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Original Research

Real-time cell analysis of the inhibitory effect of vitamin K_2 on adhesion and proliferation of breast cancer cells

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A B S T R A C T

Breast cancer is the most prevalent cancer type worldwide. Continued efforts to improve treatment strategies for patients with breast cancer will be instrumental in reducing the death rates associated with this disease. In particular, the triple-negative breast cancer subtype of breast cancer has no targeted therapy available so it is essential to continue to work on any potential therapies. Vitamin K (VK) is known for its essential role in the clotting cascade. The antitumor properties of VK derivatives have been reported in both hepatocellular carcinoma and glioblastoma. Our hypothesis was that menaquinone-4, the most common form of vitamin K_2 (VK_2), is an effective anticancer agent against breast cancer cell types. In this study, we used a novel impedance-based live cell monitoring platform (xCELLigence) to determine the effects of VK derivatives on the triple-negative breast cancer cell line, MDA-MB-231, and the HER2+ breast cancer cell line, MDA-MB-453. Cells were treated with varying concentrations of menaquinone-4 (VK_2) previously reported to have an antiproliferative effect on human glioblastoma cells. After initial testing, these concentrations were adjusted to 100, 125, and 150 μmol/L. A significant dose-dependent, growth inhibitory effect was found when cells were treated at these concentrations. These effects were seen in both adhesion and proliferation phases and show a dramatic reduction in cell growth. Additional analysis of MDA-MB-231 cells treated with VK_2 (100 μmol/L) in combination with a low-glucose nutrient media showed a further decrease in adhesion and viability. This is the first study of its kind showing the real-time effects of VK derivatives on breast cancer cells and suggests that dietary factors may be an important consideration for patients.

Abbreviations: CI, cell index; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RTCA, real-time cell analysis; TNBC, triple-negative breast cancer; VK, vitamin K; VK_2, vitamin K_2.

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1. Introduction

Breast cancer is the most prevalent cancer type in most countries worldwide [1]. Current treatments are not sufficient against all breast cancer types, and both improving current treatments and developing novel strategies should be a priority in dealing with this disease. Triple-negative breast cancer (TNBC) is an aggressive subtype that is devoid of the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (Her2/neu receptor). There are no targeted treatments currently available for this cancer subtype. Triple-negative breast cancer accounts for approximately 15% of all breast cancer diagnosed, represents poor survival [2], and has a higher incidence among younger women, especially those of African and Hispanic descent as well as those in lower socioeconomic groups [3]. Targeted pharmaceutical agents that act independently of receptor status are required to treat TNBC more effectively.

The HER2+ breast cancer subtype accounts for 15% to 20% of breast cancer cases [4]. Patients diagnosed as having HER2+ tumors have benefitted greatly in recent years from the development of the targeted HER2 therapy trastuzumab [5], which has reduced the recurrence and mortality rates associated with this subtype [6,7]. However, resistance to trastuzumab has become an increasing issue and alternate therapies; for example, lapatinib is being explored [7].

Vitamin K (VK) is a fat-soluble vitamin that is historically known for its role in blood coagulation where it acts as a cofactor in γ-carboxylation of clotting factors II, VII, IX, and X [8]. It exists in 3 forms—vitamin K_1 (VK_1 or phylloquinone), vitamin K_2 (VK_2 or menaquinone), and vitamin K_3 (VK_3 or menadione). Vitamin K_1 is the main dietary source, found in green, leafy vegetables and certain oils of vegetable origin. Vitamin K_2 is also known as menaquinone, or MK-*n*, where *n* stands for the number of isoprenyl units in its side chain. The most common storage form of VK in animals is menaquinone-4 (MK-4) which is metabolically converted from other VK isoforms. Vitamin K_2 is also synthesized by the gut microbiota and is obtained in smaller amounts from fermented dietary products. Vitamin K_3 is a synthetic compound that does not occur naturally but that is used widely in animal feed [8].

In addition to its established function as an antihemorrhagic agent, VK_2 has showed promise as an anticarcinogenic agent in studies involving many cancer cell types including leukemia and cancers of the liver, stomach, lung, ovary, glioblastoma, and prostate [9–15]. The anticancer activity of VK_2 has also been demonstrated in in vivo studies of colon and prostate cancer [14,16]. A number of mechanisms underlying the anticancer properties of VK_2 have been suggested including apoptosis and cell cycle arrest [17–19]. Despite its potential as an alternative to some currently used cancer therapeutics, VK_2 is not currently used as an anticancer treatment in clinical practice due to a paucity of evidence [15].

