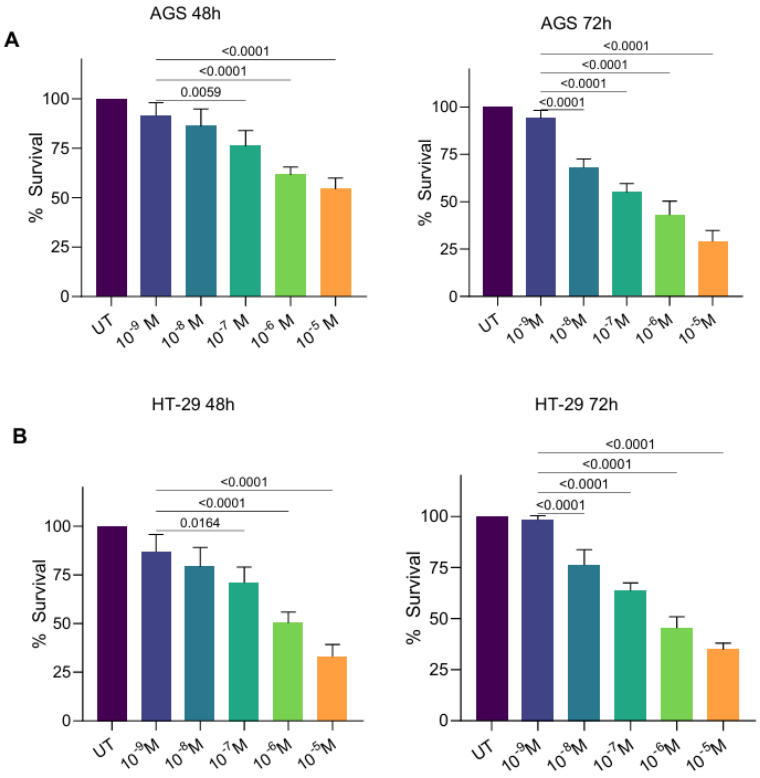

Fig. 1. Cytotoxic effects of GPER1 agonist G-1. MTT analyses at 48 and 72 hours in AGS (A) and HT-29 (B) cells. Cells were treated with increasing concentrations of G-1 (10^{-9} to 10^{-5} M). Data represents the mean ± SD of three independent biological replicates, each performed in triplicate. 17β-estradiol (10^{-9} M) was used as control. $p<0.05$ (one-way ANOVA, followed by Dunnett's post hoc test). UT: Untreated

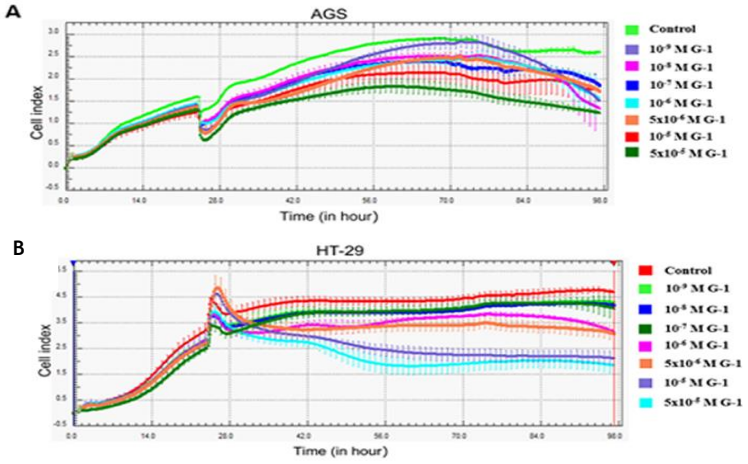

Fig. 2. The effect of the GPER1 agonist G-1 on cell proliferation at different concentrations. Cell index graphes obtained from RTCA software for **A:** AGS cell series, **B:** HT-29 cell series.

