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RESEARCH ARTICLE

PROLONGED β-HYDROXYBUTYRATE-MEDIATED KETOSIS ENHANCES PONATINIB RESPONSE OF K562 CHRONIC MYELOID LEUKAEMIA CELLS.

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ABSTRACT

Purpose: Ketosis is a metabolic state characterized by production of ketone bodies, including acetoacetate, β-hydroxybutyrate (BHB), and acetone, in response to reduced blood glucose levels. BHB stands out as the principal ketone body in nutritional ketosis which has diverse therapeutic implications for metabolic, nondegenerative and neoplastic disorders. In current study we investigated the impact of β-hydroxybutyrate mediated ketosis on viability and ponatinib response of K562 chronic myeloid leukaemia cells.

Materials and Methods: We investigated the impact of BHB-mediated ketosis on the viability of K562 cells, an *in vitro* model of chronic myeloid leukaemia (CML), and explored the influence of BHB on the sensitivity of these cells to ponatinib, a multi-targeted tyrosine kinase inhibitor used in CML treatment. We used MTT assay to measure cell viability and Hoechst/PI assay to measure cell death.

Results: Our findings reveal that BHB concentrations ranging from 1 mM to 5 mM, which fall within the physiological range of ketosis, elicit a minimal yet concentration-dependent reduction in cell viability. We also observed that while a 24-hour pre-treatment with BHB did not enhance the response of K562 cells to ponatinib, prolonged ketosis (4 days) improved response of cells to the drug by decreasing final cell viability from 25.15% to 13.12%. The primary mode of viability inhibition by ponatinib was cell death which was further intensified by exposure to prolonged ketosis.

Conclusion: Ketosis induced by ketogenic diet of ketone body supplementation is considered as safe and effective adjuvant cancer therapy options and here, we report its potential effectiveness in the context of CML.

Keywords: Ketogenic diet, ketosis, chronic myeloid leukaemia, β-hydroxybutyrate, Ponatinib

INTRODUCTION

Ketosis is the metabolic shift from catabolism of carbohydrates to lipids as a response to decreased blood glucose levels, leading to an increased rate of fatty acid β-oxidation within the liver. Excessive acetyl-CoA produced by β-oxidation serves as a substrate for the synthesis of a class of water-soluble acidic metabolites known as ketone bodies (acetoacetate, β-hydroxybutyrate, and acetone) in a process called ketogenesis within the mitochondrial matrix (1). Acetoacetate and β-hydroxybutyrate (BHB) can subsequently be catabolized back into

acetyl-CoA molecules, which are further oxidized via the Krebs cycle in extrahepatic tissues, providing an alternative energy source to glucose. BHB, in particular, emerges as the principal ketone body during natural ketosis (2) and can be employed as a supplement to induce a controlled state of artificial ketosis (3). Which will be referred as BHB-mediated ketosis in the current study.

Physiological or nutritional ketosis arises as a response to dietary modifications and is predominantly characterized by reduced carbohydrate intake. This results in elevation of ketone body levels, notably plasma BHB concentrations rising up to 7-8 millimolar (mM) range (4). This form of ketosis can also be achieved through ketone body supplementation and has been shown to exhibit multiple health benefits for patients with epilepsy, Alzheimer's disease, Parkinson's disease, and metabolic syndrome (3).

Therapeutic potential of ketosis for treatment of cancers is currently under investigation (5). Ketogenic diet has been demonstrated to sensitize most cancers to standard treatments by interfering with the regular metabolic functions of cancer cells (6,7). This anti-tumour activity often leads to an improved drug response and increased survival rate which has been observed across various cancer types, including glioblastoma, pancreatic, colorectal, breast, liver, and lung cancers, among others (5). However, data remains scarce for certain cancers, such as leukaemia. Notably, there are no preclinical or clinical studies focusing on the impact of ketosis on chronic myeloid leukaemia (CML) patients and/or cells. In current study we decided to investigate the effects of BHB-mediated ketosis on the viability of CML cells as well as the drug response of the cells in ketosis against ponatinib, a multi-targeted tyrosine kinase inhibitor primarily employed in the treatment of CML (8).