Calorie restriction without malnutrition can increase life span and protect against cancer [20–23]. Reduced consumption of food has been shown to decrease levels of growth factors, reduce oxidative stress, increase cell repair mechanisms, and possibly inhibit glycolysis [23–25]. Glucose is a main source of calories in humans, and glucose restriction has been shown to inhibit lung cancer cell growth and induce apoptosis with no effect on normal cells [26]. It is suggested that normal cells can adapt to conditions of nutrient deprivation, but cancer cells cannot [22,26]. Glucose restriction in cancer cells is a recognized metabolic stressor that triggers several cellular signaling pathways [26].

Our hypothesis was that MK-4, the most common form of VK_2, is an effective anticancer agent against breast cancer cell types. Our study used the real-time cell analysis (RTCA) xCELLigence platform to analyze the anticancer effects of VK_2 on breast cancer cells in real time as superior method of cell monitor cell growth compared with traditional end point assays. The specific objectives were (1) to investigate if VK_2 has an inhibitory effect on breast cancer cells, and (2) to investigate if a combination of low-glucose media and VK_2 has an added inhibitory effect on breast cancer cells. To the best of our knowledge, this is the first study of its kind documenting the effects of VK_2 on breast cancer cells using the RTCA xCELLigence platform.

2. Methods and materials

2.1. Materials

Vitamin K_2 (VK_2 or menaquinone-4) was purchased from Sigma-Aldrich Ltd (Wicklow, Ireland). E-plates for the RTCA xCELLigence platform were purchased from ACEA Biosciences, (Cambridge, UK). Dulbecco modified Eagle medium (DMEM) with a high glucose concentration (4500 mg/L, 25 mmol/L), DMEM with low glucose (1000 mg/L, 5.5 mmol/L), RPMI, fetal bovine serum (FBS), penicillin/streptomycin antibiotic mix, L-glutamine, and trypsin/EDTA were purchased from Sigma-Aldrich Ltd. MDA-MB-231 cells were obtained from Patrick Kiely from the University of Limerick, Ireland, and MDA-MB-453 cells were obtained from Joe Duffy from the University College Dublin, Ireland.

2.2. Cell culture

MDA-MB-231 cells were maintained in DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% FBS. MDA-MB-453 cells were maintained in RPMI supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% FBS.

2.3. Monitoring cell adhesion and proliferation using the xCELLigence system

MDA-MB-231 cells were harvested with trypsin/EDTA, washed with DMEM, and resuspended in the DMEM with 10% FBS. The cells were counted using a hemocytometer. Cells were seeded in each well of the E-plate [27]. The impedance values of each well were automatically monitored by the xCELLigence system and expressed as a cell index (CI) value. The baseline impedance is recorded using control wells containing DMEM only with no cells. Vitamin K_2 was dissolved in ethanol and diluted to the required concentrations. The maximum amount of ethanol was added to cells (0.7%) and found to have no effect (data not shown). The appropriate concentration of VK_2 was

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added to the wells of the E-plate. Cells were seeded onto the E-plate at a density of 20000 per well. The E-plate was then placed into the xCELLigence system. Scans were run with sweeps every minute for the first 8 hours to detect early stages of cell adhesion and spreading. Subsequent sweeps were taken every 15 minutes for the duration of the experiment.

2.4. *Cell adhesion*

Twenty thousand cells were plated into a 96-well plate with media containing VK_2 at concentrations of 0 (control), 100, 125, and 150 μmol and incubated at 37°C in 5% CO_2 for the indicated times. Cells were then washed 3 times with phosphate-buffered saline (PBS) and fixed in 100 μL of methanol at −20°C for 5 minutes. The methanol was removed and cells were stained with 0.1% crystal violet for 15 minutes at room temperature. Cells were carefully washed with water and left overnight to dry. The plates were then read at 590 nmol/L on a spectrophotometer [27].

2.5. *Cell proliferation*

Twenty thousand cells were plated into a 96-well plate with media containing VK_2 at concentrations of 0 (control), 100, 125, and 150 μmol/L and incubated at 37°C in 5% CO_2 for 48 hours. The cells were then counted with a hemocytometer using the trypan blue exclusion method to distinguish between live and dead cells [28].

2.6. *Statistical analyses*

All statistical analyses were performed using SPSS version 20 statistical package (SPSS, Chicago, IL, USA). Differences between groups were determined using Welsh analysis of variance, and multiple groups were compared using Bonferroni correction. A *P* value less than .05 was considered statistically significant. Data are presented as means ± SEM. All experiments were done in triplicate.

