The Effect of the Oncometabolite Fumarate in the Response to DNA Damage: An analysis of the role of fumarate in the response of cells A2780 to DNA damage induced by cisplatin

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Abstract

Previous research has been conducted on the effect of oncometabolites on DNA damage repair; however, these studies have traditionally focused on the response to damage caused by DNA double-strand breaks, whereas this study involves cisplatin-induced damage that creates DNA cross-links. This study reports on the effect of the oncometabolite fumarate on the response of A2780 cells to DNA damage produced by cisplatin. Three assays were used to complete this study: comet assay, cell cycle assay and apoptosis assay. The comet assay revealed that fumarate influenced the response of cells to DNA damage and, at a lower 1mM concentration, appeared to protect cells from further DNA damage. When looking at cell cycle progression, it was found that fumarate did not change the cell cycle nor modify the effect of cisplatin. The apoptosis assay showed that fumarate also did not induce apoptosis nor alter cisplatin-induced apoptosis. This investigation contributes to existing knowledge of the role of oncometabolites, specifically fumarate, on DNA damage repair responses.

Resumen

Se han realizado investigaciones previas sobre el efecto de los oncometabolitos en la reparación del daño del DNA; sin embargo, tradicionalmente estos estudios se centran en la respuesta al daño causado por las roturas de la doble cadena del DNA, mientras que este estudio implica el daño inducido por el cisplatino que crea enlaces cruzados en el DNA. Aquí se reporta el efecto del oncometabolito fumarato en la respuesta de las células A2780 al daño en el DNA producido por el cisplatino. Se utilizaron tres ensayos para completar este estudio: ensayo de cometa, ensayo de ciclo celular y ensayo de apoptosis. El ensayo de cometa reveló que el fumarato tiene un efecto en la respuesta de las células al daño en el DNA y, a una menor concentración, parece proteger a las células de un mayor daño en el DNA. Al observar la progresión de los ciclos celulares, se encontró que el fumarato tampoco cambia el ciclo celular ni modifica el efecto de cisplatino. El ensayo de apoptosis mostró que el fumarato tampoco induce apoptosis y no altera la apoptosis inducida por el cisplatino. Esta investigación se suma a la información conocida sobre el papel de los oncometabolitos, específicamente el fumarato, en las respuestas de reparación del daño del DNA.

1 INTRODUCTION

Cancer refers to a number of conditions characterized by abnormal and uncontrolled cell growth. This uncontrolled cell behavior is due to the accumulation of genetic and epigenetic alterations in the genome that target tumor survival and metastasis[1]. Cancer is a very complex disease that can be caused by a variety of genetic or metabolic mutations. In recent years, there has been a shift in cancer research to better understand the links between cancer and altered cellular metabolism[2]. Dysregulated metabolism is understood to be central to cancer cells' ability to survive, proliferate and metastasize. It appears that many cancer genes and mutations affect three major metabolic pathways: aerobic glycolysis, glutaminolysis, and one-carbon metabolism[3]. In place of normal ATP production, cells are forced to generate large quantities of nutrients necessary for the rapid cell growth and division of a cancer cell[4]. In terms of patient care, detection of simple metabolic changes in the body may be able to indicate early tumor and cancer development[4]. More specifically, bioinformatics analyses of gene expression data from cancer patients have associated deletion of mitochondrial genes with poor clinical outcomes[5]. When mutated, mitochondrial

genes encoding enzymes such as fumarate hydratase (FH) can lead to cancer development[2].

The role of hydratase in the Krebs cycle, or tricarboxylic acid (TCA) cycle, is the transformation of fumarate to malate. When fumarate hydratase is mutated and this transformation does not occur, there is a buildup of fumarate in the cell. The accumulation of metabolites like fumarate appears to play a causal role in the development of a wide variety of tumor types. Thus, fumarate is known as a oncometabolite and considered to be a potential biomarker of prognosis, treatment efficacy and early disease recurrence[6]. Oncometabolites, in short, are conventional metabolites that when abnormally accumulated in large amounts, exhibit prooncogenic functions[7]. Fumarate can competitively inhibit α-ketoglutarate (α-KG) dependent dioxygenase enzymes due to their similarities in structure as shown in Figure 1[8]. Dioxygenases are involved in the regulation of hypoxia conditions and epigenetic changes and, when inhibited, can alter gene expression and chromatin structure[8]. Oncometabolites can then modify the response to agents that induce DNA breaks, either by increasing or blocking the ability to repair these breaks. This study focuses on the impact of fumarate and the consequences of inhibition of these dioxygenase enzymes on the cell's response to DNA damage.

