9-(2-Phosphonyl-methoxyethyl)-adenine Promotes Erythrocytic Differentiation and Disrupts Cell Replication in Chronic Myelogenous Leukemia K562 cells

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Abstract

Disruption during cellular differentiation can cause hematopoietic stem cells to proliferate uncontrollably, resulting in the development of cancer. Differentiation therapies are being investigated as a type of cancer treatment which involves inducing agents that promote the differentiation of cancer cells into those with similar properties to normal blood cells. These cells can then undergo apoptosis at an accelerated and controlled rate compared to cancer cells, making this a potential therapeutic technique. In this study, the ability of human chronic myelogenous leukemia K562 cells to undergo cellular differentiation in response to the inducing agent 9-(2-Phosphonyl-methoxy ethyl)-adenine (PMEA) is investigated. PMEA has previously been shown to disrupt cell replication and promote erythrocytic differentiation in K562 cells. In order to further test the effectiveness of this inducer, cell proliferation was measured with a cell growth curve, hemoglobin presence was measured with benzidine staining, and gamma-globin expression (a protein subunit of fetal hemoglobin) was measured in both induced and uninduced K562 cell cultures via RT-qPCR and western blotting. The results indicate that PMEA slows cell replication and promotes hemoglobin (and subsequently gamma-globin) expression in treated cells. In summary, the findings support the conclusion that PMEA is able to promote erythrocytic differentiation in K562 cells and provides information that supports differentiation therapies as a method for cancer treatment.

Key words*: K562, cellular differentiation, PMEA, gamma globin, differentiation therapy*

Introduction

Hematopoiesis occurs throughout both fetal development and adulthood and involves the formation of new blood cells from hematopoietic stem cells (HSCs) that are present in bone marrow (Jagannathan-Bogdan and Zon, 2013; Tsiftsoglou et al., 2003). These cells can differentiate according to various cellular pathways depending on the specific patterns of gene expression that occur, including either the myeloid or lymphoid pathways (Jagannathan-Bogdan and Zon, 2013; Andersson et al., 1979). The myeloid pathway can then further differentiate into other types of cells, such as red blood cells (erythrocytes). Erythrocytes express higher levels of hemoglobin and its constituent protein subunits compared to other hematopoietic progenitors, which can then be used as markers to measure cellular differentiation (Hatse et al., 1998; Hatse et al., 1999).

If HSCs are disrupted during differentiation, it can lead to uncontrolled cellular proliferation and the development of cancer (Tsiftsoglou et al., 2003). Chronic myelogenous leukemia (CML) in particular, which is a type of leukemia present in adults and children, shows

a marked increase in the rate of cell division compared to normal HSCs, as a result of a translocation mutation between the 9th and 22nd chromosomes of these cells (Lozzio and Lozzio, 1975; Tsiftsoglou et al., 2003). This mutation causes the excision and joining of the *bcr* and *c-abl* genes from either chromosome and the expression of the tyrosine kinase oncoprotein Bcr-Abl. This oncoprotein stimulates uncontrolled cellular proliferation, which disrupts the normal rate of cell replication and leads to the development of cancer. K562 cells are a human CML cell line with similar properties to red blood cells which can be induced to differentiate into erythrocytic-like cells along the myeloid differentiation pathway (Hatse et al., 1998; Hatse et al., 1999; Tsiftsoglou et al., 2003; Andersson et al., 1979). Erythrocytic differentiation causes these cells to express various types of hemoglobin that would not normally be expressed, such as HbF (fetal hemoglobin) (Tsiftsoglou et al., 2003). HbF contains alpha and gamma globin protein subunits, which can be used as specific markers of erythrocytic cellular differentiation (Chang et al., 2011; Hatse et al., 1998; Hatse et al., 1999; Tsiftsoglou et al., 2003). Since cancer cells are otherwise immortal, differentiation into erythrocytic-like cells slows the rate of tumor formation and cancer progression by increasing the rate of cell mortality (Tsiftsoglou et al., 2003). The current drug used to treat CML (imatinib mesylate) functions by specifically targeting and inhibiting tyrosine kinases such as the Bcr-Abl oncoprotein found in K562 cells. Though this drug has been effective as a therapeutic agent, multidrug resistance has become an issue since it reduces the effectiveness of this method of treatment. This makes finding inducing agents that can safely and reliably be used for differentiation therapies to induce cellular differentiation in cancer cells an important avenue to explore more in cancer research. This is especially important as a treatment option for CML which is otherwise commonly treated with methods that can be toxic, invasive, or cause side effects, such as chemotherapy, radiation, surgery, or stem cell transplants (Canadian Cancer Society, 2021).