3. Results

3.1. *Optimization of conditions to facilitate monitoring cell behavior in real time*

The first objective of this study was to use the RTCA xCELLigence system to monitor the effect of VK_2 on adhesion and proliferation of breast cancer cells. It was necessary to determine a suitable seeding concentration to allow for analysis of the cells over the time course of experiments. MDA-MB-231 cells were seeded in wells of an E-plate at numbers ranging from 5000 to 20000 cells and monitored every minute for the first 8 hours and every 30 minutes up to 48 hours. Readouts from RTCA systems are expressed as CI values. As shown in Fig. 1a, 2 distinct patterns can be seen on the representative xCELLigence graph, which can be attributed to cell adhesion and spreading (0-8 hours) and cell proliferation (8-24 hours). The rate of proliferation was calculated for each seeding number by analyzing the slope of the line between 0 and 24 hours, and based on the patterns observed, we determined that the optimum cell seeding density to monitor cell behavior of MDA-MB-231 cells was 20000 cells/well. To investigate whether VK_2 had any effect on the cancer cells' ability to adhere and grow, cells were seeded in E-plate wells with DMEM containing VK_2 concentrations of 35, 50, and 75 μmol/L. Modest effects were seen after 16 hours of monitoring cell growth at these VK_2 concentrations (Fig. 1b).

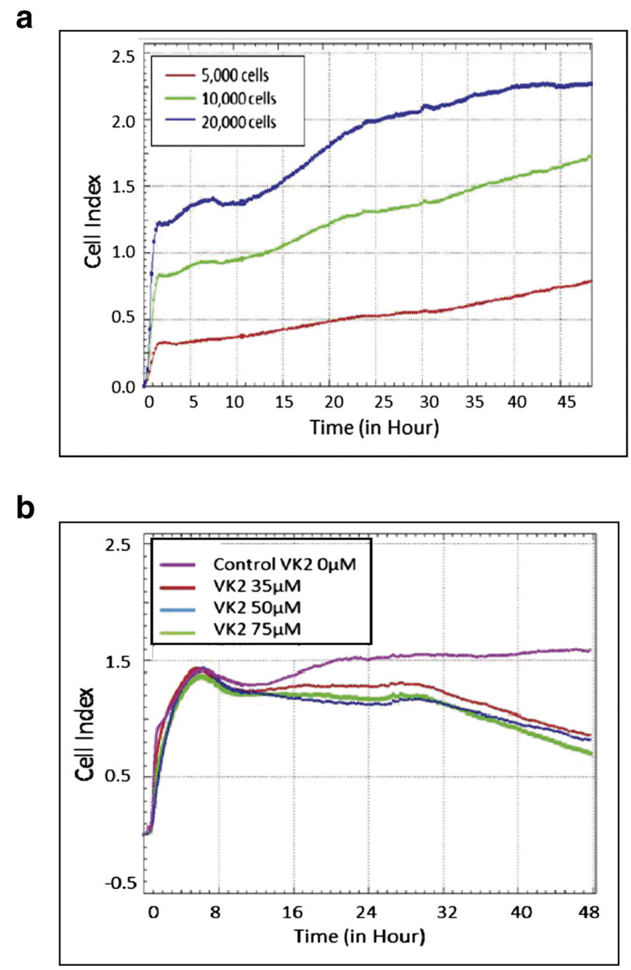

Fig. 1 – Optimization of cell number to facilitate monitoring cell behavior in real time. MDA-MB-231 cells were seeded at 5000, 10000, and 20000 cells in each well of an E-plate. Cell behavior was monitored in real time using the xCELLigence system. Readings were taken every minute for the first 8 hours and every 15 minutes for the subsequent 40 hours with readings expressed as CI values. a, The xCELLigence graph is representative of duplicate wells comparing the growth curve of MDA-MB-231 cells at 5000 cells (red line), 10000 cells (green line), and 20 000 cells (blue line). b, To investigate whether VK_2 had any effect on the cancer cells ability to adhere and grow, cells were seeded in E-plate wells with DMEM containing VK_2 concentrations of 35, 50, and 75 μmol/L. Modest effects were seen after 16 hours of monitoring cell growth at these VK_2 concentrations.

