Arsenic treated human acute promyelocytic leukemia cells (HL-60 cells) inhibited cell line proliferation in vitro

BIOL 302L-H01 Final Report

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Abstract

We wanted to examine the popular leukemia therapy compound arsenic and its effect on the proliferation of human acute promyelocytic leukemia cells (HL-60 cells). We hypothesize that cell proliferation will be inhibited in correlation to the concentration of arsenic in respect to the ideal concentration (0.1μM). We examined the cell percent viability (trypan blue count), checked the differentiation of these cells (may grunwald/giemsa stain), and evaluated the apoptotic response within HL-60 cells (caspase 3/7 glo assay). Our arsenic treated cells had not been as successful at proliferating compared to the controls (~95% in UT (untreated) and H2O versus ~69.4%-38.8% in A,B,C and D). There was an outlier of group C having a high amount of neutrophil morphological expression, but in the overall picture no major differentiation differences were observed. Significant amount of caspase 3 or caspase 7 enzyme activity was observed in untreated and H2O treated cells, therefore mostly due to the significantly less percent viability of arsenic treated cells. Due to such a significant mortality in all arsenic groups, there is no significance between the experimental groups. However, we do demonstrate inhibited proliferation of HL-60 cells in response to arsenic, but further testing with different cells would have needed to be done to support that claim in significance. Because we might be observing the specification for cancerous cells due to arsenic, it would be important to continue this experiment in calibrating the clastogenic and co-mutagen properties of the compound.

Introduction

Human acute promyelocytic leukemia cells (HL-60 cells) have long been used to study the background mechanisms involved in proliferation and differentiation of the hematopoietic lineage. This lineage is an immortal cell line “stem-like” that can be induced to differentiate into

several types of white blood cells through different reagents in the extracellular matrix (Cirtain, Mittelstadt, Higgins, Polson, 2019). These cells mirror the properties of stem cells, but are considered multipotent.

This differentiation and proliferation is all controlled by the signal transduction pathways. The ligand induced differentiation is the cause of several pathways communicating to cause different gene expression of these particular cells by controlling the central dogma of the cell. This is the general pathway from DNA to RNA, through transcription, then into RNA to protein, through translation (Cirtain et al., 2019). All along the central dogma, there are control mechanisms that are trying to be understood, yet almost insurmountable by their complexity (Xie, Laouar, Huberman, 1998).

Apoptosis is another critical factor in understanding these pathways. This is a programmed death of the cell through a particular genetic expression. A particular form of programmed cell death, important in our cell line is autophagy due to it being a very common response when the cells are incapable of going through the standardized apoptotic pathway. It is the self-ingestion of the cell by lysosomes during times when the path for apoptosis is inhibited, like in cancerous HL-60 cells (Cirtain et al., 2019). Necrosis is another promoter of cell death by an extracellular variable common in a lot of *in vivo* cells. Arsenic may flood the cells and cause many mutations by oxidation that would simply not allow the cell to do the same function and stay alive, thus breaking down into the extracellular matrix. To simplify the findings we focus primarily on apoptotic response based on the addition of arsenic and research done that supports this cell death pathway induction by arsenic (Bain, Liu, League, 2016).

We used arsenate to see what effects this compound would have on the signal transduction pathway of HL-60 cells due to its abundance of prior studies and relevance at being a form of acute leukemia therapy in the United States in 2006 (Bain et al. 2016). It has also been used in unapproved medicinal practices for over 2,400 years in China and other places around the world (Yedjou, Moore, Tchounwou, 2006). It is often considered a toxin, however, arsenic is everywhere in the environment and used in manufacturing (Bain et al., 2016).This makes it crucial for us in understanding its effects and mechanisms on cells.

Arsenic began to be used as a therapy in acute leukemia therapy due to it being a clastogenic: a mutagen known to disrupt and break chromosomes (Zhang, Schmitt, Mumford, 2003). Acute leukemia is caused by in part by the oncogenic PML-RARa fusion protein. PML is a tumor suppressor gene and RARa is helpful in the transcription of certain genes for differentiation (Zhang et al., 2003). The fusion of these proteins results in a useless protein incapable of normal function, leading to the cancerous properties of HL-60 cells. This fused protein is due to the translocation of chromosomes 15 and 17 (Zhang et al., 2003). Therefore theoretically, arsenic at low concentrations would be capable of disrupting this translocation. The ideal concentration is 0.1 μM. It is known to reduce proliferation at higher concentrations (Zhang et al., 2003).