The objective of this project was to test the effectiveness of the inducing agent PMEA (9-(2-Phosphonyl methoxyethyl)adenine) on cellular differentiation in the human K562 leukemia cell line. PMEA is known to influence the erythrocytic differentiation of K562 cells by promoting hemoglobin expression, particularly HbF, and by increasing the rate of cell death by stalling cellular replication (Hatse et al., 1998; and Hatse et al., 1999). The expression of the gamma-globin protein subunit from HbF was measured as a marker of cellular differentiation to confirm whether or not PMEA was successful as an inducing agent on K562 cells (Tsiftsoglou et al., 2003; and Salvador et al., 2013). In this study, we used a cell growth curve to measure cell proliferation, tested for hemoglobin expression via benzidine staining, and then looked specifically at gamma-globin expression via both RT-qPCR and Western Blotting procedures. It was predicted that because of PMEAs ability to induce HbF expression, those cells that were exposed to it should express gamma-globin more readily at both the level of RNA and protein, and thus undergo a higher rate of cellular differentiation into erythrocytic-like cells, compared to those K562 cells not exposed to PMEA (Hatse et al., 1998; Hatse et al., 1999). By measuring for the expression of gamma-globin after exposure to PMEA, we could further elaborate on the effectiveness of PMEA as an inducing agent on cellular differentiation and cell cycle arrest in K562 cells. However, this research will provide insight not only to the effect of PMEA as an inducing agent on K562 cells but will also provide relevant information about how inducing agents function in general to influence cancer cells, as well as why differentiation therapies

should be considered further in future cancer studies, or as an alternative method for CML cancer treatments.

Materials and Methods

Cell Culture and Harvesting

Human CML K562 cell cultures were prepared according to an existing protocol (Hills et al., 2020). A total of $1x10^5$ K562 cells were suspended in 35mL of RPMI 1640 culture medium (Millipore Sigma) with either 500uL of PMEA solution added to the treated cultures (45mM final concentration) or 500uL of deionized water added to the untreated control cultures. Four PMEA treated and two untreated control cultures were prepared. After induction, the cells were cultured at 37℃ and 5% CO_2 for 7 days. Passaging took place on day 3, and TC10 cell counts were taken daily using 10uL samples from a designated treated cell counting culture and a water control culture. This was done to measure the rate of cell proliferation and viability, where the data gathered was used to construct the growth curve seen in Fig 1. Following the culturing procedure $4x10^6$ live cells from either treatment were harvested and stored at -20℃ for subsequent experiments.

Benzidine Staining

Prior to harvesting, 1mL of cell culture was taken from both the PMEA treated and the untreated samples to be used for staining. A benzidine stain containing 0.4% (10uL) of 35% H_2O_2 (v/v) to 2.5mL of benzidine stock solution (0.2% w/v of benzidine dihydrochloride in 0.5M acetic acid) was prepared immediately prior to staining. A volume of 0.1mL of stain solution was added to each 1mL cell culture sample and then incubated for five minutes prior to counting. Cell counts were performed in triplicate using a hemocytometer, with 200-300 cells counted per replicate (Fig 2).

RT-qPCR

Total RNA was extracted from one treated and one untreated cell pellet sample, as outlined in the RNeasy ® Mini Kit (Qiagen). The purity and concentration of RNA obtained from the extraction was determined using a NanoPhotometer N60-Touch (Implen) which measured the concentration and the A_{260}/A_{280} and A_{260}/A_{230} ratios for both the treated and untreated cells (Tbl 1).

Denaturing gel electrophoresis was performed using the Formaldehyde-Free RNA Gel Kit (Amresco), a 2% agarose gel, and a 0.5-9kb RNA ladder. The gel was run at 5-8V/cm for ~1.5-2 hours, and then the gel was imaged using the UVP BioSpectrum Multispectral imaging system using UV transillumination to confirm that the RNA was intact following extraction.

