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Pro-GA, a Novel Inhibitor of γ-Glutamylcyclotransferase, Suppresses Human Bladder Cancer Cell Growth

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Abstract. *Background/Aim: γ-Glutamylcyclotransferase (GGCT), a key enzyme involved in glutathione metabolism, catalyzes a specific reaction that generates 5-oxoproline and free amino acids from the γ-glutamyl peptide. Inhibition of GGCT is a promising therapeutic strategy for the treatment of various cancers. Materials and Methods: Immuno-histochemistry was used to evaluate GGCT expression in bladder tumors. The growth inhibitory effect of pro-GA, a novel GGCT inhibitor, in the presence or absence of mitomycin C (MMC) was assessed in three distinct bladder cancer cell lines. Results: Over half of the clinical bladder tumor samples overexpressed GGCT. Pro-GA reduced the growth of all bladder cancer cell lines in a dose-dependent manner, and increased the anti-tumor effect of MMC. Conclusion: Inhibition of GGCT using pro-GA provides a novel therapeutic strategy for the treatment of bladder cancers.*

Bladder cancer is the fourth most common cancer in men in the United States, and although it is less common in women, almost 20,000 new cases are diagnosed each year (1). Approximately 75% of patients with this malignancy present with non-muscle invasive bladder cancer (NMIBC) (2). In NMIBC cases, bladder preservation therapy with transurethral resection of the bladder tumor (TURBT) is usually performed as the initial treatment. Intravesical instillation therapy of anticancer drugs or bacillus Calmette-Guerin (BCG) is performed for preventing intravesical recurrence of post-TURBT, although the efficacy of this approach is limited. Therefore, innovative new therapeutic drugs are desired.

In earlier studies, we analyzed the proteome of bladder cancer tissues to identify potential therapeutic targets (3-5). We identified chromosome 7 open-reading frame 24 (C7orf24), which was registered as a hypothetical protein with unknown function (6). Later, Oakley *et al*. identified C7orf24 as γ-glutamylcyclotransferase (GGCT) (7), one of the major enzymes involved in glutathione metabolism (8, 9). GGCT catalyzes a specific reaction that generates 5-oxoproline and free amino acids from the γ-glutamyl peptide. GGCT is overexpressed in various cancers, and its knockdown has antiproliferative effects in tumor cell lines (6). This positions GGCT as a potential therapeutic target. Through the screening of small molecules, we identified *N*-glutaryl-L-alanine (GA) as an efficient inhibitor of GGCT enzymatic activity (10). Moreover, we have developed a novel cell-permeable diester-type pro-drug, which we named 'pro-GA, (11). The anticancer activity of pro-GA was demonstrated in human cancer cells, and its administration exhibited anticancer effects in a prostate cancer xenograft mouse model (11). Therefore, targeting GGCT with pro-GA may provide a promising therapeutic strategy for the treatment of a wide range of aggressive malignancies expressing GGCT.

In this study, we examined the expression of GGCT in NMIBC tissues obtained by TURBT, and verified the anti-cancer effect of pro-GA in bladder cancer cells.

Materials and Methods

Patients. One hundred and thirty patients underwent TURBT for the treatment of NMIBC at the Shiga University of Medical Science Hospital from 2001 to 2015, and were analyzed in the present study. The patient's demographics were those of a typical NMIBC cohort. Median age was 67 years (range=25-88 years). Genders were 106 males and 24 females. Ta and T1 were 98 and 32, respectively. Histological grades (WHO classification 1973) were 48, 69 and 13 in G1, G2 and G3, respectively. All patients had primary bladder tumors and no history of urothelial cancer. Clinico-pathological data were obtained from medical records of the patients. The clinical stage was determined by UICC TNM classification (12). The study was approved by the Ethical Committee of Shiga University of Medical Science (No. 29-081).

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Immunohistochemistry. Surgical specimens obtained by TURBT were transferred to 10% buffered formalin and fixed overnight. The fixed samples were embedded in paraffin and sliced into 5-μm sections. After dewaxing, sections were autoclaved at 120˚C for 1 min in 10 mM sodium citrate buffer (pH 6.0) and immersed in 0.3% H_2O_2. The sections were incubated overnight at 4˚C with primary antibody against GGCT (clone 6-1E, TSS-M01, Cosmo Bio Co., Tokyo, Japan; diluted 1:5,000) established by Kageyama *et al*. (6). They were rinsed with PBS and incubated with a secondary antibody conjugated to horseradish peroxidase (SimpleStain MAX-PO, MULTI; Nichirei, Japan) at room temperature for 1 h. The sections were then stained with 3,3'-Diaminobenzidine tetrahydro-chloride and counter-stained with hematoxylin.