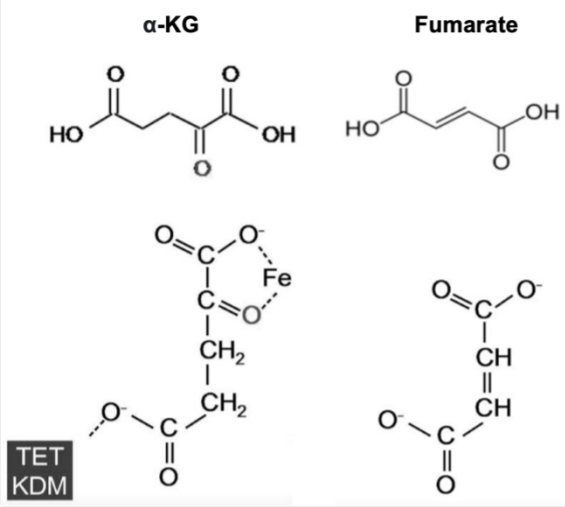

Figure 1. Similarity in α-KG and fumarate structure[8]

The introduction of cisplatin can be used to induce DNA damage and along with the induction of fumarate, to study the subsequent effect of fumarate on repair. Cisplatin is a platinum-based chemotherapeutic drug used to treat solid tumors such as those of ovarian, prostate, and lung cancer[9]. The drug primarily forms intra- and inter-strand cross-links within the DNA. The cross-link between two adjacent guanines is believed to be the critical lesion responsible for the cytotoxicity of cisplatin[10]. The formation of cisplatin-DNA adducts interferes with DNA replication and transcription, altering the DNA structure. These alterations are then recognized by cellular proteins which alert damage repair systems[9]. The efficacy of cisplatin as a chemotherapeutic agent is therefore dependent on the cell's ability to detect and respond to DNA damage[11]. The cell's response to repair the damage is not guaranteed, in which case the cell may undergo apoptosis. The signaling pathways that control apoptosis then also significantly impact a cell's responsiveness to cisplatin[10].

In this study, cisplatin was introduced into the A2780 line of ovarian cancer cells to induce inter-strand crosslinks in the DNA. DNA damage repair systems under the influence of fumarate were called upon to repair the damaged DNA. The response of these repair systems was then measured using a series of three damage-detection assays: comet assay, cell cycle progression analysis, and apoptosis analysis.

The comet assay or single gel electrophoresis is a relatively simple and sensitive method for the detection of DNA damage and repair[12]. This technique has several advantages, such as a relatively low cost, use of small cell samples, analysis at the single cell level, and efficiency[9]. The comet assay is a widespread and useful tool in genotoxicity testing in cells both *in vitro* and *in vivo*[13]. When the electric field is applied to lysed and stained cells suspended in a thin agarose gel during electrophoresis, negatively charged DNA is attracted to the positively charged anode. Undamaged DNA strands are too large to be moved, while smaller fragments are attracted from the core to the anode[12]. The migration of DNA from the nucleus resembles the shape of a comet, hence the name of the assay (Figure 2). The amount of DNA damage is strongly correlated with the extent of DNA migration and can be analyzed using an electronic system such as the Komet 5.

Cell cycle and apoptosis assays are based on the principle of DNA replication and the amount of DNA in a cell at any given point in its life. In this study, cell cycle analysis was used to quantify cells in one of three phases: Gap 1 (G1), Synthesis (S), and Gap 2 (G2). The G1 phase consists only of cell growth during which only one chromosome is present in the cell. In the S phase, the cell has begun replication. In the G2 phase, replication is complete, and two chromosomes are present in the cell. Apoptotic cells, in comparison, have reduced DNA content, as they are on their way to cell death. By quantifying the DNA content of each cell, the phase in which the cell was arrested can be determined. Visualization of these phases is simplified by the use of a fluorescent dye and is performed by flow cytometry to detect varying levels of fluorescence. The fluorescence is bound by the cell stoichiometrically (in proportion to the level of DNA present in the cells) and these levels of fluorescence are a direct indicator of the cell's cycle[14].