Reverse transcription of RNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen). A gDNA Wipeout Buffer (7x) was used to remove genomic DNA from the samples, and then the reverse transcription reaction proceeded according to protocol (Hills

et al., 2020), for a total of four reactions (one reaction with reverse transcriptase present and one without for each RNA sample).

qPCR was performed on the obtained cDNA samples using a QuantiTect SYBR Green PCR Kit (Qiagen), along with the C1000 ThermoCycler with the CFX96 Real-Time System (BioRad). Six reactions were set up using the four cDNA samples: (1) Treated 18S rRNA control (2) Untreated 18S rRNA control (3) Treated ɣ-globin (4) Untreated ɣ-globin (5) Treated ɣ-globin minus-RT control (6) Untreated ɣ-globin minus-RT control. 10X QuantiTect Primer Assays (Qiagen) were used for amplification of the 18S cDNA and gamma-globin cDNA during qPCR: (1) ɣ-Globin: Hs_HBG1_1_SG QuantiTect Primer Assay (200) - QT00041384 (2) 18S rRNA: Hs_RRN18S_1_SG Quantitect Primer Assay (200) - QT00199367. Additionally, an 18S and gamma cDNA control (no template added) were set up using the same reagents. The data obtained was normalized using the 18S control values, and the relative fold change in gene expression of gamma-globin was calculated using the $2^{(-\Delta\Delta Ct)}$ method.

The qPCR products were run on a 2% agarose gel for 30-45 minutes at 100V according to protocol in Hills et al. (2020), then subsequently visualized using the UVP BioSpectrum Multispectral imaging system using UV transillumination to ensure that appropriate amplification took place.

Western Blotting

Total protein was isolated from one treated and one untreated pelleted K562 cell sample according to protocol (Hills et al., 2020). The cell pellets were washed and resuspended in 500uL of ice-cold PBS, centrifuged at 4℃ and 1000RPM for 5 minutes, and then 250uL of lysis buffer (0.5% Triton X-100, 300mM NaCl, 50mM Tris-HCl (pH6.8) and 1mM PMSF) was added for 30 minutes, with regular vortexing to ensure full homogenization. The cells were then centrifuged again at 4℃ and 14,000 x g for 20 minutes before the supernatant was collected for further analysis.

Protein concentration was measured using a BioRad Protein Assay and compared to a BSA standard curve. Each concentration for the standard curve was prepared in triplicate, and the protein samples for the treated and untreated cells were prepared in duplicate. 250uL of dye solution and 5uL of each BSA standard or unknown protein sample was pipetted into each well on the microplate. The procedure was carried out according to protocol (Hills et al., 2020). The Beckman Coulter DTX 880 Multimode Detector was used to measure the absorbance (A_{595}) of both the BSA standards and the treated/untreated K562 protein samples.

Total protein samples of 20ug (15uL each) for both the treated and untreated protein samples were analyzed in triplicate using a 15% SDS-PAGE gel (+ 4% stacking gel) at 200V for 30 minutes. Then a membrane transfer procedure was carried out according to protocol (Hills et al., 2020) to a PVDF membrane using the Bio-Rad PowerPac 200 at 100V for 1 hour. After the transfer, the membranes were stained using a Ponceau S stain solution for 10 minutes, and then visualized to ensure proper protein transfer had occurred (Fig 3A).

A blocking procedure was carried out for 30 minutes using 10mL of a prepared buffer (20mM Tris, 500mM NaCl, pH 7.4, 1% w/v casein). The washing, antibody and staining procedures were performed according to protocol (Hills et al., 2020). The membrane was cut into three prior to antibody staining so that ɣ-globin as well as a α-globin and a GAPDH control

could be probed separately. Primary antibodies against ɣ-globin (1:200; mouse monoclonal, sc-21757, Santa Cruz Biotech), α-globin (1:200; mouse monoclonal, sc-514378, Santa Cruz Biotech), and GAPDH (1:400; Cat. No. 68795, Sigma-Aldrich) were left on the membranes at 4℃ for 48 hours before being washed with TBST and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1:1000; goat anti-mouse IgG HRP conjugated, Bio-rad 1705047) for 1 hour. Chromogenic detection was carried out using a CN/DAB substrate kit (ThermoFisher) until color developed (Fig 3B). The procedure was carried out according to protocol (Hills et al. 2020).