Cell lines. Bladder cancer cell lines (UMUC3, T24 and RT112) were used for the experiments. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin. These cells were cultured in a humidified 5% CO_2/95% air incubator at 37˚C.

Western blot analysis. Cells were generally lysed for western blotting as described by Sarbassov, *et al*. (13). After clearing lysed materials by centrifugation at 15,000 × *g* for 10 min, the supernatants were boiled in SDS sample buffer. Proteins resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (4-15% gel) were transferred to polyvinylidene difluoride (PVDF) filters. After blocking of the filters with TBS-T (10 mM Tris-HCl (pH 7.6), 150 mM sodium chloride, 0.1% Tween 20) containing 1.5% skim milk, the filters were incubated overnight at 4˚C with the indicated anti-GGCT antibody (clone 6-1E) diluted 1:10,000 and anti-β-actin antibody (clone AC15, Sigma-Aldrich, St. Louis, MO, USA) diluted 1:10,000 in TBS-T containing 1.5% skim milk. The filters were then washed in TBS-T and incubated for 1 h in horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, Hatfield, UK) diluted 1:10,000 in TBS-T containing 1.5% skim milk. After several washes with TBS-T, the immunoreactivity was detected using the Amersham ECL Prime (GE Healthcare) and LAS4000 biomolecular imager (Fujifilm, Tokyo, Japan), according to the procedures recommended by the manufacturers.

Introduction of siGGCT. Synthesized siRNA targeting GGCT (siGGCT) and negative control siRNA (AllStars Negative Control siRNA) were purchased from Qiagen (Hilden, Germany). The sequence of siGGCT is as follows: 5'-UGACUAUACAGGAA AGGUCTT-3', which was used in the previous studies (6, 14, 15). Using Lipofectamins RNAi MAX (Invitrogen), siRNA was transfected into cells at a final concentration of 10 nM, according to manufacturer's instructions.

Proliferation inhibitory assay using pro-GA. Cells were seeded at 1,000 cells/ well in 96-well plates. One day later, the medium was changed to D-MEM containing 50 and 80 μM pro-GA. After 2-6 days, cell viability was assessed using a WST-8 assay.

Determination of pro-GA IC_{50}. Cells were seeded at 3,000 cells/ well in 96-well plates. DMEM, with 10% FBS, HEPES, 100 μg/ml penicillin and 100 μg/ml streptomycin, was used as the culture medium. One day later, the medium was changed to DMEM containing various concentrations of pro-GA (0 to 180 μM), a specific inhibitor of GGCT that was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). After 72 h, cell variability was assessed using the WST-8 assay with Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan). The experiments were repeated in triplicate. The IC_{50} value (drug concentration producing 50% cell kill) was determined from a plot of percentage cell survival *vs*. drug concentration.

Combined treatment between pro-GA and mitomycin C. Cells were seeded at 2,000 cells/well in 96-well plates. One day later, the medium was changed to DMEM containing 0.7 μg/ml mitomycin C (MMC) and incubated for 1 h. Cells were then washed twice with 1× PBS, and the culture medium was refreshed. On day 3, the culture medium was changed again with a fresh one containing 100 μM pro-GA. Cell viability was assessed by WST-8 assay on days 5-7.

Statistical analysis. The correlation between GGCT staining grades and pathological features (T stage and pathological grade) were analyzed by Fisher's exact test. Recurrence-free survivals were estimated using the Kapan–Meier method and statistical differences were analyzed using the log-rank test. To analyze the effects of pro-GA with or without MMC, one-way factorial ANOVA accompanied by Tukey's significance test was used. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using EZR software (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html) (16).