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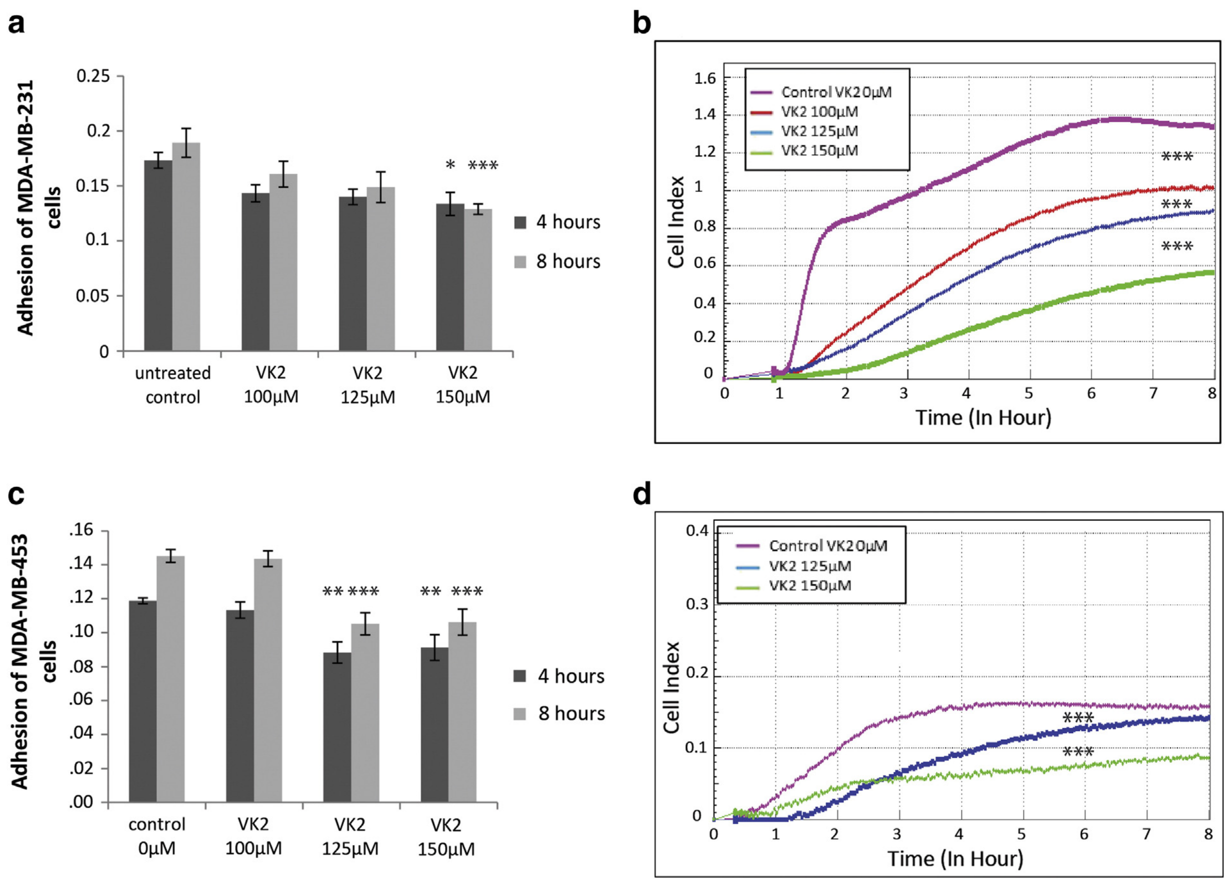

Fig. 2 – Vitamin K_2 has a significant inhibitory effect on the adhesion of breast cancer cells. Cells are plated in a range of VK_2 concentrations (0-150 µmol/L) and allowed to adhere for the indicated times. Cells are then fixed, stained, and measured using a spectrophotometer. Data shown are a bar chart with MDA-MB-231 cells (a) and MDA-MB-453 cells (c) treated with VK_2 compared with the untreated control. Cells were plated at a seeding density of 20 000 in wells of an E-plate in DMEM containing a range of VK_2 concentrations (0-150 µmol/L). The cells were monitored in real time using the xCELLigence system. Readings were taken every minute for 8 hours with readings expressed as CI values. b, The xCELLigence graph is representative of duplicate wells comparing the effect of different VK_2 concentrations on MDA-MB-231 cell adhesion. d, The xCELLigence graph is representative of duplicate wells comparing the effect of the indicated VK_2 concentrations on MDA-MB-453 cell adhesion. Data are represented as means ± SEM. *$P < .05$ **$P < .01$ ***$P < .001$. n = 3.

