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Viability Profiles of Normal and Cancer Bladder Cells With Metformin, Nitrate and Adenosine Monophosphate-Activated Protein Kinase Inhibitor

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Abstract

Background: There is no literature report on how metformin and adenosine monophosphate-activated protein kinase (AMPK) inhibitor affect normal and cancer bladder cells under the presence of nitrate.

Methods: Various treatment concentrations and methods were used to study the effects of nitrate, metformin, and/or AMPK inhibitor on normal and/or cancer bladder cells. Normal bladder cells were exposed to nitrate or metformin alone or in combination. The effects of AMPK on normal bladder cells were investigated with nitrate and metformin pretreatment. The effects of varying metformin concentrations on cancer bladder cells were examined as well.

Results: Metformin has produced almost no changes in cell viability of normal cells with various concentrations. Addition of both nitrate and metformin at the same time resulted in less than 17% cell viability as compared to the controlled values; however, this value is about 10% better than nitrate alone for 24 h and approximate 27% better for 48 h. Pretreatment of normal cells with AMPK inhibitor for 6 h prior to addition of metformin and nitrate reduced the cell viability greatly. The treatment of cancer bladder cells with metformin indicated an inverse relationship between metformin concentration and cancer bladder cell viability.

Conclusion: Metformin assisted normal bladder cells in surviving in the presence of nitrate, but its total survival was greatly reduced by AMPK inhibitors. Metformin inhibited the growth of bladder cancer cells.

Keywords: Metformin; Nitrate; Adenosine monophosphate-activated protein kinase; Bladder cancer; Epithelial bladder cells

Introduction

Bladder cancer is one of the most common cancers worldwide. The quest to preventive and treatment of bladder cancer continues to be the topic of current research interest. Thus, this paper attempts to understand the role of metformin and adenosine monophosphate-activated protein kinase (AMPK) inhibitor (also known as dorsomorphin or compound C) on normal and/or cancer bladder cells. It has been well-documented in the literature that nitrate is a cancer inducer. Previous studies had shown that a prolonged exposure of nitrate resulted in increasing risk of bladder, thyroid, esophageal, colon, kidney, and stomach cancers [1, 2]. Thus, in this study, nitrate in the form of sodium nitrate ($NaNO_3$) is used as a cancer-causing agent to normal bladder cells in the presences of metformin and/or AMPK inhibitor. According to our knowledge, there is no literature report on how metformin affects normal and cancer bladder cells under the presence of a cancer-causing agent.

Metformin commercially known as "glucophage" is a biguanide class medication for type 2 diabetes [3]. This drug is a natural derivative of galegine, commonly used in Chinese medicine [4]. Glucophage treats diabetes by lowering glucose production of the liver while enhancing insulin sensitivity [5, 6]. Previous studies reported that metformin shows anti-cancer effects in a variety of cancer cell lines and animal models [7-13]. Metformin inhibits cancer growth in a variety of cancers including breast cancer, prostate cancer, and in glioma cells mainly through AMPK [9]. Studies have proposed that metformin acts through multiple pathways by interacting with multiple targets at the cellular and molecular level. Such interaction includes inhibition of the mitochondrial respiratory chain complexes, activation of AMPK, and inhibition of the reduced form of nicotinamide adenine dinucleotide phosphate [14-16].

It has been proposed that AMPK is involved with how metformin functions in a variety of cancer conditions including breast cancer, prostate cancer, and glioma cells [9-13]. AMPK can carry out different functions depending on its microenvironment setting. For example, when metformin causes lowering cellular glucose level and increasing insulin sensitivity, AMPK activates the downstream of phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3k/AKT/mTOR) oncogenic pathway [16, 17]. Note that this pathway is known for promoting cell survival through the activation of AKT. Metformin action can be anti-cytotoxic by reducing oxidative stress through mitochondrial NADPH inhibition, complex I inhibition, and permeable pore transition

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protein inhibition at the cellular level [18]. Metformin phosphorylates and activates the check point homologue kinase-2 protein which subsequently mediates the ataxia-telangiectasia mutated protein kinase, a master regulator of the DNA damage response [19, 20]. In this paper, the reagent called dorsomorphin or compound C is used as the AMPK inhibitor to examine the effect of this specific inhibitor on the function of metformin on normal bladder cells.