Results

GGCT expression and clinico-pathological features of TURBT specimens. GGCT expression was immunohistochemically analyzed in 130 cases of TURBT. Immunohistochemical grading was performed by two independent researchers who had no information of the clinical parameters. Intensity of GGCT expression was allocated into one of four grades; negative (–), weak (–/+), moderate (+) and strong (++). Representative specimens are shown in Figure 1A. For further analysis, we categorized grades of GGCT expression into two groups; high- (+, ++) and low-GGCT (–, –/+). In terms of pathological stage, the rates of high GGCT were 57% and 66% in Ta and T1, respectively (p=0.417). Regarding pathological grade, the rates of high GGCT were 52%, 62% and 77% in G1, G2 and G3, respectively (p=0.255). Regardless of pathological stage and grade, high GGCT expression was present in more than half of the cases (77 of 130). In all patients, there was no difference in recurrence-free survival between low- and high-GGCT groups (Chi-square=1.7, p=0.191; Figure 1B). Otherwise, in Ta patients (n=98), there was a trend toward better recurrence-free survival in the low-GGCT group (log-rank test, chi-square=2.9, p=0.088; Figure 1C).

Growth inhibition of GGCT knockdown in bladder cancer cells. Three independent bladder cancer cell lines, UMUC3, T24 and RT112, expressed high levels of GGCT that were