3.2. Apoptosis results

Based on the flow cytometric analysis following Annexin V/PI staining, increasing the concentration from 10^{-9} to 5×10^{-5} M resulted in a concentration-dependent increase in necrosis, late apoptosis, and early apoptosis. After 48 h exposure to 10^{-9} M G-1, AGS cells exhibited 45.6% necrosis, 3.7% late apoptosis, and 2.3% early apoptosis, whereas HT-29 cells showed 14.3% necrosis, 0.8% late apoptosis, and 0.9% early apoptosis. At the 5×10^{-5} M concentration, AGS cells showed 49.1% necrosis, 9.9% late apoptosis, and 4.1% early apoptosis, while HT-29 cells exhibited 32.6% necrosis, 2.6% late apoptosis, and 1.5% early apoptosis (Fig. 3).

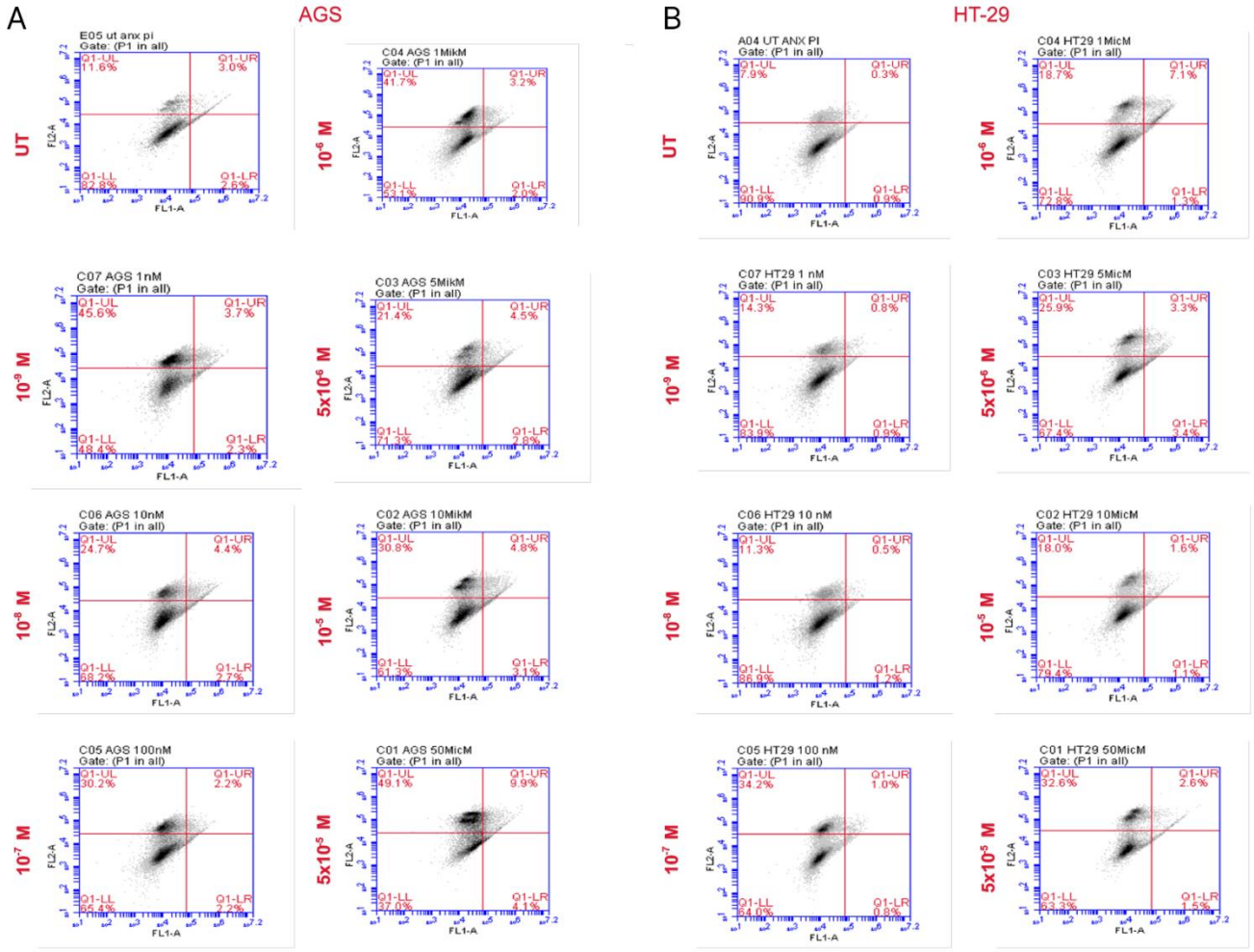

Fig 3. Annexin/PI staining findings in cells treated with G-1 agonist **A:** Flow cytometry graphs of AGS cell series and **B:** Flow cytometry graphs of HT-29 cell series.

