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Investigating the Effects of Vitamin K1 and K2 on the Triple Negative Breast Cancer Cell Line SUM159PT

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ABSTRACT

Vitamin K serves as a cofactor in the post-translational carboxylation of glutamate to γ-carboxyglutamate (GLA) by the enzyme gamma-glutamyl carboxylase (GGCX). Vitamin K epoxide reductases (VKOR) then oxidize the vitamin K to regenerate the compound's reduced form. While most of the known γ-carboxylated proteins function in coagulation and bone homeostasis, the presence of GGCX and VKOR in a wide variety of tissues suggests that the vitamin and its pathway has more extensive physiological roles. Our previous research has assessed the expression of the vitamin K pathway in triple negative breast cancer cells (TNBCs) and compared the effects of vitamin K1 to those of vitamin K2. These data indicated that K1 promoted, while K2 suppressed, TNBC cell growth and aggressive phenotypes. Here, using cell culture techniques, western blotting, cell-based assays, and qPCR, we expanded on this research by assaying for the effects of K1 and K2 on GLA expression, cell density, viability, cycle kinetics and the expression of the oncogenes cyclin D1 and c-Myc in the TNBC cell line SUM159PT. Our data demonstrated that short term (24h) treatment with K1 did not alter GLA modified protein expression in SUM159PT cells. Despite lack of evidence for γ-carboxylation, K1 exerted biphasic effects on the density of SUM159PT cultures, with minimal impact at low at concentrations and a reduction in density at higher concentration. Neither K1 nor K2 affected cell viability, but the compounds had opposite effects on cell cycle progression. Vitamin K1 tended to reduce the percentage of cells in S and G2/M phases with increasing treatment concentrations, whereas K2 induced dose-dependent entry and accumulation of cells in G2/M coupled with exit from G0/G1. The expected changes in expression of cyclin D1 and c-Myc by 5 μg/mL K1 or K2 treatment were not correlated with the effects of K1 and K2 on cell cycle. In summary, these data highlight novel actions of vitamins K1 and K2 in TNBC cells that may be independent of γ-carboxylation.

Keywords: vitamin K, SUM159PT, triple negative breast cancer (TNBC), cell viability, cell cycle kinetics

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INTRODUCTION

Breast Cancer and Metabolism

Breast cancer is the most diagnosed cancer in females worldwide. As of 2020, 2.3 million new cases are diagnosed globally each year, with incidence and death rates having increased over the last three decades. Risk factors include age, family history, obesity, drug and chemical exposure, race/ethnicity, and others. More specifically, autosomal dominant inheritance of BRCA1 and BRCA2 are primarily linked to increased risk of carcinogenesis (Łukasiwicz et al., 2021). Breast cancer is heterogenous, with multiple subtypes characterized based on molecular composition. The triple negative breast cancer (TNBC) is an invasive breast cancer lacking estrogen and progesterone receptors and HER2 receptors and is therefore not amenable to therapeutics that target these proteins in other subtypes. About 80% of TNBC patients also harbor a TP53 mutation. (Łukasiwicz et al., 2021). In general, TNBC has a worse prognosis with quicker metastasis than other subtypes.

Recent research on cancer and malignancy has shifted focus to the roles of metabolic changes. In the past century, studies on causes and functions of the Warburg Effect, for example, have aimed to provide new insights into cancer progression. The combination of a hypoxic tumor microenvironment and oncogenic mutations contributes a series of changes in cellular metabolism in transformed cells. In malignant transformation, the changes to metabolism can serve as "hallmarks" of that malignancy (Miller et al., 2013). One metabolic pathway being investigated for its potential effects on transformation and malignancy is the vitamin K pathway.

The Vitamin K Pathway

In the vitamin K cycle (Fig 1), vitamin K takes on a role as a cofactor in γ-carboxylation, a rare post-translational modification. The two dietary forms of vitamin K are phylloquinone (K1) and

menaquinone (K2), which are first reduced to the hydroquinone forms, VKH_2. The reduced form serves as a cofactor to the enzyme γ-carboxyglutamyl carboxylase (GGCX), which converts peptide bound glutamates (GLU) to γ-carboxyglutamate residues (GLA) (Gröber et al., 2014; Shearer & Okano, 2018). Clusters of GLA residues create Ca^{2+} binding motifs, which leads to conformational changes required for function of these vitamin K-dependent GLA proteins.

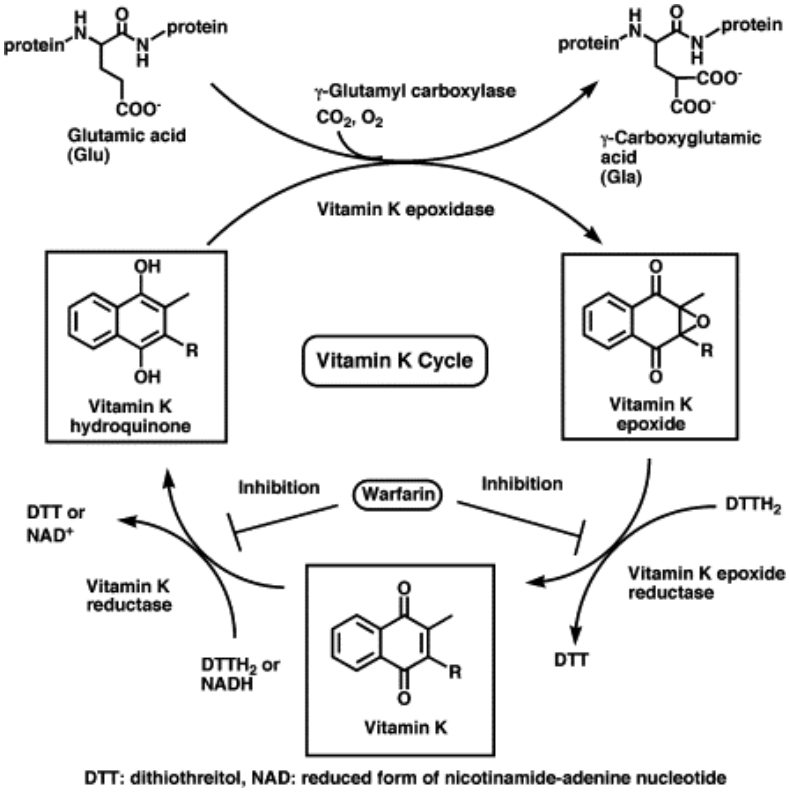

Figure 1. Utilization of Vitamin K in γ-Carboxylation. Vitamin K acts as a cofactor in the conversion of Glutamic acid (GLU) to γ-carboxyglutamate (GLA) by the enzyme γ-glutamyl carboxylase. Oxidation of the hydroquinone form to the epoxide form provides energy to deprotonate the GLU residue and yield the GLA residue. Vitamin K epoxides are converted back to the quinone form (depicted as "Vitamin K") by a Vitamin K epoxide reductase. The quinone form is then further reduced to the hydroquinone form by a reductase. *Note*. Reprinted from Suhara et al., 2006.

During γ-carboxylation, the VHK_2 form is oxidized to the vitamin K epoxide forms, which are then reduced back to the quinone form via vitamin K epoxide-reductase (VKOR) enzymes (Gröber et al., 2014; Shearer & Okano, 2018). VKORs are inhibited by vitamin K antagonists such as warfarin and other coumarin derivatives (Gröber et al., 2014; Suhara et al., 2006).

Ultimately, the coupling of GGCX and two known VKORs (VKORC1 and VKORC1L1) constitute a cycle which drive GLA modifications while conserving vitamin K within cells.

Phylloquinone (vitamin K1) and menaquinones (vitamin K2) are the naturally occurring forms of vitamin K present in most human diets. Regardless of dietary intake, the major form of vitamin K found in tissues is K2, as it is produced from K1 via the intermediate menadione (often called vitamin K3). The enzyme UBIAD1 (Fig 2) uses a side-chain substrate geranylgeranyl pyrophosphate (GGPP) to prenylate menadione, yielding the lengthened compound.