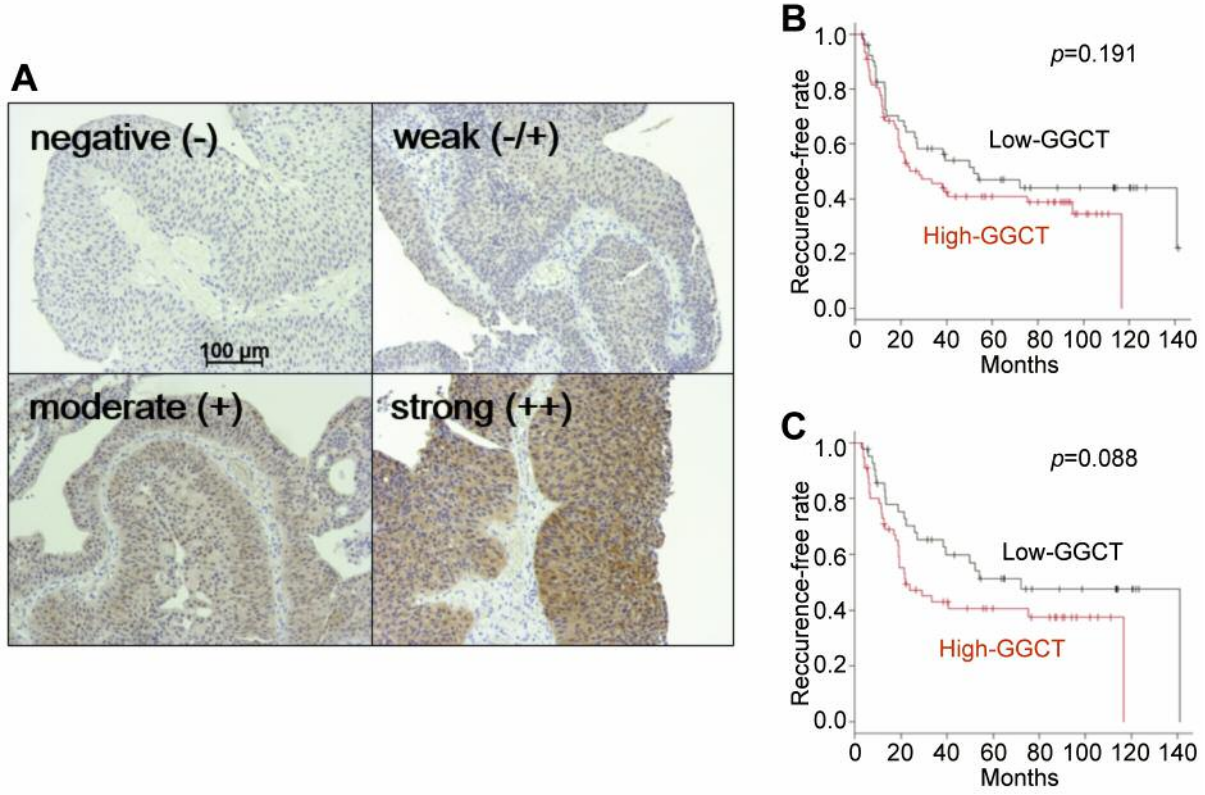

Figure 1. *GGCT expression and prognosis. A: Representative immunohistochemical GGCT staining in TURBT specimens. GGCT expression was classified in four grades; negative (-) (upper left), weak (–/+) (upper right), moderate (+) (lower left) and strong (++) (lower right). The grades of GGCT expression were categorized into two groups; low- and high-GGCT from the expression grades of –, –/+ and +, ++, respectively. Scale bar: 100 μm. Magnification was ×100. B: Recurrence-free survival in all patients of the cohort (n=130). There was no difference in recurrence-free survival between the low-GGCT group (black line) and the high-GGCT group (red line). Log-rank test; Chi-square=1.7, p=0.191. C: Recurrence-free survival in Ta patients (n=98). There was a trend toward a longer recurrence-free survival in the low-GGCT group compared to that of the high-GGCT group. Log-rank test; chi-square=2.9, p=0.088.*

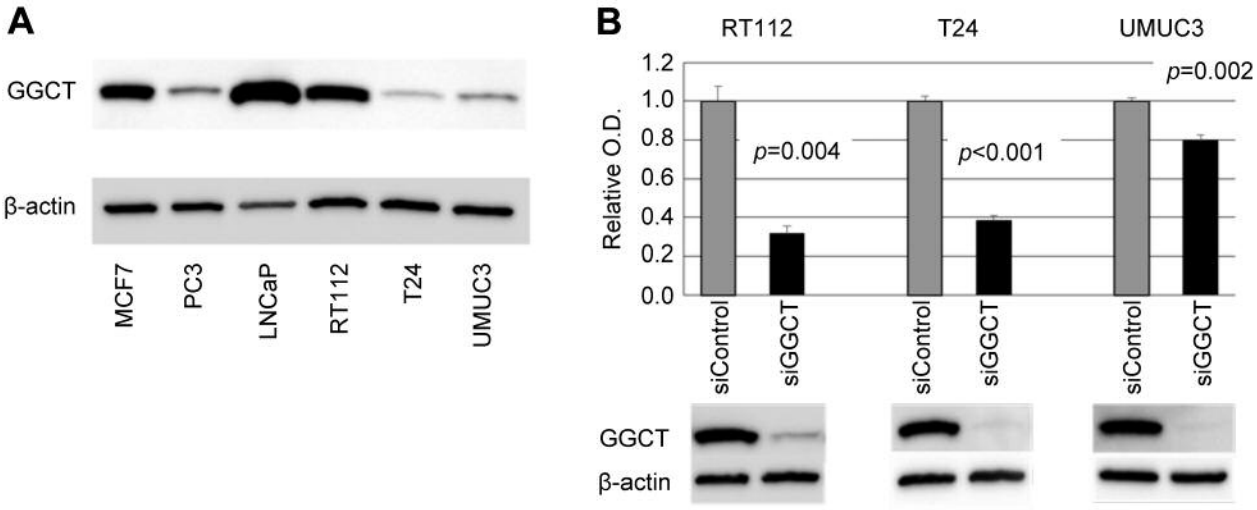

Figure 2. *GGCT expression and the effect of GGCT knockdown on the growth of bladder cancer cells. A: Evaluation of GGCT expression in MCF7, PC3, LNCaP, RT112, T24 and UMUC3 cells, using western blots. B: Growth inhibition by the introduction of siGGCT in RT112, T24 and UMUC3 cells. Representative blots of the treated cells are shown in lower rows.*