Arsenic is also known to be a co-mutagen: a carcinogen. So even though it may disrupt t(15,17) translocation, other cell properties may be halted or set off an apoptotic pathway in vivo (Yedjou et al., 2006). In vitro, with the HL-60 cells we would expect apoptosis. HL-60 apoptosis is a restricted pathway in this cell lineage through a series of factors which make cancerous cells able to proliferate without regulation. This carcinogen can result in an increase in proliferation of

cells at lower concentrations because it hinders this apoptotic pathway further. In class and previous labs, we examined a specific signal transduction with PMA ligand-receptor interaction that differentiated cells and here we are trying to examine the opposite effect for the inhibition of cells (Cirtain et al. 2019).

We plan to treat HL-60 cells with arsenic because we are observing the effects of this compound on apoptotic response and proliferation, especially since prior research seems to be uncertain about the properties and mechanisms of this compound. We hypothesize that HL-60 cells with higher concentrations of arsenic (0.2042M) will result in higher cell mortality rates whereas cells treated with lower concentrations of arsenic (0.0152M, 0.0258M and 0.1027M) will undergo increased rates of proliferation compared to the control.This is due to the fact that arsenic, at lower levels, is a carcinogen that can induce proliferation of these cells (Tong-Cun, 2003). However, as the concentration increases, arsenic has been known to cause damage to DNA, altering the signal transduction pathways and inhibiting proliferation, which induces apoptosis (Bain, 2019). Thus, it can be predicted that the rates of apoptosis in the arsenic treated cells will increase.

Methods

We made six different cell groups. Two of the groups were controls: negative (untreated cells with only the media solution) and positive (H2O treated). THis was to see how the cells responded to the media they were in and the media they were in after adjusting pH balance for the dissolving of arsenic in the cell media, respectively. This did allow us to see the effects of the arsenic directly without the confounding variable of the cell media. Four arsenate treated cells

were used with varying concentrations by adding varying amounts of our 1M arsenic solution (3µL:A-0.0152M, 6µL:B-0.0258M, 12µL:C-0.1027M and 24µL:D-0.2042M). All the methods below follow the pattern of taking each cell treated group through the same three procedures.

Trypan Blue Stain

A trypan blue stain was conducted first with 0.2 mL of cells and 0.4% trypan blue at a 1:1 ratio (dilution factor of 2) (Cirtain et al., 2019). The mixture was vortexed and sat for 5 minutes at room temperature. Solution was then vortexed again.15 µL was put on a hemocytometer from each cell group. Under a light microscope, three mm squared squares were counted (top left, middle and bottom right) in a zig-zag, top-to-bottom pattern. The percent viability was calculated (Total # viable cells/ Total # viable + nonviable cells) X 100% = % Viability).

May-Grunwald/Giemsa Stain

Formaldehyde fixation of the cells had to be conducted first. They were spun down and resuspended in 9.6 mL of fresh medium. 0.4mL of cells were mixed with 4% formaldehyde, mixed and sat for approximately 15 minutes with periodic resuspension (3 minutes) (Cirtain et al., 2019). Cells were centrifuged and medium was removed. The cells were resuspended in PBS and 10 µL smears were created on slides.

These slides were then flooded with 500 µL May-Grunwald stain and incubated for 5 minutes at room temperature. 500 µL of 1:1 May-Grunwald stain and May-Grunwald/Giemsa oxidizing buffer mixture flooded the slides. It then sat for 7 minutes. The slides were flooded with 500 µL May-Grunwald/Giemsa oxidizing agent and was then immediately flooded with 500 µL deionized water where they were placed on a heating mantle until dry at 80 degrees Celsius.

Finally, we counted approximately 75 cells under a light microscope, recording their morphology.

Caspase-Glo 3/7 Assay

The caspase-glo 3/7 solution (100 µL) and buffer (100 µL) were mixed in a microtest tube. 25 µL of cells were mixed with 25 µL of the solution (Cirtain et al., 2019). The mixture was placed in an eppendorf tube. An additional negative control was done when we took 25 µL of plain cell media and 25 µL of the solution. All seven groups settled for 45 minutes in darkness with a cooler. The control tube was used as the baseline metric in the luminometer. The rest of the samples were placed in the luminometer sequentially and metrics were recorded.

Results

The percent viability for each experimental group is in figure 1. The cell count was above 1,200,000 cells in the control groups. Only neutrophils and undifferentiated cells were found in the may-grunwald staining and representations of the counts are shown based on the percentage of the total observation (figure 2). The arsenic treated groups had caspase activity ~2690 RLU/s. Controls had ~23187 RLU/s (H2O) and ~7468 RLU/s (UT) (figure 3).