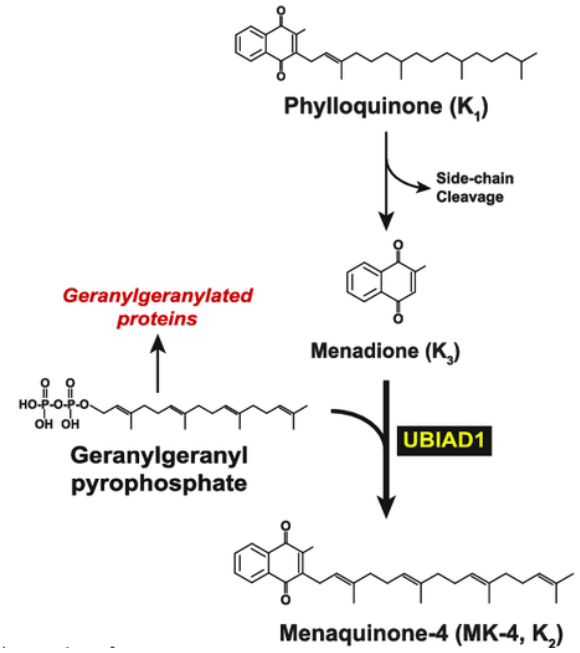

Figure 2. Biosynthesis of Menaquinone from Phylloquinone. Dietary phylloquinone (K1) is converted to menaquinone-4 (MK-4, K2) through an intermediate menadione (K3). The carbon side chain is cleaved off of K1 to yield K3. Geranylgeranyl pyrophosphate is added as a replacement side chain via the enzyme UBIAD1 to yield K2.
Note: Adapted from Schumacher et al., 2015

GLA-modified proteins produced in the liver and bone are essential for control of coagulation and skeletal health. However, because GLA proteins and the vitamin K pathway enzymes are present in other tissues, it is hypothesized that γ-carboxylation has more extensive

physiological roles (Beaudin et al., 2019). For example, VKOR activity was found to be upregulated in the glandular tissues, including the female salivary and mammary glands, as well as in the sex organs of mice (Caspers et al., 2015). Vitamin K compounds and pathway components have also been implicated to have roles in cancers; for example, GLA carboxylation has been linked to prostate and bladder cancers; however, mechanisms are not clearly defined (Kote-Jarai et al., 2011; Cheng et al., 2016).

In our laboratory's previous research, the effects of chronic exposure to vitamin K1 and K2 on expression of GGCX, VKORCs, and GLA protein modifications were compared in various TNBC model systems. Key findings included 1) GGCX, VKORC1, and VKORC1L1 were identified in literature and data surveys to be upregulated in invasive breast cancers, hinting at the relevance of vitamin K in the disease; 2) Each K vitamin had distinct effects on the TNBC cell lines, with K1 promoting cell growth and γ-carboxylation and K2 suppressing growth; 3) GGCX was highly expressed in three TNBC cell lines (MDA-MB-231, Hs578T, and SUM159PT); 4) GLA modifications were induced after treatment with K1 in all three TNBC lines (Beaudin et al., 2019). In sum, it was determined that several breast cancers express γ-carboxylated proteins, and that the vitamin K pathway is upregulated in aggressive breast cancers.

Objective

We hypothesize that the vitamin K pathway represents a therapeutic target for breast cancer. Understanding the effects of the two forms of vitamin K on breast cancer development and progression may lead to revised dietary guidelines or new treatment approaches for patients. To test this hypothesis, we examined the effects of vitamin K compounds on the TNBC cell line SUM159PT with four main experiments.

1. Our previous research included western blotting to detect potential changes in the expression of GLA-modified proteins in response to vitamin K1 treatment. To confirm that these observed effects resulted from the vitamin itself, we compared two different commercially available K1 preparations' effects on GLA expression.
2. To test the impact of vitamin K1 on SUM159PT cell growth, we conducted cell density assays. The acute effects of K1 on SUM159PT cell density were examined following treatment with vitamin K1 (1, 5, 10, 25, and 50 μg/mL) or ethanol (EtOH) vehicle. Density was quantitated on the IN Cell Analyzer after Hoechst staining for DNA.
3. While the cell density assay provides details on the total cell count, it does not report the state of those cells. Thus, to complement the cell density assays, cell viability and cell cycle kinetic assays were also conducted in response to treatments of vitamin K1 (1, 5, 10, 25, and 50 μg/mL), K2 (5, 10, and 20), and EtOH vehicle using the MUSE Cell Analyzer.
4. Cyclin D1 and c-Myc are two oncogenes that are often deregulated in breast cancer due to their roles in cell proliferation and cycle regulation. We used qPCR to analyze whether vitamin K1 or K2 altered the expression of either cyclin D1 or c-Myc in SUM159PT cells.

MATERIALS AND METHODS

Cell Culture

SUM159PT cells were grown and maintained in Ham's F12 media with 5% fetal bovine serum (FBS), 5 μg/mL insulin, and 1 μg/mL hydrocortisone. Passaging occurred every 3-6 days.

Western Blots

Cells were plated in 100 mm dishes and treated with either K1 V3501, referred to as 3-K1, or K1 47773, referred to as 4-K1. While both products are chemically phylloquinone, V3501 is the reagent utilized in previous studies, while 47773 is a validated analytical standard. Both vitamin K1 compounds were dissolved in a viscous buffer (Tween 80, glucose, and 0.9% benzyl alcohol) that is used clinically for injections of K vitamins. In parallel, additional cells treated with the injection buffer served as vehicle controls. 24h post-plating, whole cell lysates were collected in 2X Laemmli buffer, sonicated, and assessed for protein concentration by Pierce BCA protein assay (ThermoFisher Scientific, Waltham, MA). Samples containing 50 μg of protein and 5% β-mercaptoethanol (Millipore Sigma) were separated on SDS-PAGE gels, wet-transferred to PVDF membranes, and blocked for 1h in 5% skim milk/ PBS. The membrane was incubated overnight with a monoclonal mouse primary antibody that specifically recognizes the GLA domain (Sekusui Diagnostics, Stamford, CT) at a 1:1,000 dilution in 5% bovine albumin serum (BSA) solution with 0.02% sodium azide at 4ºC overnight while rotating. The blot was then rinsed 3 times with 1X PBST in an orbit shaker. After washing, the membrane was incubated with an anti-mouse enhanced chemiluminescence HRP-linked secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:5,000 dilution in a solution of 5% skim milk in PBS/Tween (PBST) at room temperature for 1 hour while rotating. SuperSignal West Dura Extended Duration Substrate (ThermoFisher Scientific) was used for imaging of blots on an

iBright CL1000 system (ThermoFisher Scientific). To strip the membranes for determination of GAPDH as loading control, blots were incubated in stripping buffer for 10 min, followed by a wash in 1X PBS for 5 min on an orbit shaker. Membranes were then re-probed with GAPDH primary antibody (AbD Sertotec, Raleigh, NC) diluted 1:16,000 in 5% BSA solution with 0.02% sodium azide at 4ºC for 1 hour while rotating. Secondary antibody incubations and band detection were as described for the GLA antibody.

Cell Density Assays

Cells were grown in media with EtOH vehicle (5 µL per 10 mL media) and passaged at least three times prior to treatment and analysis. SUM159PT cells were plated at a density of 10,000 in 24-well plates in the presence of vitamin K1 (V3501, Millipore Sigma, St. Louis, MO) dissolved in EtOH at a final concentrations of 1, 5, 10, 25, and 50 µg/mL K1, or EtOH vehicle alone. After the indicated time course (48h or 72h), cells were washed with PBS, fixed with 4% formaldehyde for 30 min on an orbital shaker, and incubated with 0.1 µg/mL Hoechst (bisbenzimide H33258, Millipore Sigma) for 15 min on an orbital shaker. The stain was then removed, and the cells were washed and incubated with PBS 3 times for 5 min on an orbital shaker. Plates were read on the IN Cell 2000 high content cell analyzer (GE Healthcare, Issaquah, WA) and data was analyzed with the IN Cell Analysis Workstation.

Cell Viability Assays

SUM159PT cells were grown in media with EtOH (5 µL per 10 mL media) and passaged at least three times prior to treatment and analysis. Cells were plated at a density of 50,000 cells/well in 6-well plates in the presence of vitamin K1 (1, 5, 10, 25, or 50 µg/mL), K2 (5, 10, or 20 µg/mL; Millipore Sigma, St. Louis, MO), or EtOH vehicle. After 48h or 72h, cells for each treatment were pelleted at 4ºC at 1500 rpm for 3 min, then resuspended in 1% FBS/PBS. Cell

suspensions were then incubated with the Luminex Muse Count and Viability Reagent Kit (MHC100102, Luminex Corporation, Austin, TX) at room temperature protected from light. Two samples for each concentration were prepared and analyzed for viability using the Guava Muse Cell Analyzer (Millipore Sigma, Hayward, CA).