Materials and Methods

Materials

Primary bladder epithelial cells (BdECs), cancer bladder cells (5637), prostate epithelial cell basal medium, Dulbecco's phosphate-buffered saline (PBS), Roswell Park Memorial Institute medium (RPMI), trypsin neutralizing solution, trypsin-ethylenediaminetetraacetic acid (EDTA) for primary cells, trypsin-EDTA for bladder 5637 cells, penicillin-streptomycin-amphotericin B solution, and corneal epithelial cell growth kit were purchased from American Type Culture Collection (USA). AMPK inhibitor (dorsomorphin or compound C) was purchased from Sigma Aldrich (USA). Metformin hydrochloride 98% and sodium nitrate 98% were purchased from Fisher Scientific (USA).

Methods

Human primary bladder epithelial cells were removed from liquid nitrogen (-130 °C) storage tank. The normal bladder cells (BdECs) with stock density of 1.0×10^6 cells per vial were carefully thawed with gentle 1 - 2 min agitation. External surfaces of all growth kit components and the basal medium bottle were decontaminated with 70% ethanol. The corneal epithelial growth kit components were also thawed and then added to the prostate epithelial basal medium in concentrations as indicated by ATCC product sheet information. Strict aseptic techniques were used under a certified laminar flow hood. Following thawing, cells were seeded at a density between 3,000 and 4,000 cells/mL in 37 °C prostate epithelial basal medium for primary cells that were initially stored at 4 °C. Human bladder cancer cells (5637) were also cultured with the same methods as indicated for the primary cells and then were grown in RPMI medium with 10% bovine serum.

All cells were cultured in 25 cm^2 culture flasks and then incubated at 37 °C in 5% CO_2 until ready for subculture. All cell culture media were replaced every 48 h. Cellular morphology and growth were observed until cells reached about 80% confluency. For sub-culturing, cells were rinsed with PBS without calcium or magnesium followed by trypsin for 2 - 3 min. Trypsin was neutralized with trypsin neutralizing solution upon cells detached. Cells were spun for 3 min at 150 g to remove the supernatant and re-suspended in fresh media. Cell count was performed before all steps as a standard procedure with a Bio-Rad TC20 automated cell counter. Following plating and adherence to the flask's surface, cells were allowed to reach about 70% confluency before they were washed three times with PBS and then treated with target compounds in newly replaced culture medium.

Experiments including the addition of $NaNO_3$, metformin, and compound C were performed simultaneously and divided into eight different experiments: (a) control, (b) 300 μM $NaNO_3$, (c) 1.0×10^3 μM metformin, (d) 1.0×10^3 μM compound C, (e) 300 μM $NaNO_3$ at 24 h, then 1.0×10^3 μM metformin at 48 h, (f) 1.0×10^3 μM metformin at 24 h, then 300 μM $NaNO_3$ at 48 h, (g) 1.0×10^3 μM compound C at 6 h, 300 μM $NaNO_3$ at 24 h, then 1.0×10^3 μM metformin at 48 h, (h) 1.0×10^3 μM compound C at 6 h, 1.0×10^3 μM metformin at 24 h, then 300 μM $NaNO_3$ at 48 h.