In apoptosis analysis, the percentages of apoptotic cells measured by flow cytometry is divided between early apoptosis and late apoptosis. This division is determined based on the levels of annexin V and propidium iodide (PI) that are able to enter each cell. A cell in early apoptosis is characterized by an increase in cell membrane permeability and translocation of phosphatidylserine residues from the inside of the cell membrane to the outside[15]. The presence of these phosphatidylserine residues outside the cell membrane is irreversible and indicates that the cell is engaged in apoptosis. Annexin V is a Ca2+-dependent phospholipid-binding protein that binds tightly to these residues and thus indicates the presence of cells in the early stages of apoptosis. For PI to stain the cell, it must be able to cross the cell membrane. Since the membrane of a living or early apoptotic cell is still intact, entry of PI is prohibited. In a late apoptotic or necrotic cell, a decrease in plasma membrane integrity has occurred, allowing PI entry and staining of the cell[16]. Knowledge of cell arrests, both through cell cycle and apoptosis analyses, give rise to a better understanding of DNA cross-link damage repair in the presence of the oncometabolite fumarate.

2 OBJECTIVE

The principal objective of this work was to determine the effect of the oncometabolite fumarate on the response of A2780 cells to DNA damage produced by cisplatin. While much is unknown about DNA damage response in the presence of oncometabolites, it was hypothesized that fumarate would influence DNA damage repair systems. This study was performed by analyzing genomic instability (comet assay), cell cycle progression, and the induction of apoptosis (flow cytometer). Six conditions of differing levels of fumarate and cisplatin were tested using these three methods.

3 METHODS

3.1 Cell Culture

The human ovarian cancer cell line A2780 is known for its use in toxicity testing and cancer genetic studies (Sigma-aldrich, 2021). This cell line is a model in cisplatin treatments. Cells were cultured in a medium composed of 89.8% RPMI medium, 10% FBS, and 0.2% Plasmocin®. All cells were cultured in an incubator at a temperature of 37°C.

3.2 Cell Treatment

For each assay, A2780 cells were plated in a six-well plate and received the following treatments: control, 1 mM fumarate, 5 mM fumarate, 20 µM cisplatin, 1 mM fumarate + 20 µM cisplatin, 5 mM fumarate + 20 µM cisplatin. Cells were exposed to the treatment for three hours before performing the corresponding assay. After the three-hour treatment, excess medium was removed, and the cells were washed with cold PBS (phosphate buffered saline). The cells were then subjected to another brief incubation with the addition of trypsin to ensure their detachment from the plate. After centrifugation and removal of excess medium, cells were resuspended to reach the desired concentration.

3.3 Comet Assay

The first step in performing the comet assay was the preparation of the agarose layers. To ensure the sterility of the slides, they were immersed in ethanol for a minimum of 24 hours at a temperature of 20°C. The first of the two agarose layers were prepared the day before the experiment using 0.5% normal melting point agarose (NMPA). To dissolve the agarose in water, it was heated in a microwave and then kept in a hot water bath. On each slide, 150 µL of solution was spread with a sterile finger. The slides were placed in the oven at 37°C until cell collection.

On the day of the experiment, the second layer was prepared with 0.5% low melting point agarose (LMA). The agarose was heated in the microwave and placed in a bath to keep it warm. 30 µL of $1.5 \times 10^6 - 2 \times 10^6$ treated cells was mixed with 65 µL of agarose and placed on top of a slide already containing the first layer of solidified agarose. A cover slip was placed on each slide and the slides were stored in a refrigerator at 4°C in the dark for at least 20 minutes until the agarose solidified. Following the plating of cells, all steps of the assay were performed under red light to avoid further damage to the DNA.

Once the second layer had solidified and after removing the coverslips, the slides were immersed in 200 mL of a lysis solution and kept at 4°C for one hour. The lysis solution was composed of 89% lysis buffer (NaCl 2.5 M, Na2EDTA 100 mM, Tris 10 mM, NaOH 0.25 M), 10% DMSO (dimethyl sulfoxide), and 1% Triton X-100. The pH of the solution was 10.