Cell Cycle Assays

SUM159PT cells were grown in media with EtOH (5 µL per 10 mL media) and passaged at least three times prior to treatment and analysis. Cells were plated at a density of 50,000 cells/well in 6-well plates in presence of vitamin K1 (1, 5, 10, 25, or 50 µg/mL), K2 (5, 10, or 20 µg/mL), or EtOH vehicle. After 48h or 72h, cells for each treatment were pelleted at 4ºC at 1500 rpm for 3 min, then resuspended in 1% FBS/PBS. The cell suspension was then combined with 90% EtOH and incubated at -20ºC for > 3h prior to treatment with the Muse Cell Cycle Kit reagent (MHC100102, Millipore Sigma) and analysis with the Muse Cell Analyzer (Millipore Sigma). Further analysis and data presentation was conducted with the FlowJo cell cytometry analysis platform (Becton, Dickson and Company, Franklin Lakes, NJ)

Gene Expression Analyses

SUM159PT cells were plated at a density of 700,000 cells/well in 100 mm dishes in the presence of 5 µg/mL vitamin K1, vitamin K2, or EtOH vehicle. After 24h, the cells were pelleted at 1500 rpm/4ºC for 5 min and washed with wash buffer (PMSF, protease inhibitor, benzamidine, nuclease free water) twice. RNA was isolated from the cells according to the Qiagen RNeasy Kit (Qiagen, Valencia, CA), and quantified using the NanoDrop 1000 Spectrophotometer (Eppendorf, Germany). RNA was stored at -20ºC for later cDNA synthesis and collection.

RT-PCR was used to prepare cDNA from the RNA using Taqman Reverse Transcriptase Reagents (Life Technologies, Grand Island, NY): reverse transcriptase buffer (10X), $MgCl_2$ (25mM), ddNTPs (100 mM), random Hex (0.2ug/mL), RNase inhibitor and MultiScribe reverse transcriptase. cDNA was then analyzed in triplicate using PowerUp SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers specific for cyclin D1, c-Myc, and 18S genes. Primer sequences (Integrated DNA Technologies, Coralville, IA) used are shown in Table 1. qPCR amplification was performed, and relative gene expression determined using the QuantStudio 12K Flex System (Applied Biosystems). The ΔΔCt method was used for calculations and Ct values were normalized to 18S.

Table I. Primers and Sequences Used in qPCR.

PRIMER	SEQUENCE
Cyclin D1 forward	5’-GTT TGG AAA TAT TCA CAT CGC TTCT-3’
Cyclin D1 reverse	5’-AGA GCA GTA ATC ACA TTA AGT CAA AA-3’
C-Myc forward	5’-AAG ACA GCGGCA GCC CGA AC-3’
C-Myc reverse	5’-TGG GCC AGC TGC TGT CGT TG-3’
18S forward	5’-ACC CGT TGA ACC CCA TTC GTG A-3’
18S reverse	5’-GCC TCA CTA AAC CAT CCA ATC GG-3’

RESULTS

Western Blotting for GLA Expression

Following SDS-PAGE and transfer to membranes, Ponceau staining was used to assess total protein loading. Ponceau staining showed successful transfer of protein to the membrane, and bands were visible in all sample lanes, as well as in the lanes loaded with the Magic Marker DNA ladder. It is well researched that various GLA-modified proteins exist within given cells and tissues performing various roles, notably as residues for coagulants and anticoagulants. In western blotting, a single dark band at an identifiable molecular weight representing the expected protein is generally present because of the usual "one antigen, one antibody" relationship. However, the primary GLA antibody used is effectively pan-specific because it recognizes multiple proteins, and therefore deviates from this relationship (Fagète & Fischer, 2011). This makes identification of the specific proteins slightly less straightforward, and analysis is typically a process of simply identifying whether GLA residue expression has occurred or not. Nonetheless, the SUM159PT cells did express the GLA residues as expected. Multiple bands at various molecular weights were present on the membrane, representing the multiple GLA-modified proteins expressed in the cell, with the darkest band between 30-40 kDa (Fig 3).
It was expected that treatment with both forms of vitamin K1 would increase the expression of GLA-modified proteins in the cells, which would therefore be evident by more intense bands for treated cells. However, differences were not apparent in the bands' darkness with either K1 preparation compared to the control. The GAPDH loading control was constant across wells, indicating that the wells were loaded evenly.

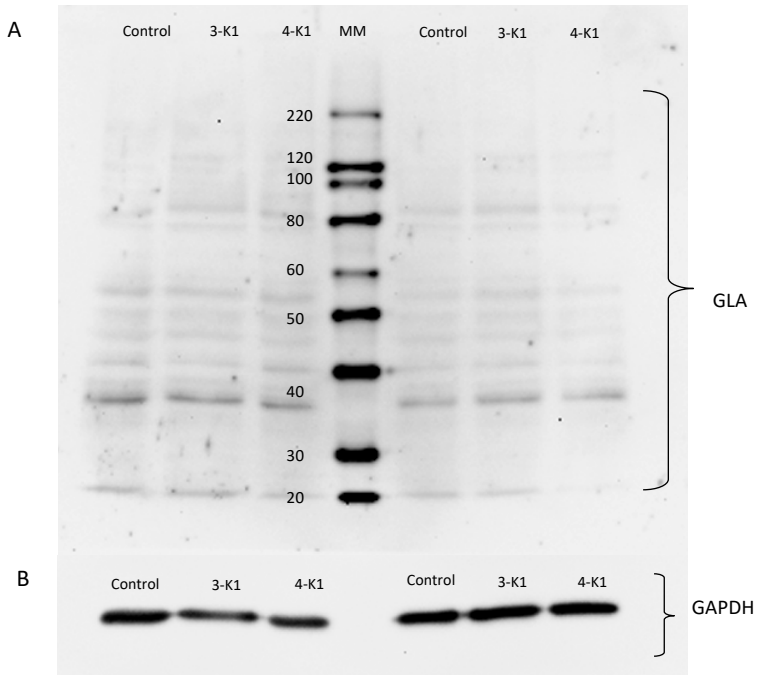

Figure 3. Effects of Vitamin K1 on GLA protein expression in SUM159PT cells. Cells were treated with 5μg/mL injection buffer, 3-K1 (V5301, Sigma Aldrich, St. Louis, MO), or 4-K1 (47773, Sigma Aldrich, St. Louis, MO) and grown for 24 hours. Once harvested, whole cell lysates were analyzed for GLA (A) and GAPDH (B) expression by western blotting. The left and right sides of the blot (on either side of the ladder) show technical duplicates from the same cell lysates.

Hoechst-based Cell Density Assay: Dose-Dependent Effects of Vitamin K1

We next determined the effects of increasing concentrations of vitamin K1 on SUM159PT cell growth measured as nuclei counts after 48h and 72h (Fig 4). Cells were treated 1 day after plating in 24-well plates with 1, 5, 10, 25, or 50 µg/mL K1 or EtOH vehicle. Using the IN Cell Analyzer, we found that average cell counts tended to increase at low concentrations of K1, but were consistently decreased at concentrations of 25 and 50 µg/mL. Similar trends were observed for both time points.