Experimental procedure

The normal bladder cells (BdECs) were plated onto a 24-well plate to reach about 80% confluency before being treated with various concentrations of $NaNO_3$, metformin and compound C. For each set of experiments, a control experiment was run and the cell viability was measured accordingly. For $NaNO_3$, seven different concentrations (30, 60, 90, 100, 200, 300, and 500 μM) were added and cell viability of each concentration was measured after 48 h. For metformin, three different concentrations (1.0×10^3, 5.0×10^3, and 1.0×10^4 μM) were added and cell viability was measured after 48 h for each concentration. For treatments with both $NaNO_3$ and metformin, three different plates were used: one for 300 μM of $NaNO_3$, one for treatment with 1.0×10^4 μM of metformin, and one for a combined of 300 μM of $NaNO_3$ and 1.0×10^4 μM of metformin. Cell viability measurements were taken at 24 and 48 h. For sequential treatments of $NaNO_3$, metformin and compound C, seven different plates were used: the first one (b) 300 μM of $NaNO_3$, (c) 1.0×10^3 μM of metformin, (d) 1.0×10^4 of compound C, (e) 300 μM of $NaNO_3$ for 24 h, then 1.0×10^3 μM of metformin for additional 48 h, (f) 1.0×10^3 μM of metformin for 24 h, then 300 μM of $NaNO_3$ for additional 48 h, (g) 6 h pre-treatment with 1.0×10^4 μM of compound C, 300 μM of $NaNO_3$ for 24 h, then 1.0×10^3 μM of metformin for additional 48 h, and (h) 6 h pre-treatment with 1.0×10^4 μM of compound C, 1.0×10^3 μM of metformin for 24 h, then 300 μM of $NaNO_3$ for additional 48 h. Cell viability measurements for (b)-(h) were taken after 72 h.

Cancer bladder cells (5637) were plated onto 24-well plates to reach about 80% confluency. At the desired confluency, cells were treated with 1.0×10^3, 5.0×10^3, and 1.0×10^4 μM of metformin. Cell viability measurements were taken after 48 h.

Statistical analysis

Statistical *t*-test analysis was calculated in all experiments. The P-values less than 0.05 were interpreted as statistically significant. The *t*-test was calculated between control and the treatment samples 1.0×10^4 μM metformin, 1.0×10^4 μM metformin + 300 μM $NaNO_3$, and 300 μM $NaNO_3$ alone. The test generated P-values of 0.27, 0.02, and 0.01, respectively.

Results indicated a similar overall pattern with 24 h treatment, and double treatment of metformin and $NaNO_3$ showed a significantly higher viability percent (P = 0.01) when compared to the $NaNO_3$ alone. The statistical analysis between the control and 1.0×10^4 µM metformin treatment had a P-value of 0.27. The generated P-value between the control and the double treatment (1.0×10^4 µM metformin + 300 µM $NaNO_3$) was 0.02. The statistical significance of P-value of 0.01 was found between the control and the double treatment group in the 48-h experiment. A statistical significance between control and the double treatment group in the 24 h experiment was not observed (P = 0.11). However, in the 48-h group, all treatments were statistically significant with respect to the control.

The study was approved by the Texas Southern University IRB committee. The study was conducted in compliance with the ethical standards of the responsible institution on human subjects as well as with the Helsinki Declaration.

Results

The results of normal bladder cells treated with $NaNO_3$ alone, metformin alone, a combination of $NaNO_3$ and metformin, and a combination of $NaNO_3$, metformin, and an AMPK inhibitor are shown in Tables 1-3 and Figures 1 and 2. The results of cancer bladder cells treated with different concentrations of metformin are shown in Table 4.

Table 1 shows the percentage viability profiles of normal bladder cells (BdECs) with different concentration of $NaNO_3$ and metformin after 24 h of incubation. Each value is calculated as the average value of three runs. The cell viability for control experiment before adding $NaNO_3$ is 82.3%. The cell viability profiles for BdECs under treatment of $NaNO_3$ were at 75.7%, 69.6%, 63.4%, 55.4%, 33.5%, and 0.0% for concentrations of 30, 60, 90, 200, 300, and 500 µM, respectively. Table 1 also shows the percentage viability profiles of BdECs under treatment of metformin after 48 h. The results are 65.4%, 63.1%, and 63.6% for concentrations of 1.0×10^3, 5.0×10^3, and 1.0×10^4, respectively. The cell viability for the control experiment before the addition of metformin was 66.4%. Figure 1 shows the inverse correlation between concentrations of $NaNO_3$ and cell viabilities of normal bladder cells after 48 h.