After lysis, the slides were placed in the electrophoresis cuvette and allowed to denature for 20 minutes. The slides were placed directly next to each other without any space between them. The electrophoresis buffer in which the pores were denatured was composed of Na2EDTA 1 mM and NaOH 300 mM at a pH > 13. Electrophoresis was performed on ice at 4°C in the dark for 20 minutes at a voltage of 0.83 V/cm and a current intensity of 300mA.

To neutralize the pores, they were placed in a cuvette and washed 3 times for 5 minutes with a neutralization buffer consisting of 0.4 M Tris at pH 7.5. The cells were fixed and dehydrated in ethanol, and the slides were

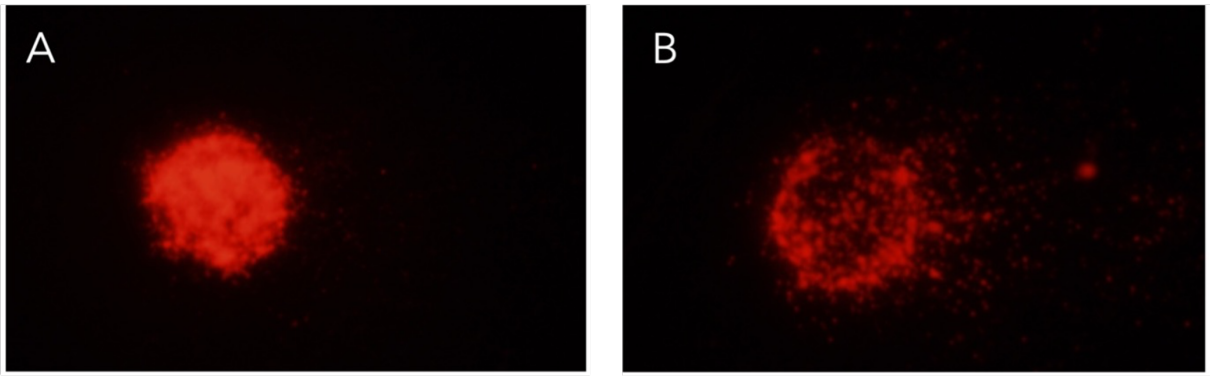

Figure 2. Image capture of two A2780 cells in a comet assay. (A) Control cells without treatment. (B) Cell showing increased DNA migration following treatment.

kept at room temperature in the dark overnight.

Each slide was coded for blinded analysis and the DNA nuclei from the lysed cells was stained with 40 µL of ethidium bromide diluted in water. Nucleoids were visualized on the Olympus BX61 fluorescence microscope, equipped with an Olympus DP-70 digital color camera, belonging to the Photon Microscopy unit and Image Processing unit of the SCTs of the University of Oviedo. The cells were visualized with a 40x objective and a BP530-550 fluorescence filter. Images were taken of 50 individual cells per slide. For each treatment, two slides were prepared, resulting in a total of 100 images per condition. The nucleoid images obtained were analyzed with Komet 5 (Kinetic Imaging Limited, UK) to quantify DNA damage by the percentage of DNA in the tail. Three individual comet assays were performed in this study.

3.4 Cell Cycle Assay

For cell cycle analysis, $1.5 \times 10^6 - 2 \times 10^6$ cells were first fixed with 2mL cold 70% ethanol while the cells were constantly agitated by vortex. The cells were then left to rest for at least 24 hours in the freezer at –20°C. To remove the ethanol, the cells were centrifuged at 1,200 rpm for 5 minutes and washed with PBS. Then, to each sample, 100 µL PBS, 100 µL RNAse, and 10 µL propidium iodide (PI) were added and the samples incubated for 30 minutes. Each sample was placed in a 96 well plate and analyzed with the Cytoflex S cytometer (Beckman Culter®). Three individual cell cycle assays were performed in this study.

3.5 Apoptosis Assay

To determine apoptosis levels and status (early or late), 1×10^3 cells were collected from each treatment. Each sample was centrifuged for 5 minutes at 1200 rpm and then 200 µL of binding buffer, 5 µL annexin V, and 1 µL propidium iodide (PI) were added. The cells were incubated in the dark at room temperature for 10 minutes. Each sample was then plated and analyzed with the Cytoflex S cytometer (Beckman Culter®). Four individual apoptosis assays were performed in this study.