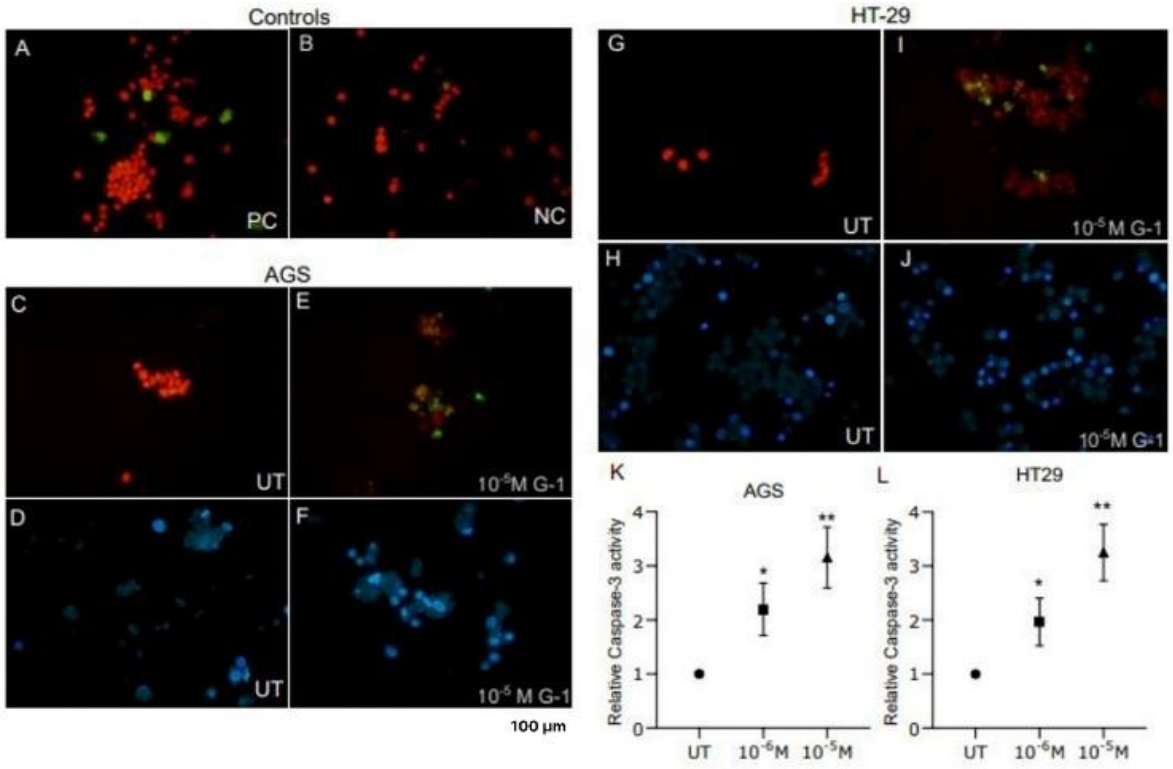

Fig. 4. DNA fragmentation in **A:** positive control and **B:** negative control samples (green cells indicate fragmented DNA-containing cells). Fluorescence imaging (scale bar = 100 µm). **C, D, E, F:** DNA fragmentation in untreated (UT) control and 10^{-5} M G-1 treated AGS cells. Green staining shows mitochondria and cytoskeleton (Alexa Fluor 488). Red staining shows apoptotic cells (Alexa Flour 594), Blue staining shows nucleus and DNA. **G, I, H, J:** DNA fragmentation in control and 10^{-5} M G-1 treated HT-29 cells. **K:** Relative caspase-3 activity in AGS cells treated with 10^{-6} M and 10^{-5} M G-1 for 72 hours, normalized to the mean of the untreated control group (*p=0.0004; **p<0.0001). **L:** Relative caspase-3 activity in HT-29 cells under the same conditions, normalized to the mean of the untreated control group (*p=0.0014; **p<0.0001). Data in (**K**) and (**L**) are presented as mean ± SD of n = 6 samples. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test to compare each treatment group to the untreated control.