Table 2 shows cell viability profiles of BdECs when treated alone with either 300 µM $NaNO_3$ or 1.0×10^3 µM metformin, and when combined both $NaNO_3$ and metformin with same concentrations. At 24 h, a combined treatment of $NaNO_3$ and metformin to BdECs resulted in a 77% cell viability as compared to 93.3% of the control experiment. Treatment alone with $NaNO_3$ resulted in 66.7% (control experiment was 93.3%) and with metformin was 92.7% (control experiment was 93.3%). At 48-h, the combine treatment yielded a 69.3% cell viability; whereas the control was 86%. Treatment alone with $NaNO_3$ resulted in 42.7% for a control value of 86.0% and with metformin was 84.3% for a control value of 86.0%. Figure 2 compares the changes in cell viability profiles of normal bladder cells with 300 µM $NaNO_3$ and 1.0×10^3 µM metformin when treated alone or combined.

Table 3 shows cell viability profiles of BdECs with timed-sequential treatment of $NaNO_3$, metformin, and/or AMPK inhibitor dorsomorphin (compound C) after 72 h. The concentration of $NaNO_3$, metformin, and compound C used in these experiments were 300, 1.0×10^3, and 1.0×10^3 µM, respectively. Viability percentages (each experiment) were 92.0% (a - control), 23.3% (b - $NaNO_3$), 91.7% (c - metformin), 88.7% (d - compound C), 84.3% (e - $NaNO_3$, metformin), 84.0% (f -metformin, $NaNO_3$), 55.3% (g - compound C, $NaNO_3$, metformin), and 56.0% (h - compound C, metformin, $NaNO_3$).

Table 4 shows viability profiles of cancer bladder cell (5367) with different concentrations of metformin after 48 h. Results

Table 1. Viability Profiles (%) of Normal Bladder Cells (PdECs) With Different Concentrations of $NaNO_3$ and Metformin After 48 h

Concentration, µM	$NaNO_3$	Metformin
Control	82.3	66.4
30	75.7	
60	69.6	
90	63.4	
200	55.4	
300	33.5	
500	0.0	
1.0×10^3		65.4
5.0×10^3		63.1
1.0×10^4		63.6

Each value is the average values of three runs.

Table 2. Viability Profiles (%) of Normal Bladder Cells (PdECs) With 300 µM $NaNO_3$ and 1.0×10^3 µM Metformin (Met) When Treated Alone or Combined

Concentration, µM	24 h			48 h		
	$NaNO_3$, %	Met, %	Both, %	$NaNO_3$, %	Met, %	Both, %
Control	93.3	93.3	93.3	86.0	86.0	86.0
300 µM $NaNO_3$	66.7			42.7		
1.0×10^3 µM metformin		92.7			84.3	
Both			77.0			69.3
Δ in cell viability	26.6	0.6	16.3	43.3	1.7	16.7

Each value is the average value of three runs.

Table 3. Viability Profiles (%) of Normal Bladder Cells (PdECs) With Sequential Treatments of $NaNO_3$, Metformin, and/or AMPK Inhibitor (Compound C) After 72 h

Addition order	Cell viability, %	Δ in cell viability, %[b]
(a) Control	92.0	
(b) 300 μM $NaNO_3$	23.3	68.7
(c) 1.0×10^3 μM metformin	91.7	0.3
(d) 1.0×10^3 μM compound C	88.7	3.3
(e) 300 μM $NaNO_3$ (24 h)[a], 1.0×10^3 μM metformin (48 h)[a]	84.3	7.7
(f) 1.0×10^3 μM metformin (24 h)[a], 300 μM $NaNO_3$ (48 h)[a]	84.0	8.0
(g) 1.0×10^3 compound C (6 h pre-treatment)[a], 300 μM $NaNO_3$ (24 h)[a], 1.0×10^3 μM metformin (48 h)[a]	55.3	36.7
(h) 1.0×10^3 compound C (6 h pre-treatment)[a], 1.0×10^3 μM metformin (24 h)[a], 300 μM $NaNO_3$ (48 h)[a]	56.0	36.0

Each value is the average value of three runs. [a]Incubation time. [b]Each value is calculated as control minus the experiment.

were 91.3% for control experiment, 81.3% for 1.0×10^3 μM, 74.9% for 5.0×10^3 μM, and 42.0% for 1.0×10^4 μM metformin.