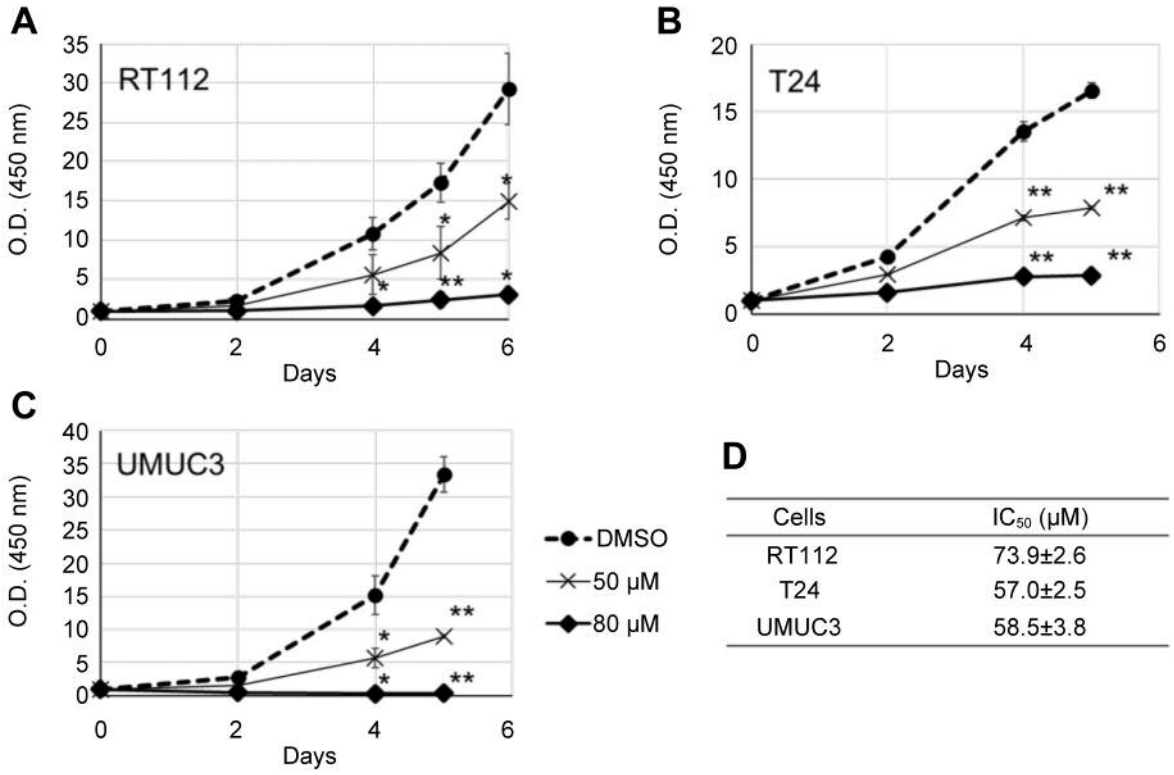

Figure 3. *Growth inhibitory effect of pro-GA in bladder cancer cells. A: RT112 cells, B: T24 cells, and C: UMUC3 cells were treated at doses of 50 μM and 80 μM. Significant differences were observed in one-way factorial ANOVA accompanied by Tukey's significance test on days 2-6. *p<0.05, **p<0.01; significant referring to DMSO control. D: IC_{50} of Pro-GA in the cells.*

similar to those observed in tumor lines with different tissues of origin (*i.e.* MCF7, PC3 and LNCaP) (Figure 2A) (6). In all of the bladder cancer lines, GGCT knockdown significantly reduced cell growth, ranging from 20 to 65% inhibition compared to control siRNA treatment. Marked reduction of GGCT was confirmed by western blot analyses (Figure 2B).

Growth inhibitory effect of pro-GA in bladder cancer cells. We performed time course analyses using the WST-8 assay at doses of 50 μM and 80 μM pro-GA in the bladder cancer cell lines. In all cases, pro-GA reduced viability in a dose-dependent manner (Figure 3A–C). Half-maximal inhibitory concentrations (IC_{50}) of pro-GA are shown in Figure 3D.

Growth inhibition of pro-GA combined with MMC in bladder cancer cells. To investigate whether pro-GA enhances the anti-tumor effect of chemotherapeutic agents, RT112 bladder cancer cells were treated with pro-GA in combination with MMC, a drug that is frequently used for intravesical chemotherapy in NMIBC. Pro-GA (100 μM) administration following treatment with 0.7 μg/ml of MMC had a significant anti-proliferative effect within 5 to 7 days. On day 7, the combined treatment of pro-GA and MMC was particularly effective when compared treatment with either agent alone (Figure 4). The enhanced effect of the combined treatment was also observed in two other bladder cancer cell lines (data not shown).

Discussion

GGCT is up-regulated in various cancers, and we previously showed that GGCT knockdown inhibited proliferation of several types of cancer cells, but not of normal human fibroblasts (6). Uejima *et al.* reported that silencing GGCT reduced invasion, as well as cell motility in human osteosarcoma cells; this suggests that GGCT plays a role not only in tumor growth, but also in invasion and/or metastasis (14). Together, these data indicate that GGCT is a promising therapeutic target for the treatment of various cancers (17). In the current study, we extended our earlier work on the cell-permeable GGCT inhibitor, pro-GA (11), by examining its effects in bladder cancer.