Results

Growth response of K562 cells to PMEA

To understand how K562 proliferation is altered in response to PMEA, we monitored both cell concentration and viability from a designated treated counting culture and a water control (uninduced) culture (Fig 1). Following the one-week culturing procedure, the PMEA treated cell culture exhibited a lower concentration of both total and live cells than the untreated cell culture (Fig 1). The growth curve in Fig 1 shows a clear trend between the treated and untreated cultures, with the PMEA treated culture exhibiting lower total and live counts compared to the water control culture for every day that was measured. Following day 1 post induction, the treated and untreated cultures diverged in cell number more evidently as cultures were able to undergo more rounds of replication.

Presence of hemoglobin in K562 cells

Following the culturing procedure, cell samples were taken from both the treated and untreated cell cultures, and then exposed to a benzidine stain to test for the presence of hemoglobin. Cells were counted in triplicate for both samples with the average percent of blue stained cells (benzidine positive) recorded (Fig 2). According to the obtained data, the treated cell sample exhibited a much higher ratio of benzidine positive cells (454/898) than the control untreated cell sample (82/1168) (Fig 2). The data indicates that those cells that were exposed to PMEA before the 7-day incubation period expressed hemoglobin at a much higher concentration than those that were not exposed to PMEA.

Gamma-Globin RNA expression

In order to determine how PMEA influenced the expression of gamma-globin mRNA (our marker of differentiation), a series of experiments were performed to analyze the RNA from both the treated and the untreated cell cultures. Whole RNA was extracted from both cell samples, with those cells that were treated with PMEA exhibiting a lower concentration of total cellular RNA than the untreated cells (Tbl 1). The absorbance values obtained indicated that there was no protein/aromatic contamination in the samples (Wilfinger, 1997). Denaturing gel electrophoresis was performed on both samples, with two bands visualized (at 285 and 185bps) which

correspond to the total rRNA and mRNA present in either sample (data not pictured). The absence of a smear at the end of the gel indicated that our RNA samples were intact.

A reverse transcriptase reaction was carried out, followed by qPCR with gamma-globin specific primers in order to measure the concentration of gamma-globin mRNA expressed in either cell sample (Tbl 2). Two rounds of qPCR were carried out, with the C(t) values normalized using an 18S RNA control. The qPCR products were run on an agarose gel, with bands of expected sizes for the samples with reverse transcriptase present (18S RNA and gamma-globin), and no bands observed for the controls (no reverse transcriptase and gamma-globin/18S cDNA primers with no template added) indicating no contamination (data not pictured). The relative fold increase in gene expression for gamma-globin in the PMEA treated samples following two rounds of qPCR was 11.70 and 11.88, indicating that gamma-globin was more heavily expressed in those cells treated with PMEA (Tbl 2).

Gamma-globin protein expression

To determine the effect of PMEA on K562 gamma-globin expression at the protein level, a series of experiments were performed to analyze the protein from both the treated and the untreated cell samples. Whole protein was extracted from both cell samples and quantified using a Bradford Assay and BSA standard curve (data not pictured). The protein extract from the treated cell samples had a concentration of $1.41x10^4$ug/uL, and the untreated samples had a concentration of $1.34x10^4$ug/uL.

A western blot procedure was performed to test specifically for the presence of gamma-globin protein expression. A Ponceau S staining procedure was performed to confirm successful electrotransfer of protein from the SDS-PAGE gel to the PVDF membrane (Fig 3A). Then, following antibody probing and chromogenic detection, bands for gamma-globin and for alpha-globin and GAPDH controls were visualized (Fig 3B). For gamma-globin, and also surprisingly for alpha-globin, the treated samples exhibited higher expression of either protein compared to the untreated samples (Fig 3B).