Discussion

Table 1 presents the viability profiles of normal bladder cells (BdECs) with varied doses of $NaNO_3$ and metformin. As illustrated in Figure 1, the results clearly show a direct adverse correlation between the concentration of $NaNO_3$ and cell viability. At 30 μM $NaNO_3$, the cell viability was found to be 75.7%, compared to 82.3% for the control, and steadily reduced to 0% after 48 h at 500 μM. Thus, when the concentration of $NaNO_3$ increased, so did the rate of cell death. In contrast to the effect of $NaNO_3$ on BdECs, Table 1 shows that varied doses of metformin do not affect BdEC cell survival. For example, at 1.0×10^3 μM metformin, cell viability was determined to be 65.4%, compared to 63.6% at 1.0×10^4 μM metformin. After 48 h, a 10-fold rise in metformin levels resulted in the death of just 1.8% of normal bladder cells.

Table 2 and Figure 2 show the cell viability characteristics of BdECs treated with $NaNO_3$ (300 μM) or metformin (1.0×10^3 μM) alone and a combination of the two. BdECs treated with $NaNO_3$ alone endured a 26.6% drop in cell viability after 24 h, but BdECs treated with only metformin had essentially unchanged cell viability. BdECs treated with only $NaNO_3$ observed a 43.3% decline in cell viability after 48 h; however, BdECs treated with metformin alone resulted in only a 1.7% drop in cell viability.

Cell viability was reduced by 16.3% when a combined dose of $NaNO_3$ (300 μM) and metformin (1.0×10^3 μM) was administered to BdECs for 24 h, according to the data in Table 2 and Figure 2. With the same treatment time and same concentration, BdECs treated with $NaNO_3$ alone resulted in 26.6% cell death, demonstrating a functional synergy between $NaNO_3$ and metformin toward BdECs. According to the findings, metformin preserves BdEC cell viability in the presence of $NaNO_3$ by around 10.3% at 24 h.

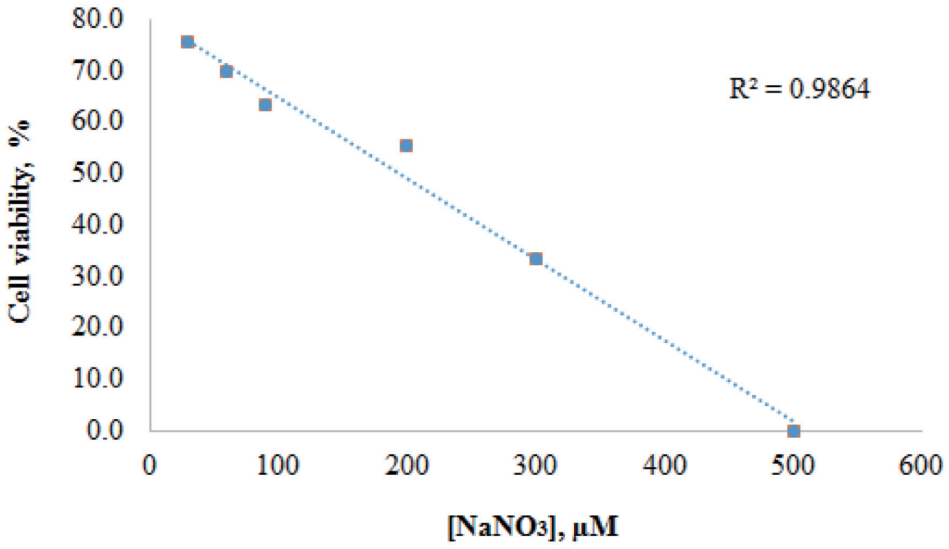

Figure 1. Correlation between concentrations of $NaNO_3$ and cell viabilities of normal bladder cells (PdECs) after 48 h.