4 RESULTS

4.1 Comet Assay

A comet assay was used to study the effect of cisplatin and fumarate on DNA damage and the role of fumarate in DNA damage repair. The following results were obtained by scoring the percentage of DNA present in the comet "tail" using the Komet 5 program. Figure 2 represents the image captures of two cells to demonstrate quantifiable "tail" observed in a treated cell.

The results presented are from three independent experiments. Figure 3 shows the respective DNA damage for each condition, indicated by the percentage of DNA particles in the comet tail of each nucleoid.

As expected, the cisplatin condition caused a significant increase in DNA damage. The percentage of tail damage was 9.65% in the control and 13.77% after the addition of cisplatin. Untreated with cisplatin, fumarate had an effect and induced damage as well. The DNA damage present in the 1mM fumarate and 5mM fumarate conditions is nearly equal, showing that this increased damage is not concentration dependent. With the addition of cisplatin to fumarate, there is a significant decrease in damage in the 1mM fumarate condition (12.14%) relative to the cisplatin control (13.77%). The 5mM fumarate condition (13.76%) shows no significant change from the cisplatin condition.

4.2 Cycle Cell Progression

Analysis of cell cycle progression was used to determine the influence of fumarate and cisplatin on cell division. Treatment of cells with PI allowed detection of the DNA content within the cell, which was directly proportional

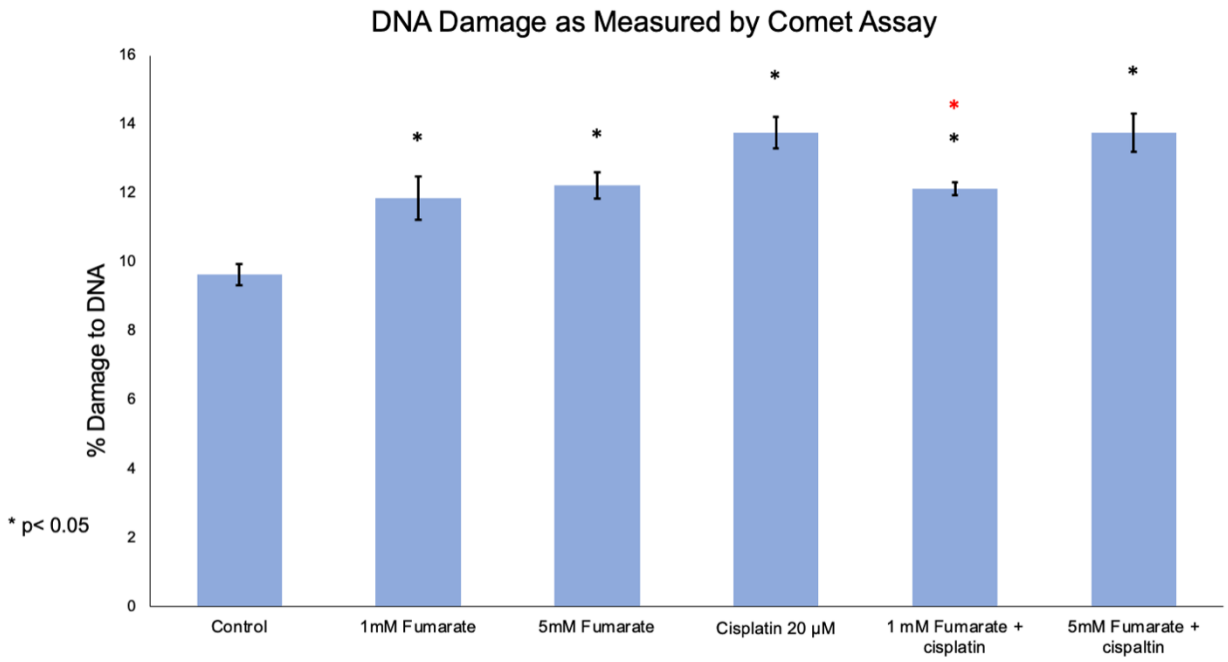

Figure 3. *The effect of fumarate treatment on A2780 cells with cisplatin-induced DNA damage as analyzed by comet assay.* All conditions are statistically significant compared to control. Compared to cisplatin, only the 1mM fumarate + cisplatin condition is statistically significant. * $p < 0.05$ comparing each concentration with control by paired t-test. * $p < 0.05$ comparing each concentration with 20 µM cisplatin by paired t-test.

to the stage at which the cell was arrested [17]. The flow cytometer was then able to quantify the number of cells within each phase according to the level of PI present. The results presented are from three independent experiments. Figure 4 shows the percentage of fumarate- and cisplatin-treated A2780 cells in each phase of the cell cycle obtained in the cytometry assays.