3.2. *Vitamin K_2 has a significant inhibitory effect on the adhesion of breast cancer cells*

Cells were seeded in E-plate wells with DMEM containing a range of VK_2 concentrations (0-150 µmol/L). Cell behavior was monitored using the RTCA platform over a period of 48 hours. To investigate any effect on cell adhesion and spreading, data were extracted from the first 8 hours of cell monitoring (Fig. 2). Initially, using an end point adhesion assay, MDA-MB-231 cells had inhibited levels of adhesion at both time points tested, 4 and 8 hours with the 150-µmol/L dose having the most significant effect (Fig. 2a). On the xCELLigence system, adhesion of MDA-MB-231 cells was inhibited at each VK_2 concentration tested in comparison to the untreated control (Fig. 2b). Comparison of the CI of untreated cells and cells treated with different concentrations of VK_2 showed that CI values were reduced by 20% to more than 95% as the VK_2 concentration was increased (Fig. 2b). This demonstrates that the TNBC cells are sensitive to VK_2 at concentrations greater than 100 µmol/L when analyzing the effect in real time. We also recorded the effect of VK_2 on the adhesion of MDA-MB-453 cells using the end point adhesion assay. Vitamin K_2 had an inhibitory effect on the adhesion of these cells at both time points tested when cultured with a VK_2 concentration of 125 and 150 µmol/L (Fig. 2c). Inhibition at these concentrations was also recorded on the xCELLigence system over the course of the 8-hour adhesion process (Fig. 2d).

3.3. *Vitamin K_2 has a significant inhibitory effect on the proliferation of breast cancer cells*

Cells were seeded in E-plate wells with DMEM containing a range of VK_2 concentrations (0-150 µmol/L). Cell behavior was monitored using the RTCA platform over a period of 48 hours, and CI values were analyzed. Results show that VK_2 significantly inhibited the proliferation of both cell lines at each VK_2 concentration tested in comparison to the untreated control (Fig. 3). Comparison of the CI of untreated MDA-MB-231 cells and cells treated with different concentrations of VK_2 showed that CI values were reduced by 30% to almost 70%, depending

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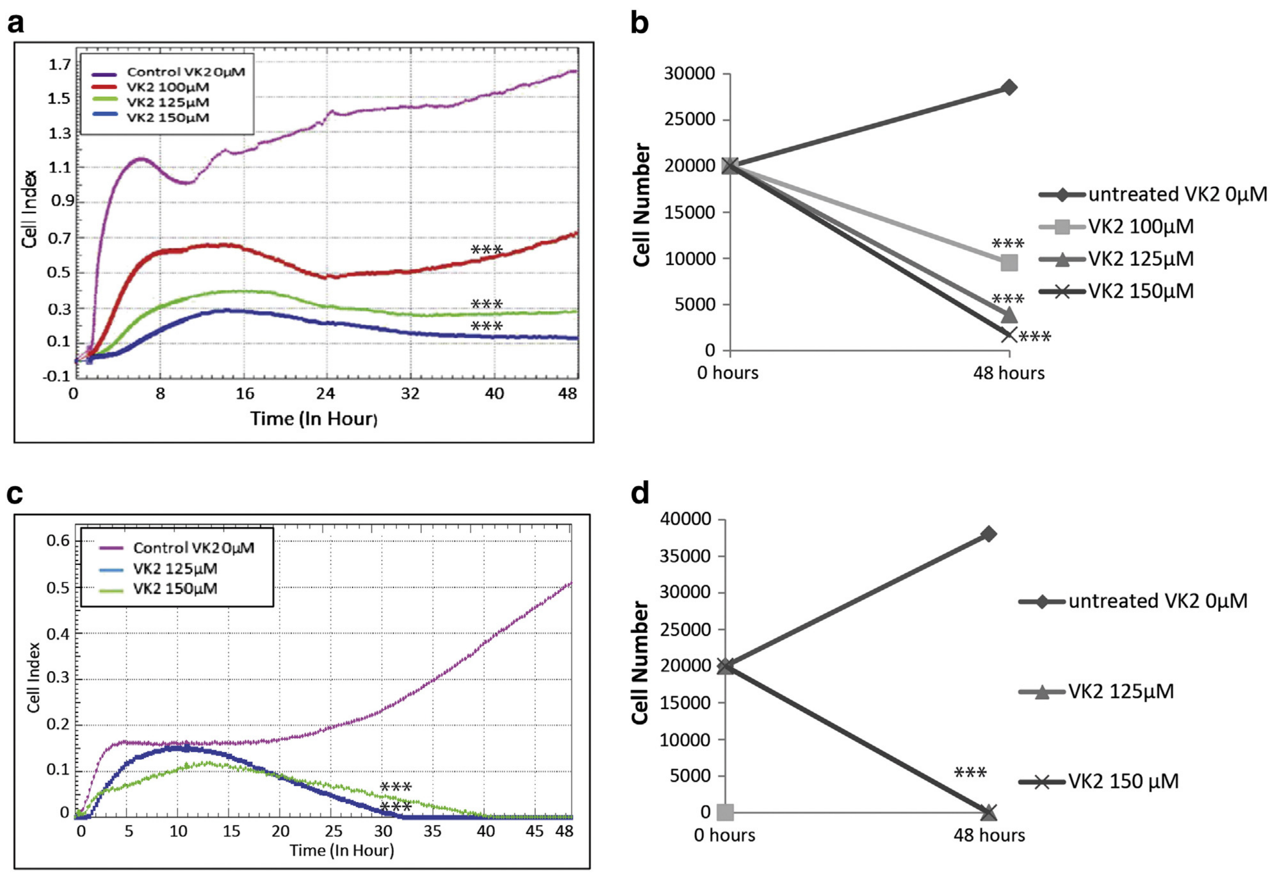