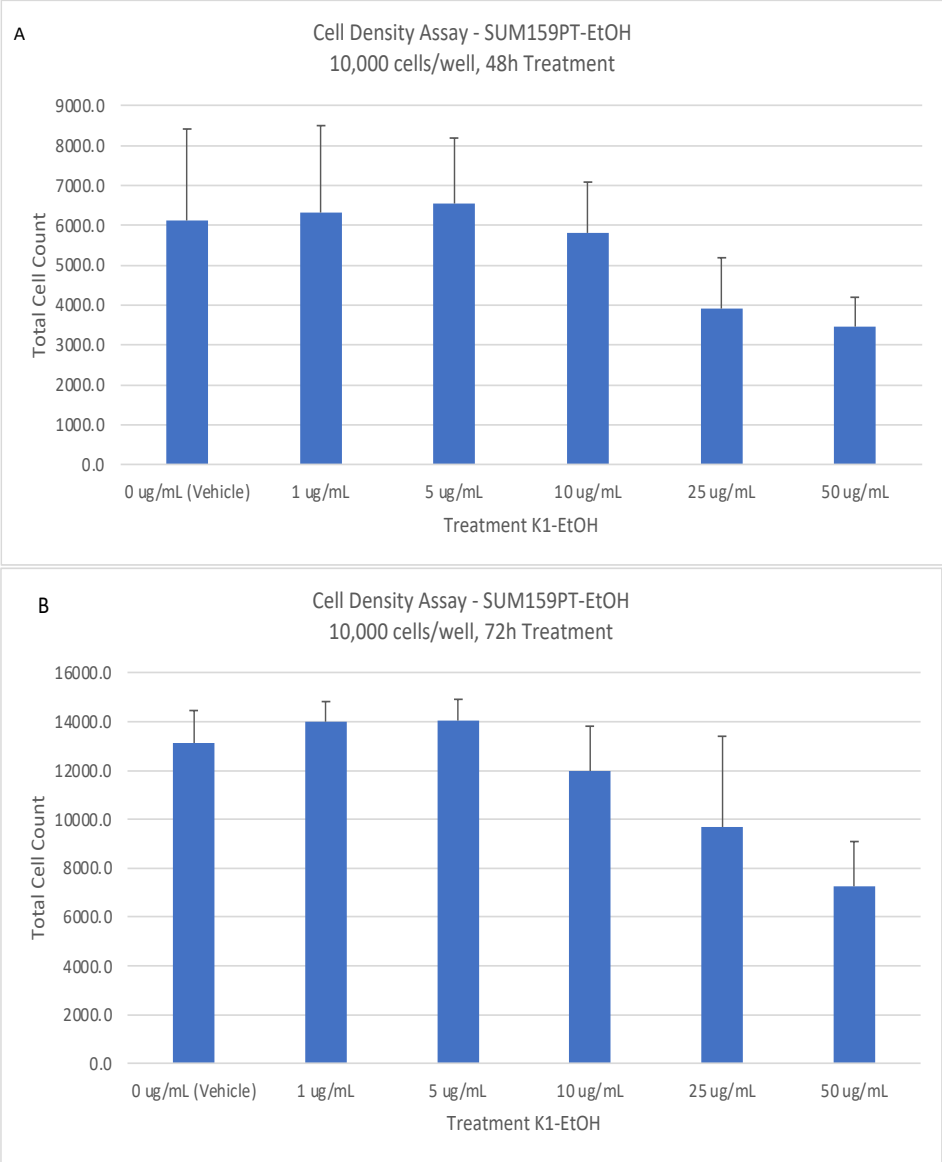

Figure 4. Cell density assays of K1-treated SUM159PT cells. Cell density assays were conducted for SUM159PT cells in 24-well plates seeded with 10,000 cells/well for 48h (A) and 72h (B) with treatments of the vehicle (EtOH, first bar) or K1-EtOH at concentrations of 1, 5, 10, 25, and 50 µg/mL. Cells were treated for the given time course before sampling and analysis with the IN Cell Analyzer. Each data point represents the average total cell count from two or three assay trials. Error bars show standard error.

Cell Viability Assay: Dose-Dependent Effects of Vitamin K1 and K2

To determine whether changes in cell density were associated with changes in cell viability, we used Guava Muse Cell Analyzer to measure the total number of viable cells and the percentage of viable cells. We also compared the effects of K1 with those of K2. Cells were treated 1 day after plating in 6-well plates with K1 (1, 5, 10, 25, or 50 μg/mL), K2 (5, 10, or 20 μg/mL), or EtOH vehicle, and assayed for percent viability after 48h (see Appendix) and 72h (Fig 5). For treatment with vitamin K1 (Fig 5A), lower concentrations slightly increased the percent of viable cells, while percent viability tended to decrease at higher concentrations. Similarly, percent viability was slightly increased relative to control in cells treated with K2 (Fig 5B) at 5 μg/mL. In general, however, the effects on cell viability relative to control were minimal and not statistically significant as determined by a student's two-tailed t-test.

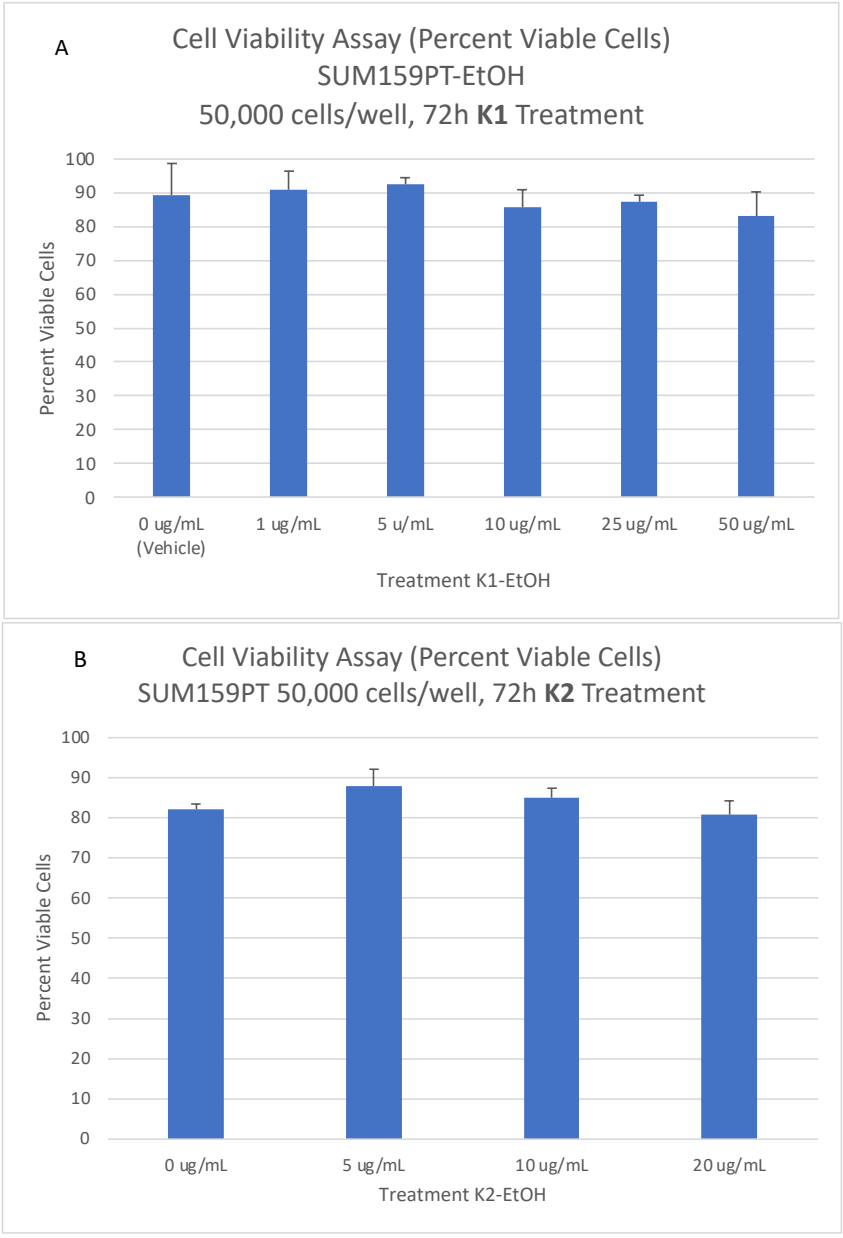

Figure 5. Cell Viability of Vitamin K-Treated SUM159PT Cells. Cell viability assays conducted for SUM159PT-EtOH cells with treatments of vehicle (EtOH, first bar) or K1-EtOH at concentrations of 1, 5, 10, 25, and 50 µg/mL K1-EtOH (A) or SUM159PT cells treated with 5, 10, and 20 µg/mL K2-EtOH (B). Cells were treated for 72h before sampling and analysis with the Guava Muse Cell Analyzer. Each data point represents the percent of viable cells from one or more assay trials.

Cell Cycle Assays: Dose-Dependent Effects of Vitamin K1 and K2

Using the Guava Muse Cell Analyzer, we then profiled the cell cycle kinetics of the cultures following treatment with vitamin K1 or K2 at 48h (see Appendix) and 72h. Cells were treated 1 day after plating in 6-well plates with K1 (1, 5, 10, 25, or 50 μg/mL), K2 (5, 10, or 20 μg/mL), or EtOH vehicle. Following the respective time course, cycle kinetics were monitored with the Muse Cell Analyzer. We performed additional, more accurate modeling of the cell cycle data using the FlowJo analysis platform (Fig 6). Two cell cycle assays were analyzed with FlowJo and values averaged (Fig 7).