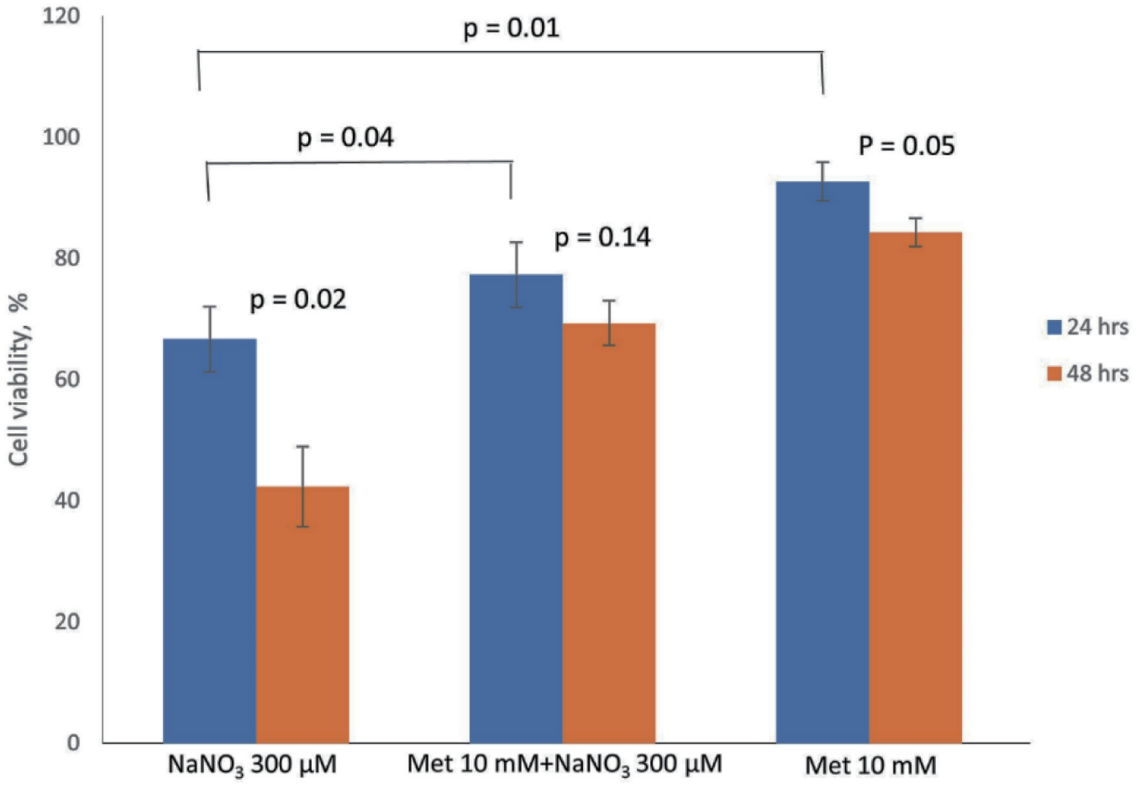

Figure 2. Comparison of changes in cell viability profiles of normal bladder cells (PdECs) with 300 µM $NaNO_3$ and 1.0×10^3 µM metformin when treated alone or combined. Both 300 µM $NaNO_3$ and 1.0×10^3 µM metformin were added to PdEC at the same time.