Figures and Tables

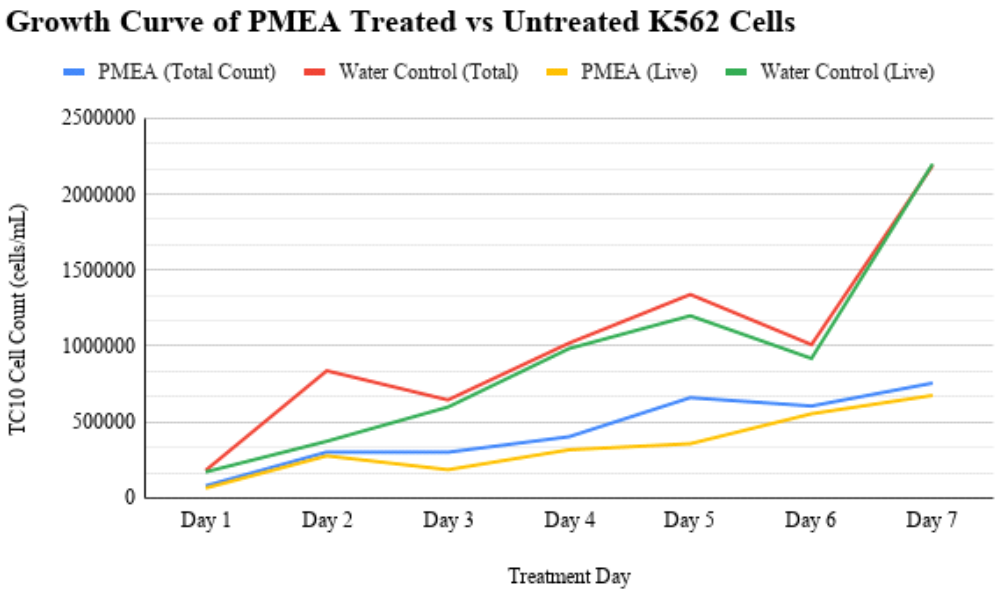
Table 1. Quantification and absorbances of extracted whole cellular RNA from K562 treated and untreated cell lines

	Treated	**Untreated**
Concentration	0.9063ug/uL	1.1251ug/uL
A_{260}/A_{280}	2.167	2.195
A_{260}/A_{230}	2.016	2.260
A_{260}	22.70	28.19

Table 2. RT-qPCR C(t) values and relative fold change in gene expression for gamma-globin in PMEA treated and untreated K562 cell lines

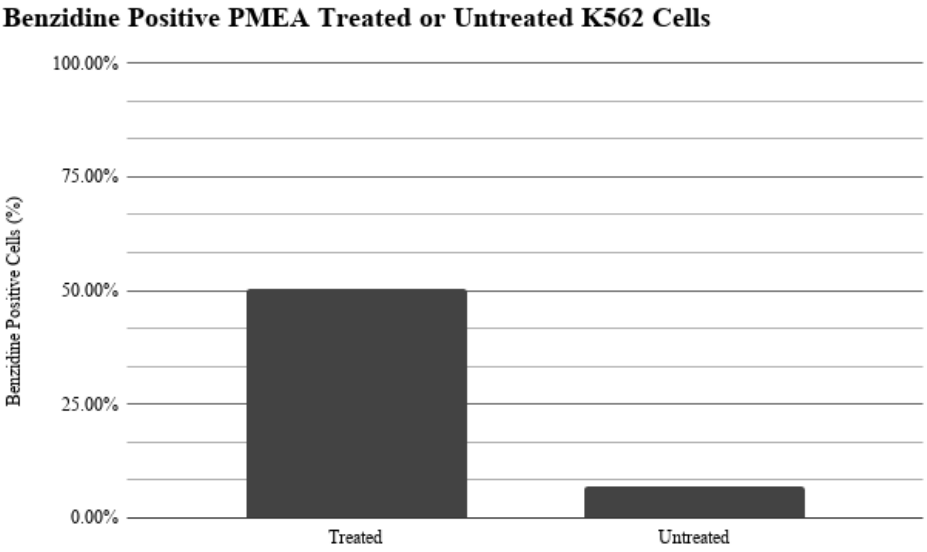
Round 1	**C(t):** Untreated 18S rRNA control (RT+): 12.12 Treated 18S rRNA control (RT+): 12.61 Untreated ɣ-globin (RT+): 15.06 Treated ɣ-globin (RT+): 12.00 Untreated ɣ-globin (RT- control): N/A (no contamination) Treated ɣ-globin (RT- control): N/A (no contamination)	**Relative fold change in gene expression**: 11.70
Round 2	**C(t):** Untreated 18S rRNA control (RT+): 13.31 Treated 18S rRNA control (RT+): 16.3 Untreated ɣ-globin (RT+): 15.17 Treated ɣ-globin (RT+): 14.59 Untreated ɣ-globin (RT- control): N/A (no contamination) Treated ɣ-globin (RT- control): N/A (no contamination)	**Relative fold change in gene expression**: 11.88

Figure 1. K562 cells treated with PMEA exhibit lowered rates of cellular proliferation.