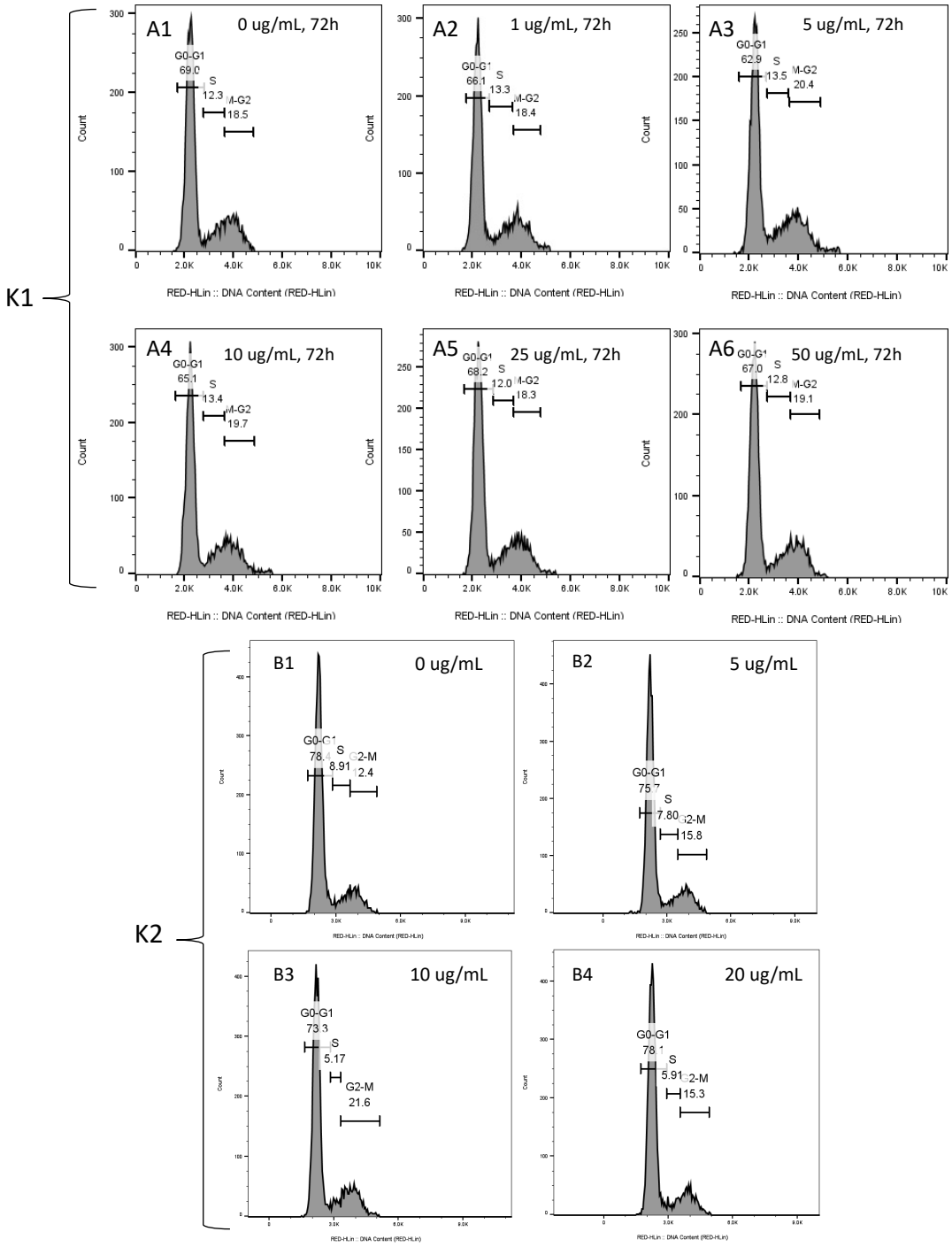

Figure 6. Representative FlowJo Analysis of Vitamin K-Treated SUM159PT Cell Cycle Kinetics. Following acquisition with the Muse Cell Analyzer, single-assay data for SUM159PT cells treated with EtOH control, 1, 5, 10, 25, or 50 µg/mL K1-EtOH at 72h (A1-6), or SUM159PT cells treated with EtOH control or 5, 10 or 20 µg/mL K2 (B1-4) was modeled with the FlowJo flow cytometry data analysis platform. The X-axis displays DNA content, while the Y-axis displays cell count. The population in each phase can be identified as successive peaks, centered approximately over 2.0K (G0-G1), 3.0K (S), and 4.0K (G2-M).

At 72h, treatment with vitamin K1 (Fig 7A) reduced the percentage of cells in S phase relative to control (15.7%), with the lowest percent detected at 10 μg/mL K1. An increased percentage of cells in G2/M phase relative to control (18.4%) was observed in cells treated with 1 to 10 ug/mL (peak of 20.6% at 10 μg/mL) while higher concentrations decreased the G2/M percentage relative to control. In contrast to K1, the major effect observed following treatment with K2 at 72h (Fig 7B) was a dose-dependent decrease in the percentage of cells in G0/G1 from 76.6.% to 67.3% with a corresponding increase in G2/M from 16.1% to 23.6%. The percentage of cells in S phase was decreased in all cultures treated with K2 relative to control, with the lowest value observed at 10 μg/mL vitamin K2 (7.4%). Statistical evaluation by a two-tailed t-test indicated that the percentage of cells in G2/M with 20 ug/mL treatment was significantly different from control at $p=0.004$.

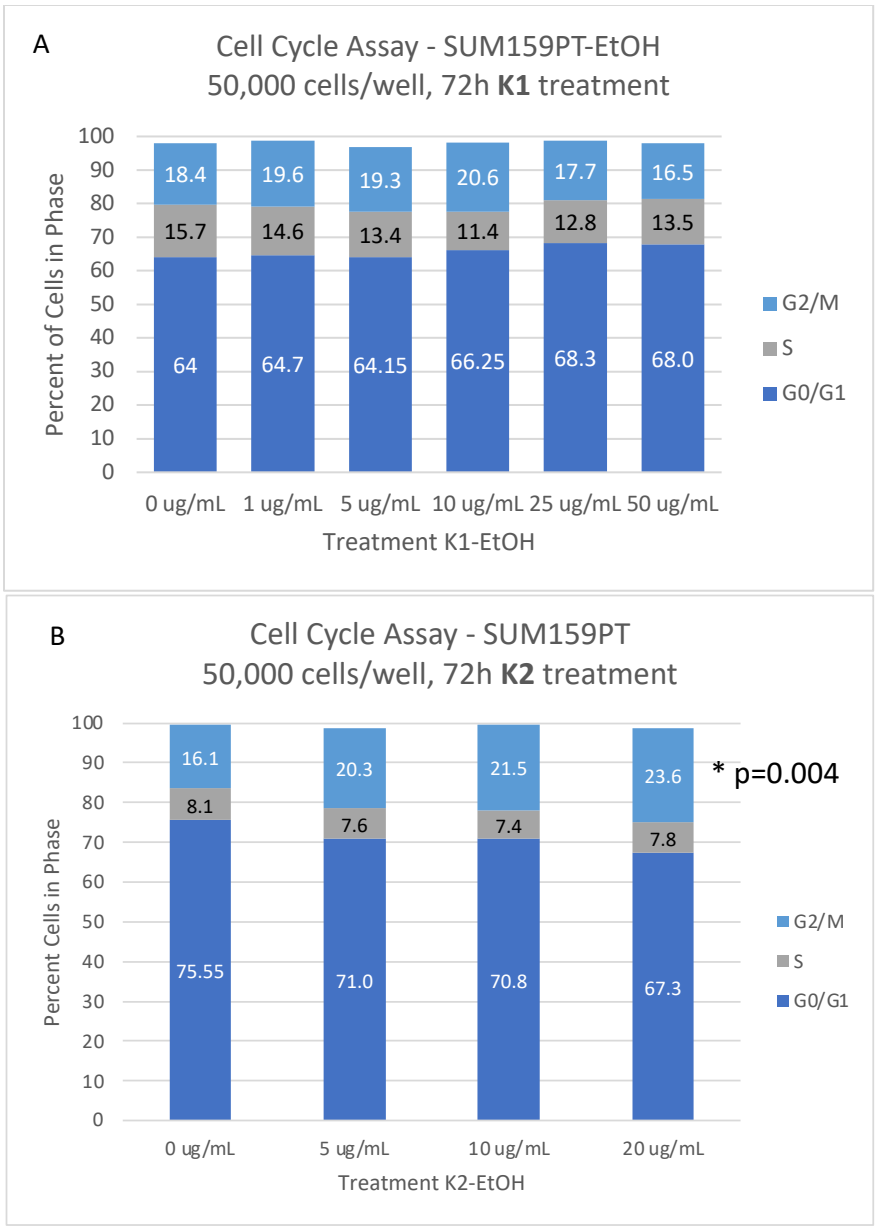

Figure 7. Comparative Effects of Vitamin K1 and K2 on SUM159PT Cell Cycle Kinetics. Following acquisition with the Muse Cell Analyzer, single-assay data for cells treated with 0, 1, 5, 10, 25, 50 µg/mL vitamin K1 at 72h (A) or 0, 5, 10, 20 µg/mL K2 at 72h (B) was modeled with the FlowJo software. Each data point shows the average of two values. *The difference between the G2/M cohort under control and K2 treatment conditions was determined to be statistically significant ($p < 0.05$) using a two-tailed paired t-Test.

C-Myc and Cyclin D1 Expression Analyses

To determine the effect of vitamin K1 and vitamin K2 on cyclin D1 and c-Myc gene expression relative to vehicle control, qPCR was conducted on cDNA collected from EtOH-, vitamin K1- or K2-treated cells (Fig 9). In each experiment, four duplicates of cDNA of each treatment from one or more cell passages were plated. Following qPCR, Ct values were analyzed for outliers using the Grubbs test ($p > 0.05$) and all samples included were combined for averaging and determination of standard deviation. Outliers were removed from the duplicates and mean fold change for treatments were calculated relative to control values normalized at 1.0.