Fig. 3 – Vitamin K_2 has an inhibitory effect on the proliferation of breast cancer cells. Cells were plated at a seeding density of 20000 cells in an E-plate in DMEM containing a range of VK_2 concentrations (0-150 μmol/L). Proliferation was monitored in real time using the xCELLigence system. Readings were taken every minute for 8 hours and then every 30 minutes up to 48 hours with readings expressed as CI values. a, The xCELLigence graph is representative of duplicate wells comparing the effect of different VK2 concentrations on MDA-MB-231 cell proliferation for 48 hours. b, Cells were plated in a range of VK_2 concentrations (0-150 μmol/L) and allowed to proliferate for 48 hours. Cells were then counted using trypan blue live/dead exclusion. c, The xCELLigence graph is representative of duplicate wells comparing the effect of different VK_2 concentrations on MDA-MB-453 cell proliferation for 48 hours. d, MDA-MB-453 cells were plated in a range of VK_2 concentrations (0-150 μmol/L) and allowed to proliferate for 48 hours. Cells were then counted using trypan blue live/dead exclusion and recorded as a bar chart. Data are represented as means ± SEM. ***P < .001. n = 3.

on the concentration of VK_2 used (Fig. 3a). We calculated the half maximal inhibitory concentration (IC_{50}) for this TNBC cell line to be 124.37 μmol/L.

3.4. *A combination of low-glucose media and VK_2 has an added inhibitory effect on breast cancer cell adhesion and viability*

Following this, we investigated if lowering the glucose concentration in the cell culture media would have an effect on breast cell growth both alone and in combination with VK_2. Both cell lines were seeded in an E-plate with either a high- or low-glucose media (Fig. 4ai, aii). Viability of the TNBC cell line was compromised when cultured in low glucose compared with high glucose. No effect was reported on the MDA-MB-453 cell line when cultured in the same conditions. The TNBC cell line, now shown to be sensitive to low-glucose conditions, was further cultured in low glucose in combination with VK_2 at a concentration of 100 μmol/L which is now known to be effective on this cell line (see Figs. 2 and 3). Adhesion and spreading were monitored over an 8-hour period on the xCELLigence system. In agreement with our hypothesis, culturing cells in low-glucose media had an inhibitory effect on breast cancer cell adhesion when compared with cells cultured in high glucose media (Fig. 4b). Over the first 8 hours of cell adhesion and spreading, cells cultured in low-glucose media had a 20% reduction in adhesion when compared with the CI values of the cells cultured in high glucose (Fig. 4b). Culturing of cells with VK_2 (100 μmol/L) in combination with the low-glucose media is shown to have a greater inhibitory effect on cell adhesion than when culturing cells with low glucose alone (Fig. 4b). Data were also analyzed over a 24-hour period. Cells cultured in low-glucose media had lower CI values when compared with the CI index values of cells cultured in high-glucose media, which indicates reduced cell viability (Fig. 4c). Viability is inhibited to the greatest extent (74%) when cells are cultured with a combination of low-glucose media and VK_2 (100 μmol/L). This is marginally above the 68% decrease in CI values seen when cells are cultured in high glucose with VK_2 (100 μmol/L).