In our study, the analysis of DNA fragmentation in cells treated with G-1 was performed using the ApoDIRECT In Situ DNA Fragmentation Assay, which operates based on the TUNEL method, and Hoechst dye, which emits blue fluorescent light when bound to double-stranded DNA. The cells stained with propidium iodide/RNase A solution were analyzed under a fluorescent microscope. DNA fragmentation was examined in positive and negative control samples included in the kit (Figure 4A-B). Comparable to the control, an increase in DNA fragmentation and the presence of condensed nucleus pyknotic cells were observed in AGS cells treated with 10^{-5} M G-1 (Fig. 4C-F). Similarly, an increase in DNA fragmentation and pyknotic cells was observed in HT-29 cells treated with 10^{-5} M G-1 (Fig. 4G-J).

The application of G-1 in AGS and HT-29 cells significantly increased caspase-3 activation. Approximately a two-fold increase in caspase-3 activation was observed in cells treated with 10^{-6} M G-1, while a three-fold increase was observed in cells treated with 10^{-5} M G-1 (Fig. 4K-L).

3.3. Effects of GPER1 inhibition on cell viability

GPER1 expression was knocked down by siRNA application in AGS and HT-29 cells (Fig. 5A). In the MTT cell viability assay results of GPER1 knockdown cells, the GPER1 agonist G-1 showed similar effects on both untreated and treated AGS and HT-29 cells (Fig. 5B-5C). According to these results, the antiproliferative effect induced by G-1 appeared to be GPER1-independent.