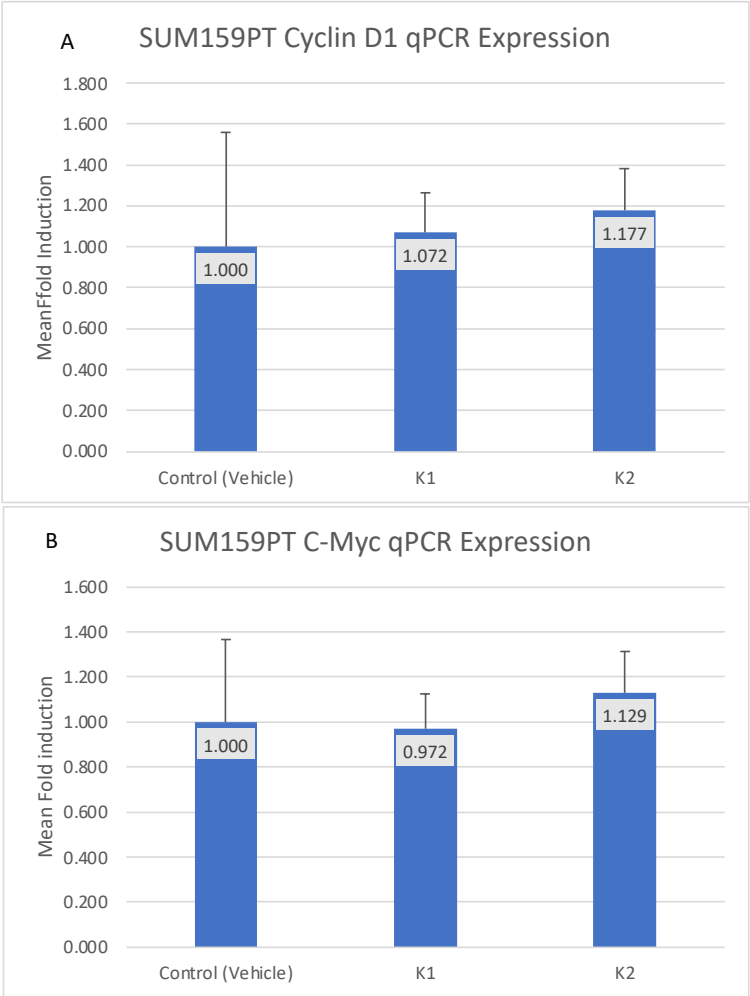

Figure 8. C-Myc and Cyclin D1 Expression in Vitamin K-treated SUM159PT Cells. Mean fold change of cyclin D1 (A) and c-Myc (B) in cells treated with vitamin K1, K2 or EtOH control, as determined by qPCR. Each data point is an average of three samples (one sample p#57, two of p#61), each with 2 to 4 duplicates. Error bars show standard deviation.

While slight increases in the expression of these genes compared to control were detected, the range of Ct values varied greatly between the samples, and the standard deviations are large. In addition, given the effects of K1 and K2 to slow the cell cycle, decreased (rather than increased) gene expression would have been expected. Thus, it is unlikely that these genes contributed to the effects of K1 or K2 on cell behavior.

DISCUSSION

In our laboratory's previous research, the density of SUM159PT cells was assayed in response to 72h of treatment with 5 ug/mL vitamin K1 or K2 compounds. It was determined that, compared to vehicle control, treating the cells in vitamin K1 increased cell counts, while K2 treatment decreased cell counts (Beaudin et al., 2019). The results presented in this thesis further characterize these differential effects and provide novel data demonstrating that vitamin K1 and K2 induce distinct changes in SUM159PT cell cycle kinetics.

At 72h, SUM159PT cultures treated with 25 ug/mL vitamin K1 exhibited the highest distribution in the G0/G1 (68.3%) as compared to control cultures (64.0%). The S and G2/M cohorts also changed as a result of treatment: at low concentrations (5, 10 ug/mL), the S phase cohort decreased while the G2/M phase cohort increased, whereas the trends reversed at higher concentrations. Upon treatment with vitamin K2, there was a clear dose-dependent increase in the percentage of cells in G2/M phase. Whereas vitamin K1 induced minor G2/M arrest (maximum difference of 2.2% vs control), the response was much greater following K2 treatment (maximum difference of 7.5%). The relationship between K2 concentration and G2/M arrest was positive and did not plateau with the doses tested. In cultures treated with 20 μg/mL K2, 23.6% of cells were arrested in G2/M vs. 16.1% of cells in control cultures. This difference was statistically significant as assessed by two-tailed student's t-test ($p=.004$).

In summary, our data showed minimal effects on cell cycle kinetics following treatment with vitamin K1, but a true dose response is seen following vitamin K2 treatment at 72h. As shown in the Appendix, similar trends for K2 treatment were observed at 48h. Thus, these data may be indicative of dose-and time-dependent effects in which vitamin K2 prevents cells from passing through the mitotic checkpoints, thus causing them to accumulate in the G2/M phase. This would account for the simultaneous decrease in G0/G1 cohort size coupled with the increase in G2/M cohort size with increasing vitamin K2 concentration at 72h.

Given the role of the vitamin K cycle in γ-carboxylation, induction of GLA-modified proteins is a possible explanation for the observed changes to cell cycle phase distribution after K2 treatment. However, while it is known that the carboxylation pathway that produces the GLA-modified proteins is functional in SUM159PT cells (Beaudin et al., 2019), previous research found the upregulation of GLA proteins to be greater in K1-treated cells than in K2-treated cells. If GLA modification was responsible for the differential effects on cycle kinetics, the dose-dependent effect would be expected in cells treated with vitamin K1. In addition, western blots presented here failed to detect increases in GLA-modified proteins upon treatment with two different vitamin K1 treatment preparations; it was expected that the control would not express any GLA residues as observed. These results suggest a possible error in the experimental procedure, specifically at the level of antibody specificity or blocking efficiency. Repeated experiments should check and optimize these factors. Additionally, the experiment could be improved with the addition of a positive control, such as blotting blood plasma, which is known to have GLA-modified proteins. Lastly, although this finding is not consistent with the previous findings (Beaudin et al., 2019) in which treatment with K1 increased GLA residue expression compared to control, is important to note that experimental methods varied. In our study, cells

were grown in standard medium without K1 prior to treatment, whereas previous were conducted in cells first passaged three times in a medium containing vitamin K1 (Beaudin et al., 2019). Therefore, treating the cells in K1 following growth in standard medium may not be sufficient to impact γ-carboxylation. Our experiments also used a different buffer for solubilization of K1, which could further impact the expression of GLA proteins. Nonetheless, the western blotting data presented here do not provide strong evidence that the observed effects of K compounds on cell cycle kinetics are related to γ-carboxylation.

Because prolonged arrest of cells in G2/M phase may also lead to changes in cell viability or apoptosis, we assessed cell viability after treatment with vitamin K1 or K2. While little research has been done on the effect of K1 on TNBC lines, cell viability assays for different vitamin K compounds on the breast cancer cell line BC-M1 demonstrated that, relative to K2 and K3, vitamin K1 had relatively low anti-tumor, cytotoxic effects (Wu et al., 1993). Our previous experiments on SUM159PT cell density at 72h showed decreased density at 5 μg/mL K2 (Beaudin et al., 2019). Here, we evaluated additional K1 treatment concentrations, as well as both 48h and 72h time points. Treatment with vitamin K1 at low concentrations (1-10 ug/mL) initially slightly increased SUM159PT cell counts relative to vehicle, then caused a decrease in cell density at higher concentrations (25, 50 ug/mL). These results indicate a biphasic effect of treatment with K1: at lower concentrations, particularly 5 μg/mL, K1 had a stimulatory effect on SUM159PT cell density, while at higher concentrations, (≥ 25 μg/mL), K1 reduced cell density. Differential effects of K1 treatments at different times were not seen; the only difference in cell count was an overall higher confluency at 72h. Further, the observed effects on viability for both treatments were minimal and not statistically significant, showing no cell death to have occurred. The observed accumulation in G2/M phase at 72h following treatment with vitamin K,

specifically K2 or moderate concentrations of vitamin K1, may thus be representative of a larger inability to complete mitosis and begin a new cycle, causing the decrease in cell density. In other words, K2 and moderate K1 concentrations might have antiproliferative, but not necessarily cytotoxic, effects on SUM159PT cells.

A final potential reason for the observed effects on cell cycle kinetics would be decreased expression of the oncogenes cyclin D1 or c-Myc. Cyclin D1 promotes cell cycle progression through the G1/S transition in many cell types by phosphorylating the tumor suppressor retinoblastoma protein (Alao, 2007). Overexpression of the product can enhance DNA synthesis and has been seen in several human cancers, including breast cancer (Ozaki et al., 2007; Alao, 2007). C-Myc encodes a transcription factor, which is frequently increased in tumor cells as a result of gene amplification or changes in upstream oncogenic pathways (Miller et al., 2012). Deregulation of c-Myc can lead to cell cycle arrest, apoptosis, and tumorigenesis (Ge et al., 2004). Overexpressed c-Myc stimulates genes involved in cancer metabolism, cell cycle and proliferation, while inhibiting expression of certain miRNAs and tumor suppressor genes. At the same time, while encouraging rapid cell division and inhibiting genes with anti-proliferative functions, c-Myc tends to induce apoptosis (Miller et al., 2012).