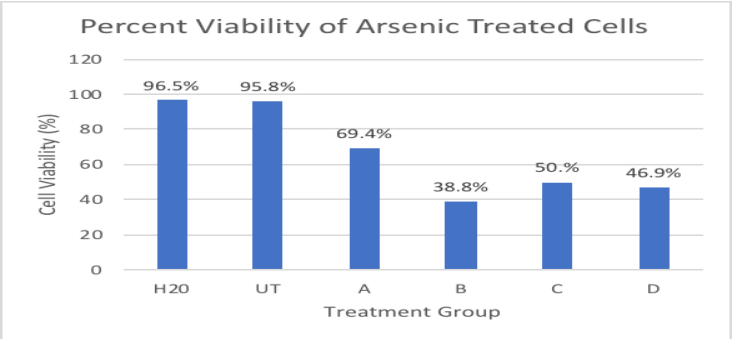

Figure 1. Percent viability in all six groups. Cell count viability in arsenic treated groups (~95% in UT (untreated) and H2O versus ~69.4%-38.8% in A,B,C and D).

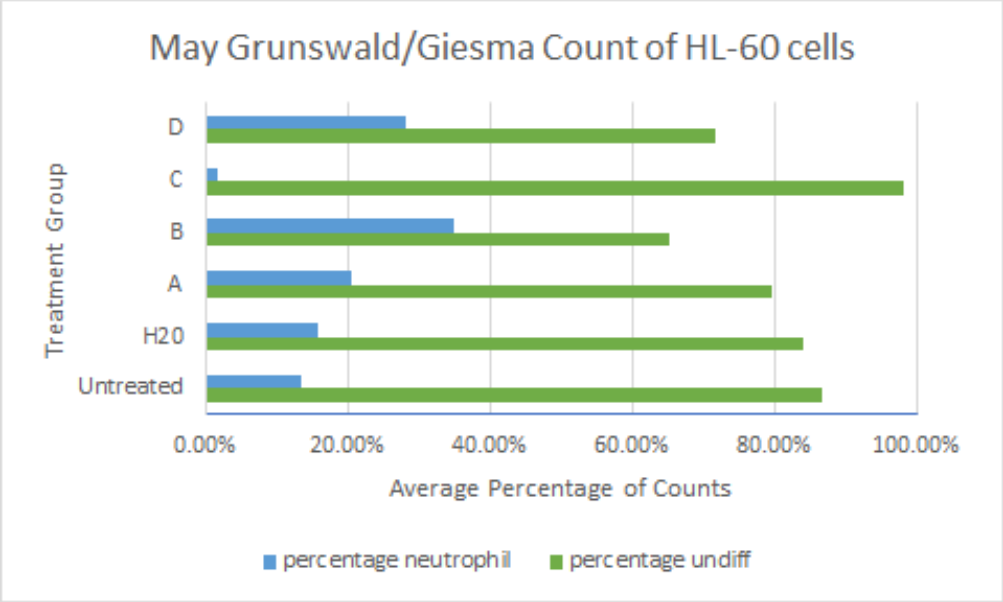

Figure 2. May grunwald/giemsa counts in each group. Percentage (green-undifferentiated and blue-neutrophils) out of all cells counted in slide (~70 cells a slide). All were roughly close in percentage (neutrophils ~20% and undifferentiated ~80%).

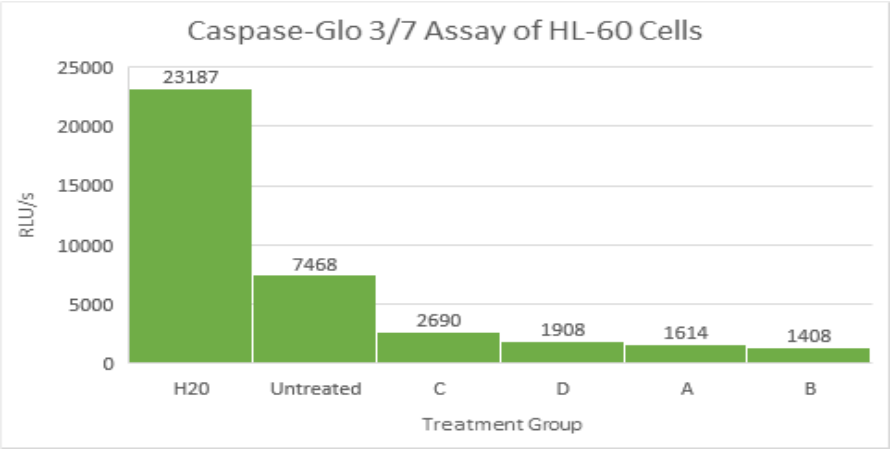

Figure 3. Treatment group over relative light units (RLU/s). Relative abundance of caspase 3 and caspase 7 activity (involved primarily in apoptosis, autophagy uses caspase 3).