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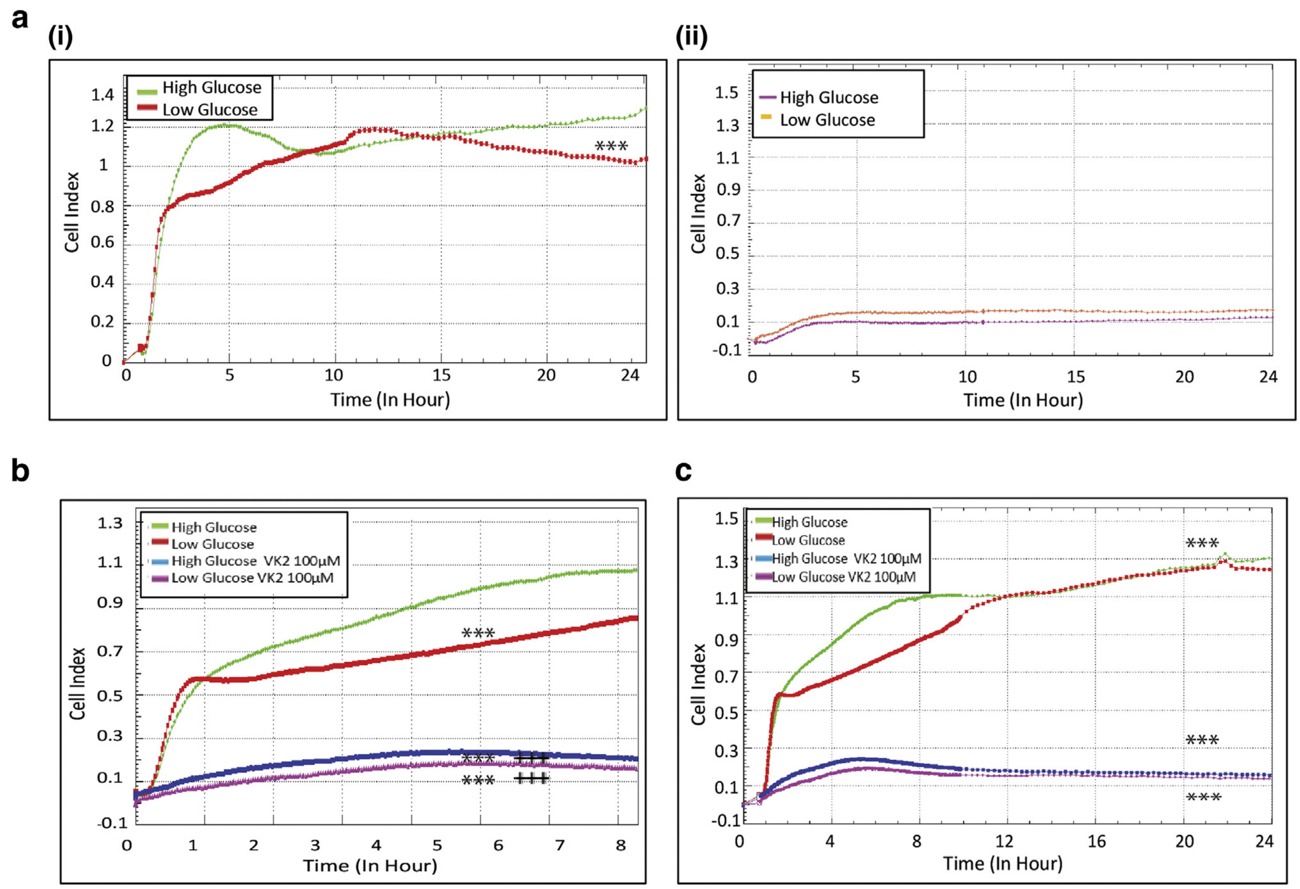

Fig. 4 – A combination of low-glucose media and VK_2 has an added inhibitory effect on breast cancer cell adhesion and viability. a, MDA-MB-231 cells (i) and MDA-MB-453 cells (ii) were plated at a seeding density of 20000 cells in wells of an E-plate in DMEM containing either high glucose or low glucose and cell behavior was monitored in real time using the xCELLigence system. b, TNBC cells were treated with 100 μmol/L VK_2 and adhesion was monitored every minute for 8 hours on the xCELLigence system with readings given as CI values. The xCELLigence graph is representative of duplicate wells comparing the combinatory effect of different glucose conditions and VK_2 treatment on cell adhesion. c, TNBC cells were plated at a seeding density of 20000 cells in wells of an E-plate in DMEM containing either high glucose or low glucose and 100 μmol/L VK_2 where indicated. Cell viability was monitored in real time every minute for 24 hours using the xCELLigence system with readings given as CI values. The xCELLigence graph is representative of duplicate wells comparing the combinatory effect of different glucose conditions and VK_2 treatment on cell viability. $^{***}P < .001$; $^{+++}P < .001$. n = 3.