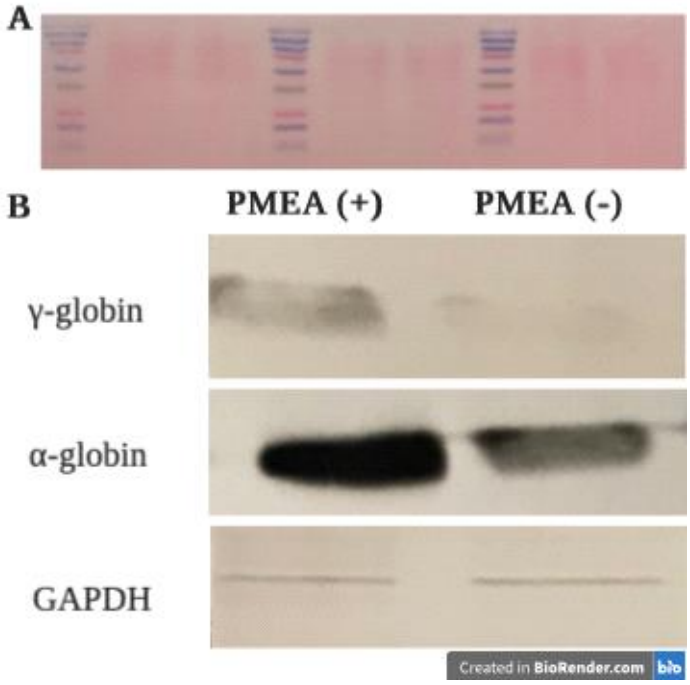
Cell counts were taken daily with a TC10 cell counter, measuring the total number and live number of K562 cells from either a designated treated counting culture, or a water control sample (untreated). The PMEA treated cells exhibited lower total and live cell counts than the untreated cells for each day of incubation. Though there is a clear trend that PMEA treated samples proliferate at a slowed rate compared to the water samples, more samples would need to be taken per day before it could be determined if the increase in cell number was significantly different between the two treatments.

Figure 2. PMEA treatment enhances hemoglobin expression in K562 cells



Benzidine positive cells were counted in triplicate using a hemocytometer for both the treated and untreated K562 cell samples at one week post induction and shown in the figure above. The treated cells (n=3, x̄= 49.93, SD: 5.4, CI(99%): 18.95-80.92%) exhibited significantly more benzidine positive cells than the untreated samples (n=3, x̄ 7.03%, SD: 1.21, CI(99%) 0.08-13.98) ($p<0.01$).

Figure 3. *K562 cells exposed to PMEA exhibit increased gamma and alpha globin expression*



(A) Ponceau staining of K562 whole cell protein extract samples (B) A western blot was probed with anti-Hbɣ, anti-Hbα, and anti-GAPDH primary antibodies. Cells treated with PMEA exhibited increased globin expression compared to the untreated cells.

Discussion and Conclusions

In previous studies, it has been shown that PMEA (9-(2-phosphonyl-methoxy ethyl)-adenine) influences human CML K562 cells to undergo cellular differentiation into homologues of erythrocytes and disrupts cell replication by halting the S-phase in cell replication (Hatse et al., 1998; Hatse et al., 1999). In order to test the efficacy of PMEA, we performed a series of experiments on K562 cells to test for evidence of cell cycle disruption and erythrocytic differentiation by measuring the rate of cell replication and the expression of HbF and its constituent gamma-globin protein subunit in comparison to cells that were not exposed to PMEA.

The growth curve generated during the 7-day incubation period supports the hypothesis that PMEA influences the ability of K562 cells to undergo replication, since cells treated with PMEA exhibited lower cell concentrations (for both live and total cells) for every day of the incubation period compared to those not treated with PMEA (Fig 1). These observations support the conclusion that PMEA disrupts S-phase in cell replication, which has been previously suggested in the available literature (Hatse et al., 1998; Hatse et al., 1999). However, the experiments performed did not specifically test for the specific phase of cell replication that was disrupted, and so although the results support previous findings, further experiments would need to be performed in order to confirm that it was S-phase that was disrupted during replication in our experiments. Additionally, although the results obtained show clear trends in

the rate of proliferation based on the growth curve obtained, more samples would need to be measured before we can confirm if the difference in number of cells between the two treatments was truly significant.