Relative to the control, the number of cells arrested in any phase of the cell cycle in the 1mM fumarate or 5mM fumarate condition is not significantly different. In all three conditions, most cells are arrested in G1 phase with few arrested in S and G2 phase. This is the expected result for normal cell division as the G1 phase is the longest phase involving cell growth. With the addition of cisplatin, there is a significant increase in the percentage of cells arrested in S phase and a significant decrease in cells arrested in G1 phase. This same trend is observed with the addition of both concentrations of fumarate to cisplatin. The addition of fumarate to cisplatin also produces no change from the cisplatin treatment.

4.3 Apoptosis Assay

Cells stained with annexin V were characterized as having undergone early apoptosis and those stained with IP as late apoptosis. Figure 5 shows the percentages of cells in early and late apoptosis after exposure to cisplatin and two different concentrations of fumarate. The results presented are from four independent experiments.

With respect to the control, both fumarate conditions showed very similar levels of apoptosis, in both early and late. In these three conditions, 8.8% of cells experienced early apoptosis and 16.4% of cells experienced late apoptosis. With the addition of cisplatin to the control, there was an increase in apoptosis, but not to a statistically significant level. With the addition of both fumarate concentrations, there is no change from cisplatin treatment, only from control. The conditions with cisplatin show that on average, 20.1% of the cells experienced early apoptosis and on average 24.2% of the cells experienced late apoptosis. Regardless of concentration, the addition of fumarate caused no significant change.

5 DISCUSSION

5.1 Comet Assay

As shown in Figure 3, the addition of fumarate produced significantly more DNA damage compared to the control. However, this was not dependent on the dose of fumarate. If fumarate alone was causing damage to the cells, a higher fumarate concentration would be expected to yield higher levels of damage. Rather than indicating that fumarate induced DNA damage, the results more likely suggested that fumarate was blocking