The conditional effects of vitamin K compounds on c-Myc and cyclin D1 have been investigated in other (non-breast) cancer cells, such as hepatocyte cancer cells. For example, in one study, HepG2 cells treated with compound 5 or vitamin K3 exhibited a decrease in cyclin D1 during G1-phase. There were no discernible effects following treatment with vitamin K2, however, and the effect on K1 was not tested (Matkovits et al., 2003). On the other hand, the vitamin K2 analog menatetrenone has been shown to have antitumorigenic effects against several cell lines and downregulatory effects on cyclin D1 expression. G1 arrest occurred in treated

HepG2 and Hep3b cells. In these cells and the Huh7 cell line, increasing vitamin K2 concentrations resulted in decreased cyclin D1 expression. In general, the growth of the cells was also inhibited in a dose-dependent manner (Ozaki et al., 2007). Many of the above studies do not mechanistically connect the roles of these regulatory proteins to the actions of vitamin K compounds, and there are no studies of cyclin D1 or c-Myc expression in TNBC cells treated with vitamin K compounds.

To elucidate the effects of vitamin K treatment on these oncogenes in TNBC cells, we profiled c-Myc and cyclin D1 expression after 24h treatment with vitamin K1 or K2. Our data show slight but not significant mean fold changes of each gene in response to both vitamin K1 and K2 treatment. As previously mentioned, there was great variation between the ranges of different samples' Ct values in RT-PCR assays. For example, in control samples targeting cyclin D1, one passage had Ct values between 21.54-21.75 while another had values between 24.33-24.79. However, none of the genes had very low Ct values; in fact, all determined were less than 25. Therefore, low cellular expression was not the cause of the variation. Because the variation was seen between the different samples, which were each different passages, it is likely that the variation is from technical differences, such as plating, media and culture conditions, etc. between the passages.

In line with the known function of these genes, the aforementioned research, and our own previous data, we can make some predictions on what regulation might have occurred. One outcome of c-Myc overexpression in cancer is upregulation of genes involved in cell proliferation. In our cell density assays, treatment with 5 μg/mL of vitamin K1 increased total cell count relative to control, while K2 decreased cell count relative to control (Beaudin et al., 2019). Therefore, because the vitamin K1 increased cell proliferation at this point, it may also

upregulate c-Myc expression. Because the K2 decreased proliferation, it may have been expected to downregulate c-Myc expression. Cyclin D1 regulates the G1-S transition, with overexpression promoting entry into the S phase (DNA synthesis). This gene expression data can thus also be considered in the context of cell cycle kinetics. In response to treatment with K1 at 72h (see Appendix for 48h), the proportion of cells in G0/G1 phase increases with increasing concentration, while the proportion of cells in S phase decreases, then increases. This disruption may be reflective of a downregulation of cyclin D1 in response to K1 treatment at lower concentrations. For treatment with K2, the G0/G1 cohort drops more significantly with and continues to decrease with increasing doses. The percent of cells in S phase also was reduced with the introduction of treatment. Based on these observations, downregulation of cyclin D1 would have been expected, as indeed was reported by Ozaki et al. in hepatocytes. Further studies of gene expression after K1 and K2 treatment at time points and concentrations that affect cell cycle phase distribution are necessary to clarify the potential roles of c-Myc and cyclin-D1 in TNBC cells.

CONCLUSION

Our experiments aimed to determine potential effects of treatment with vitamin K compounds on GLA-expression, density, viability, and cell cycle kinetics on SUM159PT cells. Accordingly, we conducted cell density, cell viability, and cell cycle assays to evaluate the acute effects. These results enhance previous findings by evaluating different conditions and/or more treatment groups.

Using cell culture and western blotting techniques, we treated SUM159PT cells, a TNBC cell line, with vitamin K1 and observe the effects on growth and GLA-modified proteins. While previous research showed that passaging cells in K1 affected GLA residue expression, our

research showed that acute treatment with vitamin K1 dissolved in a clinically relevant buffer did not affect the expression of GLA-modified proteins relative to control in cells grown in standard medium. Further studies are needed to clarify this discrepancy.

Our cell density assays both support and expand upon previous findings; at low to moderate doses of vitamin K1 had a proliferative effect on SUM159PT cells, causing cell density to increase. With increasing concentration, density decreased below that of control, suggesting higher concentrations of K1 might be cytotoxic. While increasing concentrations of both K1 and K2 did not exert any conclusive effects on cell viability, cell cycle assays revealed minor effects of K1 and a more significant dose response of K2. Specifically, vitamin K2 induced entry into G2/M phase with increasing concentrations, which may result from the inability of cells to complete mitosis and re-enter G0/G1. However, it is important to note that did not assess apoptosis or necrosis, which may be triggered by prolonged G2/M arrest. Future experiments should include these assays, and expand them to other TNBC cells lines, to achieve a more well-rounded understanding of the compounds' effects on breast cancer cells.

Lastly, we chose to target cyclin D1 and c-Myc for their roles in cell proliferation, cycle control, and apoptosis, all of which, when misregulated, can contribute to cancer progression. We treated SUM159PT cells with K1 and K2 for 24h, then performed qPCR to measure the changes in expression of these genes. Results did not show any statistically or biologically significant changes in these genes; however, these results are potentially due to variation in the Ct values stemming from variation in cultures between samples. To determine if there is truly an effect on these genes as a result of vitamin K treatment, these experiments should be repeated with samples from the same culture to eliminate the variation. Western blots may also be conducted to determine if, beyond any changes in gene expression, changes in protein expression

occur following treatment. This is especially true for cyclin D1, as cyclins are regulated at the protein level. Additional targets with similar roles, such as cyclin E/Cdk2 or PCNA, may also be targeted to gain further insight on the effects of vitamin K compounds on the cell cycle and proliferation.

These studies and others like them are important in understanding the potential effects of dietary vitamin K compounds on the progression of breast cancer development. Based on our findings, it may be more beneficial for women with breast cancer to consume foods containing vitamin K2 as opposed to vitamin K1. Confirmation of the differential effects of these two forms of vitamin K with additional breast cancer models and human patient populations is clearly warranted.

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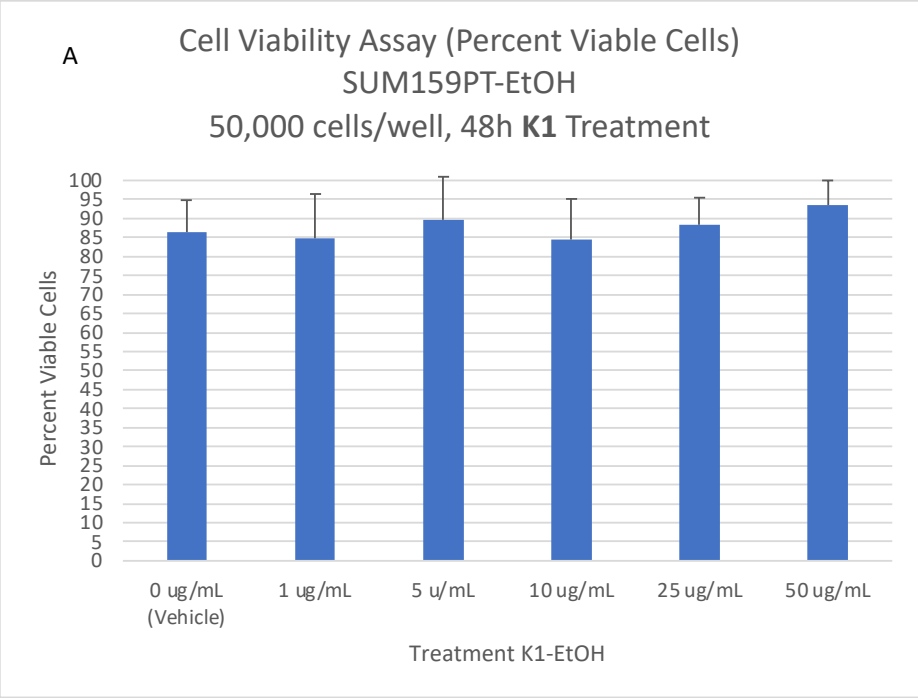
APPENDIX

For cells treated with vitamin K1 at 48h (Fig 9A), the percent of viable cells was slightly greater than control for treatments of 5, 25, and 50 µg/mL. Initially, with lower concentrations at 48h, the percent of viable cells slightly increased relative to control at 5, 25, and 50 µg/mL, while that at 1 and 10 µg/mL slightly decreased viability relative to control. In our cell density assays, lower concentrations treatments showed an initial increase in cell number relative to control, followed by a decrease at 10, 25, and 50 µg/mL vitamin K1 treatment. This may mean that while there is an there is an effect on cell number at higher doses, the viability of the remaining cells is slightly improved at 48h.