MATERIALS AND METHODS

Cell Culture Maintenance

K562 (ATCC, #CCL-243) CML cell line was used in the study. K562 cells were maintained in RPMI-1640 (Biowest, #MS015H) medium (complete medium) containing 10% fetal bovine serum (FBS) (Cytiva, #SV30160.03), 1% (2 mM) L-Gln (Capricorn, #GLN-B) and 1% (100 U/mL) Penicillin/streptomycin (Pan Biotech, #P06-07100) in a humid incubator at 37ºC 5% CO_2 pressure.

Chemical Treatments

β-hydroxybutyrate (Sigma, #166898) was dissolved in absolute Ethanol (EtOH) and subsequently diluted with cell culture media to attain varying concentrations to simulate physiological ketosis. The highest treatment concentrations contained 1% EtOH and since this concentration exceeds the recommended threshold (9) we included vehicle controls to assess and clarify any potential impact of ethanol on our study outcomes. Ponatinib (Selleck chemicals) was dissolved in dimethyl sulfoxide (DMSO) and further diluted with cell culture media to achieve different concentrations for experimental treatments. The highest treatment concentrations contained less than 0.1% DMSO.

Cell Viability Measurement

The viability of K562 cells was assessed using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, Abcam) assay to evaluate the impact of ponatinib, β-hydroxybutyrate (BHB), and their combination. Briefly, a total of $3x10^3$ K562 cells were seeded in 50 µL of culture medium into the wells of 96-well plates. After an overnight incubation, cells in the experimental wells were treated either with ponatinib (1-14 µM) for 24-hours or BHB (0.01-5 mM) for 48-hours in a total volume of 100 µL. For longer incubation periods (BHB for 6 days, ponatinib for 2 days), 1000 K562 cells were seeded into the wells of 24-well plates in a total volume of 300 µL of culture medium and total volume was 600 µL after the treatment. The non-treated (NT) and vehicle control (VC, %1 EtOH) wells received an equivalent volume of complete medium as the treatment groups. Upon completion of the treatment, 10% MTT solution with a final concentration of 500 µg/mL was added to all wells. The plates were incubated in darkness for 4 hours. Subsequently, a 10% SDS solution (dissolved in 0.01 M HCl) in a 1:1 ratio was added to all wells treated with MTT and incubated overnight. A spectrophotometric reading at 570 nm was conducted using a BMG Labtech LUMIStar Omega instrument. Cell viability in the non-treated group was considered as 100%, and the viability of cells in the treatment groups was calculated accordingly. Each experiment contained at least five (96-well plate experiments) or three (24-well plate experiments) replicate wells and the average viability and standard deviation calculated for these cells were used in graphical representations which were plotted using GraphPad Prism v9. The data is uploaded as a supporting file.

Cell Death Measurement

The Hoechst/Propidium iodide (PI) staining method was employed to assess the impact of ponatinib, β-hydroxybutyrate (BHB), and their combination on cell death in K562 cells. K562 cells were seeded in 24-well plates at a density of 1000 cells per well in a 300 µL culture medium. Following an overnight incubation, the cells were treated either with ponatinib (6 µM) or BHB (5 mM) in 600 µL total volume (BHB for 6 days, ponatinib for 2 days). Upon completing the treatment, the cells were collected and pelleted by centrifugation at 300 g for 5 minutes. Following centrifugation, the supernatant was carefully aspirated, and the cell pellet was resuspended in a phosphate-buffered saline (PBS) solution containing Hoechst-33342 (0.25 µg/mL) (ChemCruz, #sc-495790) and PI (1 µg/mL) (Biolegend, #421301) fluorescent dyes. The suspension was incubated at room temperature for 20 minutes in the dark. After the incubation period, the number of Hoechst-positive cells, representing the entire cell population, and PI-positive cells, indicative of dying cells, were quantified using fluorescence microscopy (Zeiss, AxioScope Z1). Subsequently, the cell death rate was computed based on these counts. For each sample, at least three different cell suspensions were assessed as technical replicates, and the resulting average cell death rate values and standard deviation were used to generate graphical representations which were plotted using GraphPad Prism v9. The data is uploaded as a supporting file. The study was evaluated by the Human Ethical Research Committee of Istinye University.