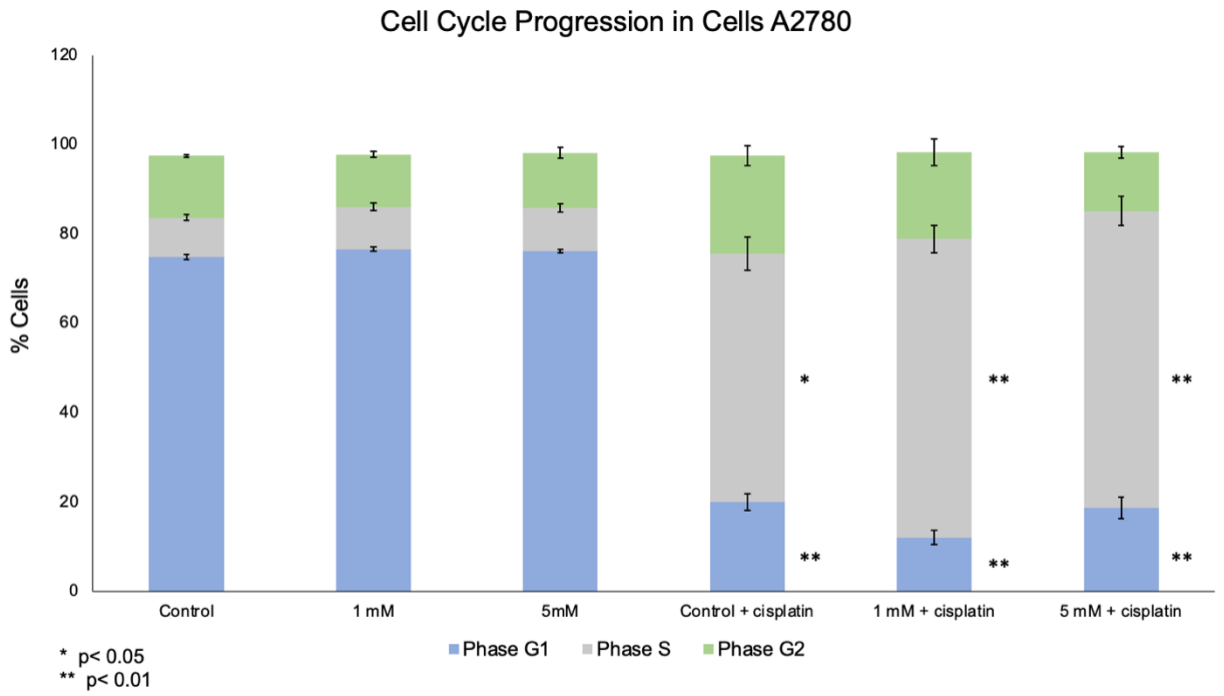

Figure 4. *The effect of cisplatin and fumarate on cell cycle progression*. The percentage of cells in cell cycle phases G1, S, G2 after fumarate and cisplatin treatments for three hours. G1 and S phases are statistically significant with respect to the control. * $p < 0.05$; ** $p < 0.01$ comparing each concentration with the control by paired t-test.

the repair of spontaneous damage. This phenomenon has been documented in other studies like that of Sulkowski et. al in 2020. Sulkowski observed that by disrupting local chromatin signaling, oncometabolites such as fumarate, succinate and 2HG, suppress DNA damage repair[18]. In his study, it also appeared that oncometabolites specifically suppress the homology-dependent repair (HDR) pathway and develop sensitivity to poly (ADP-ribose) polymerase (PARP) inhibitors; however, the mechanisms by which this does not occur are not yet clearly understood[19].

When 1mM fumarate was added to the cisplatin-treated cells, there was a significant decrease in the amount of damage relative to the cisplatin control. This same effect, however, did not occur at the higher concentration of 5mM fumarate. This indicates that, at the lower concentration, fumarate had a protective effect against cisplatin. There are two theories as to how this occurred, but further studies are needed to verify this. It is possible that fumarate caused increased DNA methylation and thus prevented cisplatin from entering DNA and inducing further damage. Another theory is that cisplatin still damaged DNA, but fumarate, at a lower dose, was able to help repair the damage caused. Although the mechanisms of this phenomenon are still unclear, it appeared that 1mM fumarate did have a protective effect against cisplatin-induced damage. A study by Gueble & Bindra[20] revealed that the physiological level of fumarate may play a role in the activation and regulation of certain DNA repair pathways. Under normal conditions, translocation of fumarate hydratase from the cytosol to the nucleus is crucial for activation of the DNA damage checkpoint[21]. Lack of FH and this translocation can then inhibit proper DNA damage repair[20].

5.2 Cycle Cell Progression

The addition of cisplatin was observed to increase the number of cells arrested in S phase, but the addition of fumarate did not change cell arrest trends compared to control or cisplatin. Traditionally, an increase in S phase cell arrest indicates high levels of toxicity caused by a block in DNA replication[22]. Several other studies have observed this phenomenon of cell arrest in the synthesis phase when treated with cisplatin. It has also been observed that as the dose of cisplatin increases, so does the number of apoptotic and necrotic cells[23]. There are two explanations for this observed increase in S phase cell arrest under cisplatin treatments. The first plausible explanation is that detection of the damage caused by cisplatin caused the cell to arrest while awaiting repair. The second is that the damage caused by cisplatin (DNA cross-linking) blocked the DNA replication process itself. Under normal conditions, as observed in cells not treated with cisplatin, most cells are in G1 phase, as this