Table 2 and Figure 2 further show that when a combination dose of $NaNO_3$ (300 µM) and metformin (1.0×10^3 µM) was given to BdECs for 48 h, cell viability was reduced by 16.7%, compared to 43.3% when treated alone with $NaNO_3$ and 1.7% when treated just with metformin, at the same time frame and concentration. Interestingly, even though the changes in cell viability under $NaNO_3$ treatment increased from 26.6% for 24 h to 43.3% for 48 h, the change in cell viability remained nearly the same with metformin, 0.6% for 24 h and 1.7% for 48 h, and the same observation was made with a combined dose of $NaNO_3$ and metformin, with changes in cell viability of 16.3% for 24 h and 16.7% for 48 h. Metformin strengthens cell survival in BdECs by about 26.6% at 48 h in the presence of $NaNO_3$, a cancer-causing agent.

Table 3 shows the cell viability of BdECs with different sequential treatments of $NaNO_3$ (300 µM), metformin (1.0×10^3 µM), and/or AMPK inhibitor (dorsomorphin or compound C, 1.0×10^3 µM). The cell viability dropped by 3.3% after 72 h when treated with compound C alone, compared to 68.7% when treated with $NaNO_3$ alone and 0.3% when treated with metformin alone (Table 3: b, c, and d). The addition of $NaNO_3$ first, then metformin, or in the reverse order, to BdECs resulted in comparable cell viability profiles with a 7.7-8.0% reduction (Table 3: e and f). Pre-treatment of BdECs with compound C for 6 h before adding $NaNO_3$ for 24 h, then metformin for 48 h, or the reverse order of 24 h for metformin, then 48 h for $NaNO_3$, resulted in nearly identical cell viability profiles with a 36.0-36.7% reduction (Table 3: g and h). The findings presented in Table 3 reveal that when BdECs are pre-treated with compound C before adding either metformin first and $NaNO_3$ or $NaNO_3$ first and metformin, there is around a 28% reduction in cell viability (Table 3: e and f vs. g and h), indicating that compound C inhibits metformin from promoting cell survival.

Table 4. Viability Profiles (%) of Cancer Bladder Cells (5367) With Different Concentrations of Metformin After 48 h

Concentration, µM	Cell viability, %	Δ in cell viability, %
Control	91.3	
1.0×10^3	81.3	10.0
5.0×10^3	74.9	16.4
1.0×10^4	42.0	49.3

Each value is the average value of three runs.

The cytotoxicity of metformin on bladder cancer cells (5367) was also investigated in this study. Table 4 summarizes the results of the viability profiles of cancer bladder cells. The cancer cells were given different concentrations of metformin (1.0×10^3, 5.0×10^3, and 1.0×10^4 µM) and cultured for 48 h. The findings demonstrated a clear and direct relationship between metformin concentration and cancer cell viability. For example, at 1.0×10^3 µM metformin concentration, cancer cell viability was reduced by 10%, 16.4% at 5.0×10^3 µM, and 49.3% at 1.0×10^4 µM. The research showed that a 10-fold

rise in metformin concentration resulted in a fivefold increase in cancer cell mortality, suggesting that metformin can prevent the proliferation of bladder cancer cells.

Conclusions

This study investigated the effects of nitrate, metformin, and/or AMPK inhibitors on normal and/or cancer bladder cells. Metformin enabled normal bladder cells to survive in the presence of a recognized cancer-causing agent, $NaNO_3$, implying that the combination of nitrate and metformin may have synergistic effects in sparing normal bladder cells while efficiently inhibiting bladder cancer growth. This study showed that AMPK inhibitors significantly reduced the synergistic effects of metformin and nitrate on normal bladder cells. More research is needed to understand the mechanism of how metformin works in normal bladder cells when administered to the cells together with $NaNO_3$ and/or AMPK inhibitors.

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Financial Disclosure

There was no specific funding source to be mentioned.

Conflict of Interest

The authors declare that they have no conflict of interest.

Informed Consent

This study does not require informed consent.

Author Contributions

TP and HA designed the study. HA performed experiments under the guidance of TP. SG analyzed data, prepared the manuscript, and did critical editing. TP revised and finalized the manuscript.

Data Availability

The authors declare that data supporting the findings of this study are available within the article.

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