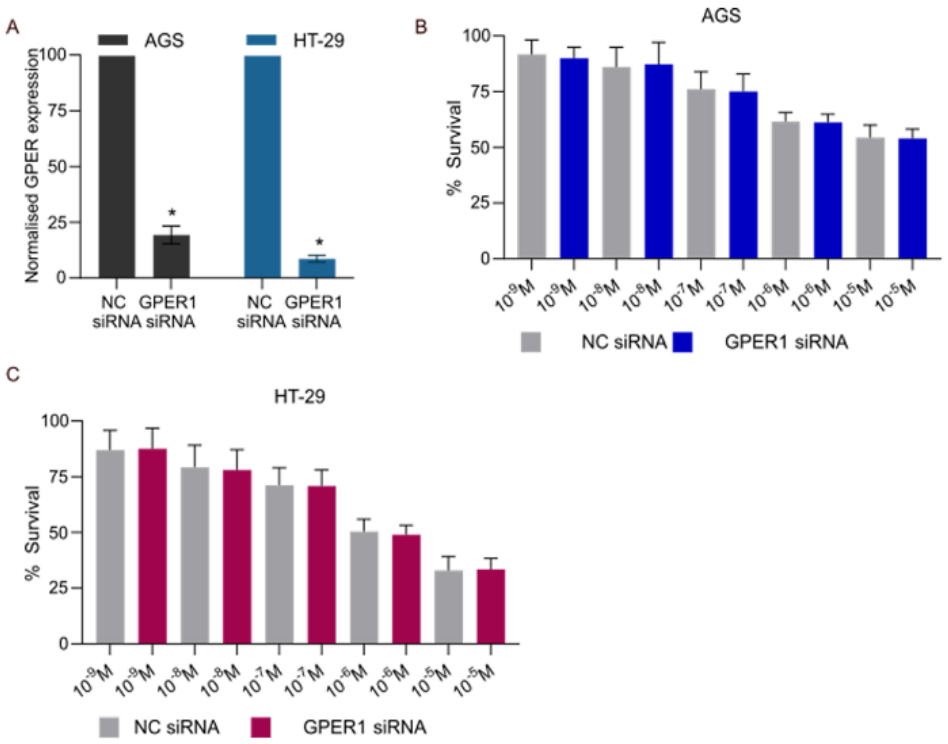

Fig. 5. Effects of GPER1 knockdown on cell proliferation **A**: GPER1 mRNA expression in cells treated with siRNA. **B**: Effect of G-1 substance on cell proliferation (%) in AGS cells transfected and nontransfected with siRNA. **C**: Effect of G-1 substance on cell proliferation (%) in HT-29 cells transfected and nontransfected with siRNA. NC: Non-targeting siRNA pool.

4. Discussion

GPER1 agonist G-1 has been previously shown to exert both GPER1-dependent and independent antiproliferative effects in various cancer models. For example, it inhibited proliferation in PC-3 prostate and MCF-7 breast cancer cells via GPER1, while inducing GPER1-independent apoptosis in KGN ovarian and MDA-MB-231 breast cancer cells [33,34]. Similarly, in our previous work on A549 lung cancer cells, G-1 significantly reduced proliferation and increased apoptosis at 2×10^{-5} M, alongside increases in nitric oxide levels and antioxidant enzyme activity [35]. Possible G-1 mechanisms independent of GPER1 should be considered. Several studies indicate that G-1 can directly target the cytoskeleton by binding to the colchicine-binding site of tubulin and inhibiting microtubule polymerization, producing mitotic arrest and antiproliferative effects similar to classical microtubule-targeting agents [36]. Moreover, G-1 has been reported to activate mitochondrial apoptotic pathways and to promote mitochondrial fission, suggesting a role for mitochondrial dysfunction in its cytotoxicity [37]. In addition, G-1 has been shown to induce oxidative stress and trigger a ROS → MAPK → Egr-1 → BAX signaling cascade in adrenocortical carcinoma (ACC) cells, an effect that has been described as at least partially GPER1-independent [38]. Notably, several reviews state that G-1's off-target activities become more likely at micromolar concentrations and therefore concentration-dependent off-target mechanisms (tubulin binding, ROS induction) should be considered when interpreting results [39].

The role of GPER1 in Shikonin (SK)-induced apoptosis was investigated in human ovarian adenocarcinoma cell lines SKOV3 and A2780. Using a combination of assays, it was reported that SK promotes apoptosis through the GPER1/EGFR/PI3K/AKT signaling pathway. These findings highlight GPER1 as a critical mediator of apoptotic responses in ovarian cancer cells [40]. G-1 exposure decreased the cyclin B expression, induced cell-cycle arrest, and led to apoptosis in ER-breast cancer cell lines [41]. Lv et al. demonstrated that G-1 effectively suppresses the growth of breast cancer cells both *in vitro* and in xenograft mouse models. Notably, this antiproliferative effect was independent of the expression status of GPER1 and classical estrogen receptors, suggesting a receptor-independent mechanism [42]. Hirtz et al. investigated the effects of the GPER agonist G-1 on glioblastoma cell proliferation and found that G-1 exerts a cytostatic effect by inducing a reversible G2/M cell cycle arrest. This was attributed to G-1's ability to disrupt tubulin polymerization during mitosis. Importantly, consistent with our findings, the observed effects were independent of GPER signaling. The authors proposed that G-1 could serve as a potential adjuvant to standard chemotherapy in limiting glioblastoma progression and aggressiveness [39].

Liu et al. investigated the expression and functional role of GPER in CRC and demonstrated that G-1, at concentrations of 8.55–11.7 μM, suppressed CRC progression [22]. In our study, we similarly evaluated the antiproliferative effects of G-1 using MTT and xCELLigence assays in AGS and HT-29 cancer cell lines. Consistent with Liu et al., we observed a reduction in cell viability at comparable concentrations. In contrast, Gilligan et al. reported that G-1 (0.001–1000 nM) increased proliferation in HCT-116 cells [43]. This discrepancy may be attributed to concentration-dependent effects of G-1, where lower doses could

promote proliferation, while higher doses induce antiproliferative responses. Furthermore, while both MTT and xCELLigence methods generally yielded consistent results in our study, minor differences at lower concentrations may stem from the methodological nature of the assays as xCELLigence measures impedance-based changes in real time, whereas MTT quantifies mitochondrial dehydrogenase activity via formazan production. Nonetheless, both approaches confirmed significant antiproliferative effects of G-1 at medium and high concentrations, aligning with findings in other cancer types such as prostate, ovarian, and breast cancers [22].