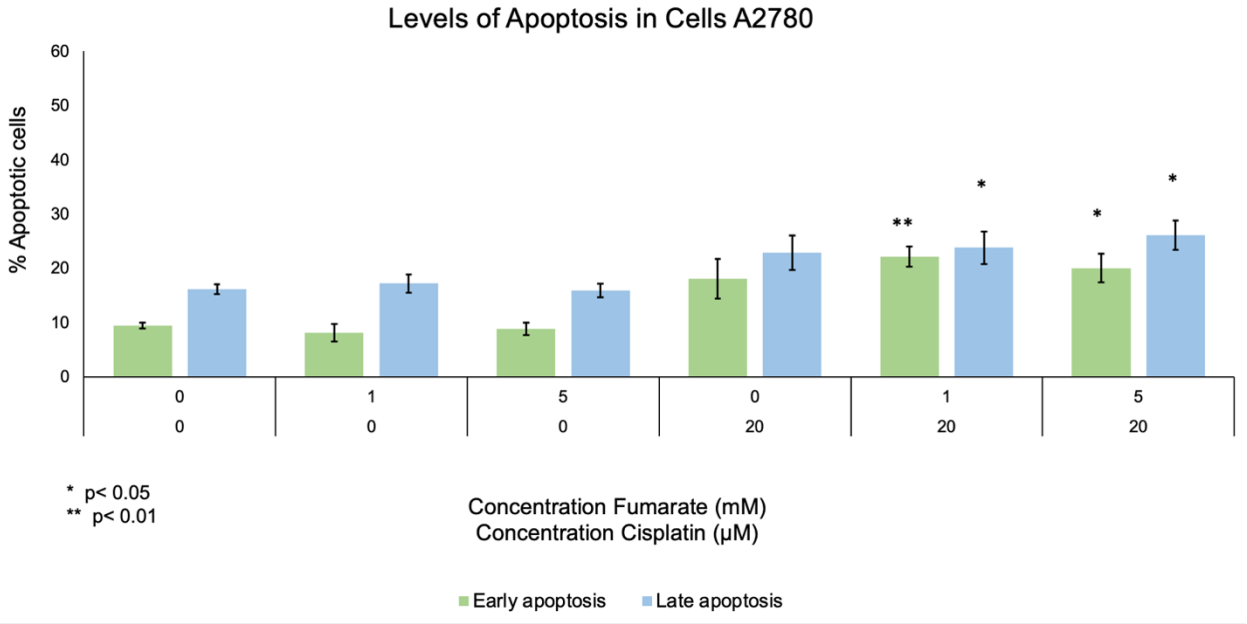

Figure 5. The effect of cisplatin and fumarate on apoptosis. The conditions of 1 mM fumarate + cisplatin and 5 mM fumarate + cisplatin are statistically significant in early and late apoptosis with respect to control. * $p < 0.05$; ** $p < 0.01$ comparing each concentration with control by paired t-test.

is the longest phase of the cell cycle as the cell collects adequate nutrients to be able to enter S phase[24].

5.3 Apoptosis Assay

A significant increase in apoptosis, both early and late, was observed in cisplatin-treated cells. This is a well-documented observation, and it has been shown that the induced apoptosis is both time and dose dependent. There is a positive correlation between the number of apoptotic cells and the treatment time and dose of cisplatin[23]. However, due to the lack of change with the addition of fumarate to the control or cisplatin condition, fumarate was observed to have no effect on apoptosis. This aligns with expectations that oncometabolites, rather than directly affecting apoptotic sequences, affect chromatin structure and access to DNA repair systems. Therefore, it can be concluded that fumarate does not induce apoptosis either in early or late, or in the conditions with and without cisplatin.

6 CONCLUSION

From this study and each assay, three major conclusions were drawn. From the comet assay, it was found that fumarate influences the response of cells to DNA damage. At a concentration of 1mM, this effect appears to protect DNA from further damage. It was also concluded that fumarate does not change the cell cycle or modify the effect of cisplatin. Lastly, fumarate does not induce apoptosis and does not alter cisplatin-induced apoptosis.

While there is still much research to be done, this study begins the process of understanding the effect of oncometabolites on DNA damage repair systems called upon by abnormal cross-links in the DNA. Metabolic reprogramming through chemotherapies that target metabolism has been an area of focus in recent years for cancer treatment[25]. Oncometabolites can modify the response to agents that induce DNA breaks through their ability to increase or inhibit the ability to repair DNA damage. This study works to address the role of the oncometabolite fumarate in this process. An understanding of the role played by oncometabolites in DNA repair processes is of interest for several reasons. Knowledge of their influence on the response to different types of tumor treatments could provide useful information in the management of patients and their response to antitumor therapy. It is also thought that genes that encode Krebs cycle enzymes could be used as therapeutic targets to modulate a patient's response to therapy. Additionally, the effect of oncometabolites has only previously been studied in response to DNA damage induced by breaks, and this study offers a new insight into their role when DNA damage is induced by cross-links.

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8 EDITOR'S NOTES

This article was peer-reviewed.

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