4. Discussion

A number of well-established end point assays are widely used to monitor breast cancer cells' behavioral response to novel compounds and are essential in the search to find potential, safe, and therapeutic strategies against the disease [29,30]. However, these traditional cell-based assays are very much hindered by dependence on end point analysis. The RTCA xCELLigence platform used in this study is highly advantageous in comparison to these assays as it facilitates label-free, continuous monitoring of cellular response to compounds [31,32]. It has been shown to correlate very well with the conventional adhesion, viability, migration, and invasion assays and proven to be a highly accurate platform to monitor cell behavior [27,33–38].

In our study, we used the RTCA xCELLigence platform to determine the effect of VK_2 on breast cancer cells, specifically a TNBC cell line, MDA-MB-231, and an HER2+ cell line, MDA-MB-453.

When our cell models were exposed to VK_2 at a range of concentrations, there was a significant decrease in cell adhesion, spreading, and proliferation. Low concentration previously used on glioblastoma cells is initially used [13]; however, higher concentrations were required to affect our cell lines. It has been shown that the IC_{50} of VK_2 can vary depending on the cancer type, for example, human hepatoma cells (150 μmol/L) [39], human glioblastoma cell lines (960 μmol/L and 970 μmol/L) [13], and hepatocellular carcinoma cell lines (9.73 μmol/L) [17]. Inhibitory doses of VK_2 have also been calculated on a number of cancer cell lines including leukemia, nasopharyngeal carcinoma, oral epidermoid carcinoma, and breast carcinoma and range from 1 to 2 mmol/L

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[40]. An IC_{50} of 9.73 μmol/L and inhibitory dose $(ID)_{50}$ of 112 μmol/L have also been reported in the treatment of hepatocellular carcinoma cell lines with VK_2 [17,41]. Therefore, our results using RTCA correlate well with previous work completed using a number of more traditional end point assays.

Several mechanisms have been proposed for the anticancer activity of VK_2, including induction of both caspase-dependent and caspase-independent apoptosis [12,17,18], generation of reactive oxygen species [42], and cell cycle arrest and differentiation [10,18,11]. More specifically, the BCL-2 family of proteins have been strongly linked to VK_2-mediated anticarcinogenesis; using an hepatocellular carcinoma cell model, inhibition of BCL-2 expression was shown to significantly enhance the cytotoxic effect of VK_2 treatment [19]. In addition, Karasawa and colleagues [15] demonstrated caspase-dependent apoptosis through mitochondrial apoptosis and activation of caspase-3. That study also specifically identified the proapoptotic protein BCL-2 antagonistic killer as a direct molecular target of VK_2. It is also worth noting that there are no reports of toxicity associated with use of VK_2. In fact, no upper limit of use has been set due to the lack of reported toxicity [43]. This indicates the potential of VK_2 as an alternative to current chemotherapeutic strategies [15].

It has long been established that cancer cells avidly consume glucose in much larger quantities than normal cells [44,45], whereas calorie restriction has also been well studied as an anticancer strategy [20–23]. Specifically, glucose restriction as an anticancer strategy has been investigated at a molecular level resulting in induction of growth inhibition and apoptosis in cancer cells through epigenetic regulation of human telomerase reverse transcriptase and p16 while having no effect on noncancer cells [26]. The inhibitory effects of a low-glucose environment on cancer cell growth predominantly focus on growth factor inhibition and enhanced oxidative stress [20,21,23,24]. In our study, adhesion and viability of TNBC cells were impaired when cultured in a low- vs high-glucose media. Furthermore, an added inhibitory effect on both adhesion and viability was found when these cells are cultured in both low-glucose media and VK_2 (100 μmol/L). Although our study supports the hypothesis that glucose restriction has potential as an anticancer strategy, some limitations should be mentioned. For example, the high-glucose DMEM contains glucose concentrations 4 to 5 times higher than the concentration that would be within normal physiological blood sugar levels in the human body (5-7 mmol/L) and therefore acts more as a cell stressor. Furthermore, caution must be exercised when interpreting results from cell culture model systems.

Our results are the first to show the anticancer effect of VK_2 in real time both alone and in combination with a reduced glucose culture media on breast cancer cells. Given that the TNBC cell line used in our study is a well-established, aggressive cell type, our results show potential as a therapeutic strategy to treat this cancer subtype. However, further mechanistic studies are required to determine the precise mode of action of VK_2 and a low-glucose environment on TNBC cells which are mediating growth arrest. Our findings add further evidence to the role of VK_2 as an anticancer therapy and highlight the need for further studies to develop novel, targeted therapeutics required to treat resistant cancer types and improve patient outcomes.

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