For cells treated with vitamin K2 at 48h (Fig 9A), only one assay was successfully conducted. The percent of viable cells increased relative to control at 5 µg/mL K2, showed little change at 10 µg/mL, and decreased slightly at 20 µg/mL. More assays are needed to make conclusions about the effects of vitamin K2 on SUM159PT cells at 48h.



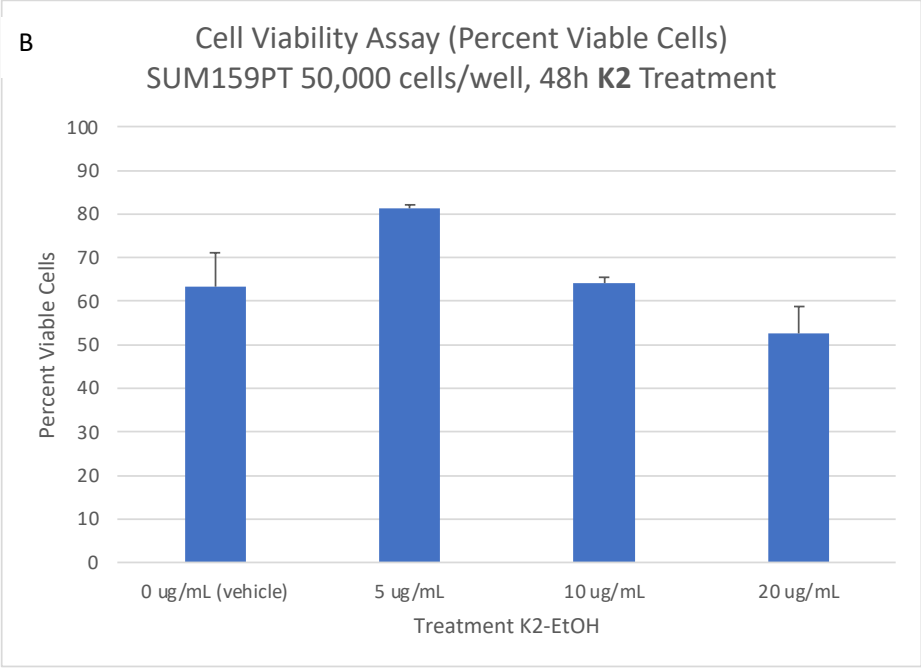


Figure 9. 48h Percent Viable Cells for Vitamin K-Treated SUM159PT Cells. Cell viability assays conducted for SUM159PT-EtOH cells with treatments of vehicle (EtOH, first bar) or K1-EtOH at concentrations of 1, 5, 10, 25, and 50 µg/mL K1-EtOH (A) or SUM159PT cells treated with 5, 10, and 20 µg/mL K2-EtOH (B). Cells were treated for 48h before sampling and analysis with the Guava Muse Cell Analyzer. Each data point represents the percent of viable cells from one or more assay trials.

For cells treated with vitamin K1 (Fig 10A), at 48h, the percent of cells arrested in S phase was always lower than that of the vehicle control (17.8%), with the lowest percent occurring at 5 µg/mL (12.6%). The percent of cells in G0/G1 phase increased relative to control (64.0%) at low concentrations, then decreased from 10 to 50 µg/mL. Similarly, with the exception of 1 µg/mL (16.7%), the percent of cells in M/G2 phase was always higher than control (17.2%). At 72h the percent of cells in G0/G1 was higher when treated than with control (64.0%), with the lowest percentage occurring at 25 µg/mL (68.3%).

For cells treated with vitamin K2 (Fig 10B), the M/G2 and S cohorts were always larger than those of the control (14.5% and 13.7%, respectfully) at 48h, reaching their peak at 5 µg/mL for S phase (17.5%) and 20 µg/mL for M/G2 (19.2%). A higher percentage of cells were arrested in S phase (ranging from 12.6% to 17.4%) and G2/M phase (16.7% to 23.7%) during the 48h time course than during the 72h time course (see Results, Fig 7B); 11.4% to 15.7% in S phase,

16.5% to 20.6% in G2/M phase). This may be due to crowding over time, causing the cycle to slow down.

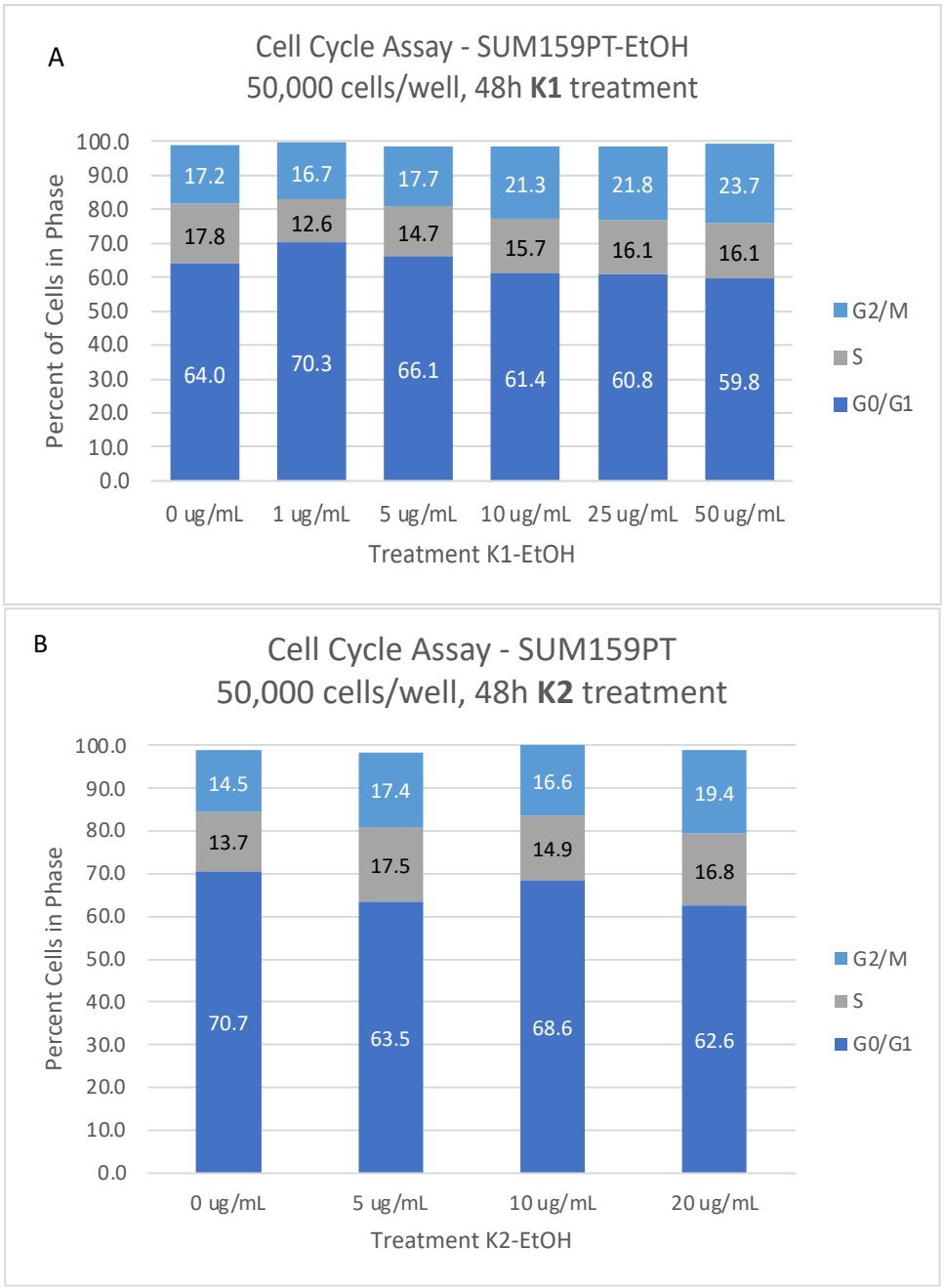


Figure 10. 48h Average Effects of Vitamin K on SUM159PT-Treated Cycle Kinetics. Following acquisition with the Muse Cell Analyzer, single-assay data for SUM159PT-EtOH cells treated with EtOH vehicle 0, 1, 5, 10, 25, 50 µg/mL vitamin K1-EtOH at 48h (A) or SUM159PT cells treated with 0, 5, 10, 20 µg/mL K2-EtOH at 48h (B) was additionally analyzed using the FlowJo flow cytometry data analysis platform. Each data point shows the average of one or two values.