Benzidine binds to hemoglobin (or heme groups) present in cells, allowing the benzidine stain procedure to act as a preliminary indicator of erythrocytic differentiation (Salvador et al., 2013; Tfistsoglou et al., 2003). Since the cells treated with PMEA exhibited a much higher mean percent of benzidine positive cells than those not exposed to PMEA, it indicates that they expressed hemoglobin at an increased rate compared to the untreated cells (Fig 2) (Salvador et al., 2013). This supports the conclusion that PMEA is involved in and promotes erythrocytic differentiation in K562 cells (Hatse et al., 1998; Hatse et al., 1999). Though the untreated samples did still exhibit some benzidine positive cells, this is most likely due to interactions between the benzidine stain and heme groups already present in these cells and is therefore not necessarily indicative of cellular differentiation (Fig 2).

Following the RT-qPCR procedure, which was done to test for the expression of gamma-globin at the level of mRNA, the PMEA treated cell samples exhibited an average relative fold increase in gamma-globin gene expression of 11.79 (Tbl 2). This indicated that the treated cells expressed gamma-globin mRNA to a much higher degree than those cells that weren't exposed to PMEA. This was expected, since PMEA has been shown to induce hemoglobin (and thus gamma-globin) expression in previous experiments (Hatse et al., 1998; Hatse et al., 1999). Based on the clear trend seen between the two rounds of qPCR (Tbl 2), the results support the conclusion that PMEA promotes erythrocytic differentiation in K562 cells.

The western blot procedure was done to further confirm how PMEA influences the expression of gamma-globin in K562 cells, since the RT-qPCR, benzidine stain, and growth curve procedures could not confirm the presence of gamma-globin at the protein level. Two western blot procedures were performed, with the PMEA treated samples exhibiting higher gamma-globin expression than the untreated samples in both. This further supports the conclusion that PMEA enhances erythrocytic differentiation in K562 cells (Hatse et al., 1998; Hatse et al., 1999). The GAPDH blot pictured in Fig 3B shows identical bands for both the untreated and treated K562 cell samples, but it is important to note that these controls, as with the alpha-globin controls, were probed separately from the gamma-globin samples (since the SDS-PAGE gel was loaded in triplicate, with each replicate subsequently probed with a separate primary antibody after the transfer procedure). Though the GAPDH control was probed separately from gamma-globin, the expression level was consistent between treatments and so it was still used (along with the results from the Ponceau S stain) to indicate consistent loading across samples. The results obtained from the alpha-globin blot show increased expression for the PMEA treated samples compared to the untreated samples, as was observed for gamma-globin (Fig 3B). This was unexpected, since alpha-globin was intended as another control where consistent expression levels were anticipated between treatment groups. These results were consistent for both western procedures, elucidating as to the possibility that PMEA promotes globin expression in general, not just gamma-globin. Since it is anticipated that PMEA increases HbF expression, which contains both gamma and alpha globin subunits, this is a possibility (Tsiftsoglou et al., 2003). Further analysis would be required to determine if PMEA influences globin expression in general, but due to limitations on reagents and time, only gamma-globin expression was specifically measured in both the RT-qPCR and western procedures. However,

since the expression of alpha-globin was higher in the treated samples for both western procedures, and the benzidine stain revealed higher hemoglobin levels for the treated cells in general, this is a possible explanation.

The overall results from the experiments performed support the conclusion that PMEA promotes erythrocytic differentiation in K562 cells and influences the rate of cell replication, as has been seen in previous studies (Hatse et al., 1998; Hatse et al., 1999). Further experiments would need to be conducted in order to measure the effect that PMEA has on the expression of other proteins, such as other hemoglobin subunits, during cellular differentiation. This would further aid in understanding the mechanisms that PMEA uses to promote cellular differentiation. It would also be beneficial to test the effect of PMEA on other types of cancer cells, or on normally functioning cells, to get a more holistic understanding of how PMEA disrupts and influences cellular processing. This would be necessary in order to measure any potential toxic or unexpected effects caused by exposure to PMEA before it could be used as an inducing agent for cancer treatments with humans. In conclusion, we have shown that PMEA promotes erythrocytic differentiation and cell cycle disruption in CML K562 cells. Therefore, PMEA has the potential to be a viable inducing agent that could be used for differentiation therapy as a form of cancer treatment in the future.

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