RESULTS

Ponatinib reduced K562 cell viability at the micromolar level. Cells were treated with different concentrations of ponatinib (ranging from 1 µM to 14 µM) for 24 hours to identify an effective concentration for subsequent experiments. MTT assay results demonstrated a concentration-dependent inhibition of K562 cell viability induced by ponatinib (Figure 1A). 6 µM ponatinib was the lowest concentration to inhibit the cell viability by 50% (specifically 51.5%) and subsequently, was selected as the optimal concentration for further investigations.

β-hydroxybutyrate treatment caused a modest reduction in K562 cell viability.

We have treated the cells for 48 hours with increasing concentrations of BHB (ranging from 10 µM to 5 mM) to assess the impact of ketosis on cell viability. Notably, the highest BHB concentration used to mimic ketosis remained below the upper physiological limit (<8 mM). MTT assay results revealed that BHB-mediated ketosis had a minimal yet concentration-dependent effect on K562 cell viability, reducing it to a minimum of 80% (Figure 1B). Consequently, we chose to proceed with BHB concentrations of 1, 3, and 5 mM for further investigations. Short-term ketosis mediated by BHB did not influence ponatinib response in K562 cells. In addition to the standard 48-hour BHB treatment (at concentrations of 1, 3, or 5 mM) and 24-hour ponatinib treatment (at 6 µM), select wells were pre-treated with BHB for 24 hours before ponatinib exposure, allowing us to elucidate the influence of ketosis on ponatinib sensitivity. The MTT assay results suggested that the 24-hour pre-treatment did not enhance the response of K562 cells to ponatinib (Figure 1C). As a result, we opted to extend our investigations to longer incubation periods using only the highest BHB concentration (at 5 mM).

Prolonged exposure to BHB-mediated ketosis enhanced ponatinib response of K562 cells. Cells were subjected to BHB treatment (at 5 mM) for 6 days, ponatinib treatment (at 6 µM) for 2 days, and a combination regimen in which cells were pre-treated with BHB for 4 days, followed by treatment with ponatinib for 2 days. MTT assay results indicated that the 4-day BHB pre-treatment improved the response of K562 cells to ponatinib, reducing final viability from 25.15% to 13.12% (Figure 1D). Nonetheless, it is worth noting that BHB itself reduced cell viability by 32.48%, suggesting that this effect may be additive rather than synergistic. We should also note that, unlike previous findings, EtOH (%1, as vehicle control) also caused a decrease in viability (79%), albeit to a lesser extent compared to BHB.

Prolonged BHB-mediated ketosis led to a mild increase in the rate of ponatinib induced K562 cell death.

In order to elucidate the mechanism of the viability inhibition, we assessed cell death levels in samples previously examined for viability (6 days of BHB treatment, 2 days of ponatinib treatment, and a combined treatment of 4 days of BHB followed by 2 days of ponatinib). Results from the Hoechst/PI assay revealed cell death as the primary mechanism of viability inhibition induced by ponatinib as 80% of the cells were PI positive. Prolonged exposure to BHB-mediated ketosis further increased the cell death to 89% (Figure 1E).