GGCT expression was found to be frequently overexpressed in the bladder cancer cells of TURBT specimens. Therefore, anti-GGCT treatment may be appropriate in many cases of

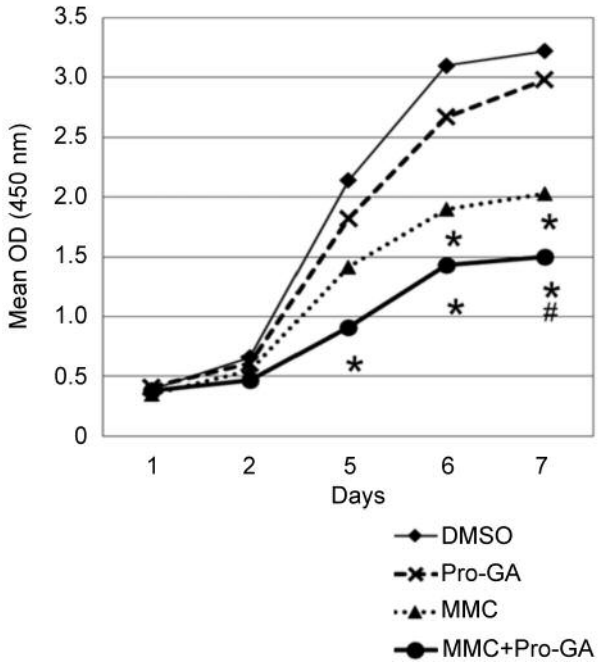

Figure 4. *Growth inhibitory effect under the combined use of pro-GA and MMC in RT112 cells. Significant differences were observed in one-way factorial ANOVA accompanied by Tukey's significance test in MMC-treated cells with/without pro-GA on days 5-7 ($p<0.01$). *$p<0.05$, significant only referring to DMSO control; #$p<0.05$, significant referring to DMSO control and to MMC.*

bladder cancers that are treated with TURBT. Although there was no difference in the recurrence rate between high- and low-GGCT groups in all patients (Figure 1B), the intravesical recurrence rate tended to be worse for the high-GGCT group in Ta patients, although statistical significance was not reached (Figure 1C). It is possible that the influence of post-operative prophylactic intravesical instillation is a more important determinant of recurrence than GGCT expression levels. Indeed, post-operative intravesical therapy was more frequently performed in T1 patients (24 of 32; 75%) than in Ta patients (34 of 98; 34%) ($p<0.001$).

Although cancer cell growth suppression due to GGCT knockdown has been reported previously, there are few reports of growth suppression using GGCT inhibitors (11). To our knowledge, this is the first report that a specific GGCT inhibitor, pro-GA, can significantly suppress growth of bladder cancer cell lines.

Currently, MMC is the world-standard drug for intravesical chemotherapy. However, the prophylactic effect obtained with MMC instillation is unsatisfactory. Therefore, to improve efficacy of intravesical MMC, several options (simultaneous hyperthermia and electromotive devise, *etc*.) and new chemotherapeutic agents such as gemcitabine and docetaxel have been explored (18, 19). Importantly, in the current study we confirmed that the antitumor effect of MMC was significantly enhanced when combined with pro-GA compared with either agent alone. GGCT is a key enzyme that regulates glutathione metabolism, which in turn acts as a buffer against cellular stress. We infer that the stress induced by MMC is augmented by pro-GA-dependent inhibition of GGCT. However, further investigation will be required in order to elucidate the precise mechanisms that underlie the efficacy of the pro-GA/MMC combination. We have previously shown that intraperitoneal pro-GA administration is both safe and effective in a mouse xenograft model of prostate cancer (11). Given our current results, the safety and efficacy of pro-GA must now be demonstrated in an orthotopic NMIBC animal model. Such studies will provide further support that GGCT inhibitors such as pro-GA can be used as potential bladder cancer therapeutics.

Conflicts of Interest

None of the Authors have any conflicts of interest to declare regarding this study.

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Authors' Contributions

EH, SKa, RM, SKu, HI, HK and TC designed the study and performed experiments. EH, SKa and TC analyzed the data and drafted the manuscript. SN, AK and TC supervised the work and helped to draft the manuscript. All Authors read and approved the final manuscript.

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