Lee et al. demonstrated that elevated GPER1 expression in gastric cancer cells enhanced G-1-induced antitumor effects via increased levels of cleaved caspase-3, -9, and PARP in a xenograft model [44]. Similarly, Weißenborn et al. showed that G-1 induced GPER1-dependent apoptosis and G2/M arrest in breast cancer cells [45], while another study reported G-1–induced caspase-3/7 activation without significant cytotoxicity in endometriotic stromal cells [46]. ß-estradiol was also found to trigger apoptosis in HeLa cells by suppressing the AKT/NF-κB pathway [46], and calycosin inhibited CRC proliferation via estrogen receptor ß-regulated pathways [47]. These findings highlight the role of estrogen-related pathways in cancer cell apoptosis. Consistently, in our study, G-1 increased caspase-3 activation and induced apoptosis in AGS and HT-29 cells, as shown by Annexin V-PI and Hoechst staining.

Wei et al. found that G-1 upregulates p53 expression in breast cancer cells [41]. Shi et al. investigated the role of GPER1 in cryptotanshinone (CPT)-induced apoptosis in MCF-7 cells and found that CPT's effects were mediated via the GPER1/PI3K/AKT pathway, confirmed using siRNA and the GPER1 agonist G-1 and antagonist G-15 [48]. Similarly, Bustos et al. showed that GPER1 knockdown in HT-29 cells abolished estrogen- and G-1-induced antiproliferative effects under normoxic and hypoxic conditions, emphasizing GPER1's regulatory role [49]. In endometrial carcinoma cells, GPER1 knockdown suppressed estrogen-induced Cav1.3 upregulation and downstream signaling, reducing cell proliferation and migration [50]. In contrast, our study showed that G-1's antiproliferative effect in AGS and HT-29 cells persisted despite GPER1 knockdown, suggesting a GPER1-independent mechanism. The discrepancy with studies by Lee et al. and Bustos et al. may be due to off-target effects of G-1 at higher concentrations [22]. Our results therefore support the hypothesis that the effect of G-1 on gastric and CRC activity follows an inverted U-shaped dose-response curve only at supraphysiological levels [51]. Our results indicate that G-1 exerts antiproliferative effects on AGS and HT-29 cells via GPER1 independent signaling pathways [52].

Initial characterization studies demonstrated that micromolar concentrations (1–10 μM) of G-1 are necessary to induce classical GPER1-mediated responses, including ERK1/2 phosphorylation and transcriptional activation [23]. Subsequent cancer studies employed similar concentrations to elicit antiproliferative or proapoptotic effects in breast, endometrial, adrenal, and prostate cancer cells [53]. The requirement for exposure to the micromolar range is thought to reflect the compound's non-steroidal structure, relatively limited membrane permeability, and rapid metabolic turnover [54]. Although higher concentrations may increase the likelihood of off-target actions, recent evaluations indicate that the 1–10 μM range remains within the pharmacologically acceptable window before microtubule disruption [55]. Therefore, the concentrations selected in this study align with the established pharmacodynamic profile of G-1 and are considered physiologically meaningful for *in vitro* GPER1 research.

5. Conclusions

In this study, G-1 showed concentration- and time-dependent antiproliferative effects independent of GPER1 activation in AGS and HT-29 cancer cell lines. Our *in vitro* study provided a preliminary data for evaluation of the reported GPER1 agonist G-1 as a potential therapeutic agent in gastric and CRC. Following *in vivo* studies performed in animal models, combined treatment strategies with chemotherapeutic agents could be developed for gastric cancer and CRC clinical trials.

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