Discussion

Our results could not concretely support nor deny our hypothesis. There was a large death rate in all arsenic groups, demonstrating no significant difference between the experimental groups based on concentration.

There is no prevalence for a clear pattern in percent viability, differentiation, and apoptosis in comparison to the controls and each other. Our arsenic treated cells had not been as successful at proliferating compared to the controls (figure 1). Cells were mostly dead in the arsenic treated cells and low numbers of viable cells observed. There is a higher amount of neutrophils in most arsenic counts (except group C) based on percentage in counts, however, no research supports a correlation of neutrophils with arsenic resistance(figure 2). Further experimentation would need to be done besides just one experimental group for each concentration. Only two morphologies were present, undifferentiated and neutrophils. Cells did not show a significant amount of caspase 3 or caspase 7 enzyme activity compared to untreated and H2O treated cells (figure 3). There is not a clear pattern within the treated cell groups and diluent showed an uncharacteristic high cell death. Arsenic treated cells demonstrate mass genocide versus the controls.

When looking at the cell viability of our experimental groups, there were almost no cells on the slide, telling us that the cells were killed through necrosis or were inhibited in proliferating. Since the caspase glo assay shows almost no programmed cell death in arsenic groups, we could support the idea that the cells were inhibited and not killed through an apoptotic pathway, yet these results do not rule out necrosis by flooding the cells with arsenic as

a major contributing factor. This would give a reason to why we observed less cells in the dish that contained arsenic.

Even though the tests were not definite in mechanics for the lack of proliferation, we had extremely low numbers in cell counts and abundance compared to the controls. We also had four experimental groups that were well above the ideal concentration of arsenic (0.1μL) seen in past experiments to inhibit cell proliferation by disrupting the translocated chromosomes (Zhang et al., 2003). This was largely due to the fact that it would take a great amount of dilution that we were not able to obtain from the original stock solution we had on hand. It would have been better if we planned out better what stock solution would be needed prior to calculating concentration needed. This gives us reason to support that an above ideal solution would cause the flooding of the HL-60 cells, inducing necrosis.

In future studies, a mechanism for determining the death pathway of necrosis versus autophagy would be to add 3-methyladenine (inhibitor of the autophagic pathway) to a replica cell culture in experimental groups A, B, C, and D to inhibit autophagy (Wu, You-Tong & Tan, Hui-Ling & Shui, Guanghou & Bauvy, Chantal & Huang, Qing & Wenk, Markus & Ong, Choon-Nam & Codogno, Patrice & Shen, Han-Ming, 2010). We would be able to deny the hypothesis that cells died due to necrosis if cells were still alive with the autophagic inhibitor. More test samples would also be needed to show any statistical significance in our findings, since we only had 1 experimental group for each arsenic concentration. We could have also experimented with the specificity of arsenic described in a lot of papers due to its unknown clastogenic properties by examining other differentiated treated cell groups by using other ligands, such as dimethyl sulfoxide (Xie et al. 1998 & Zhang et al. 2003).

We support a definite halt in proliferation of HL-60 cells whether it was either mechanism. A reason for why this experiment is important is because the mechanism for how it inhibits proliferation of HL-60 cells is still unknown and, yet, still used in leukemia therapy (Yedjou, 2006). We also wish to know the ideal concentration of arsenic to use because it is vital to inhibit the carcinogenic properties of the compound and still have the best chance to allow arsenic to have the clastogenic effects on cancerous cells. It would be astonishing if we were able to understand the mechanism and construct a synthetic compound that targets specifically the cancerous cells based on the properties of arsenic, allowing for hope of a safer procedure for patients with leukemia.

The signal transduction pathway for all ligand and cell messagers is a long and infinitely complicated process. The holy grail to understanding our cells and treating of deadly mutagens is specification in this constantly expanding network of messenger molecules. Arsenic gives us the ability and, most of all, the chance to possibly understand this specification process.

References

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