which may initially resist such changes due to their

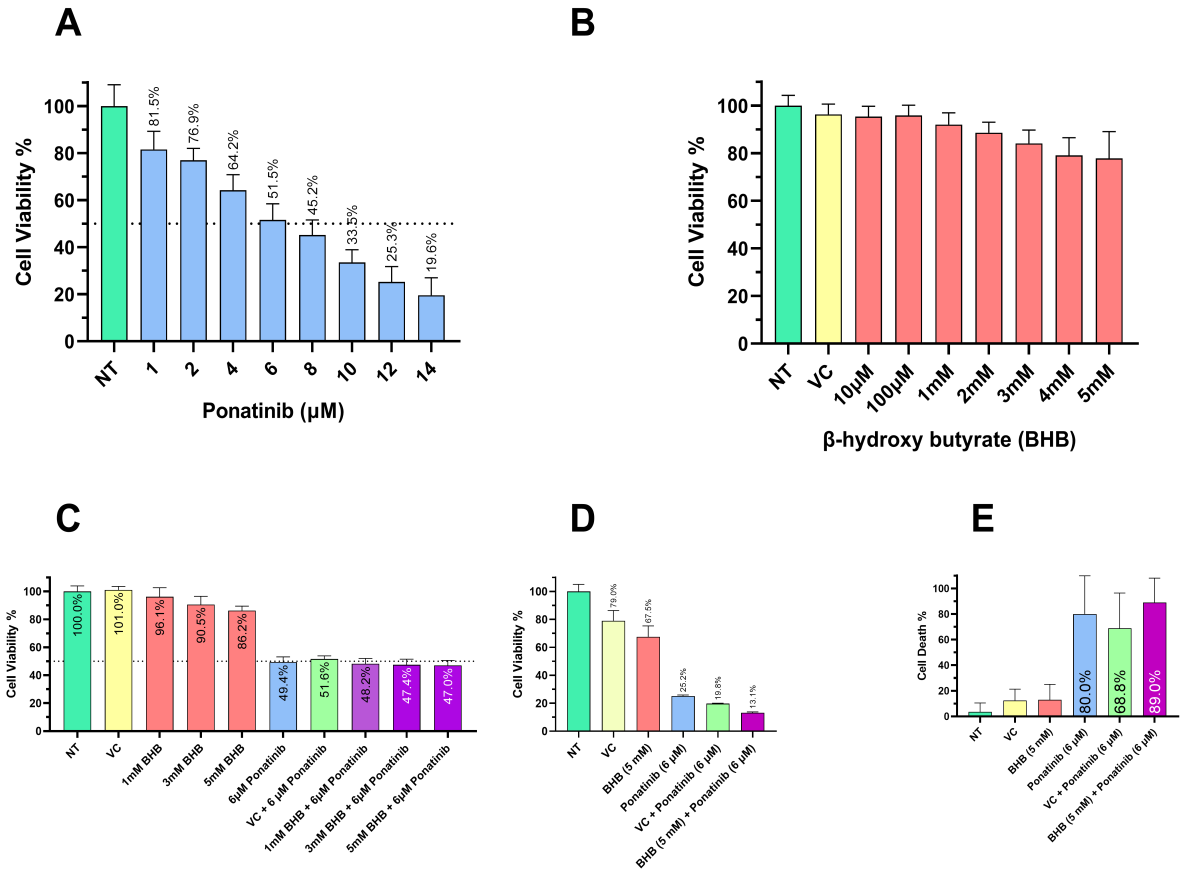

Figure 1. Effects of BHB-mediated ketosis on K562 cells. (A, B, C and D) Cell viability results according to the MTT assay. Non-treated cell viability (NT) was considered as 100% and all the others were calculated accordingly. Vehicle control is 1% ethanol, the solvent for BHB. Each value represents the average viability of at least five samples and error bars represent standard deviation. (A) Effects of 24-hour ponatinib treatment. (B) Effects of 48-hour BHB treatment. (C) Effects of combination treatment (24 hours for ponatinib, 48 hours for BHB). (D) Effects of prolonged exposure to combination treatment (2 days for ponatinib, 6 days for BHB). (E) The percentage of PI positive cells according to the Hoechst/PI assay after prolonged exposure to combination treatment (2 days for ponatinib, 6 days for BHB). Each value represents the average of at least three different samples and error bars represent standard deviation.

DISCUSSION

The utilization of the health benefits associated with ketosis achieved via ketogenic diet or ketone body supplementation, has garnered increasing interest in recent years. Notably, the ketogenic diet has been proposed as a safe and potentially beneficial approach for cancer patients (10,11). Although there are numerous studies focusing on different cancers to elucidate the impact of ketosis *in vitro*, *in vivo* and in clinical settings, the data is still scarce for some cancers including leukaemia. This lack of studies on the impact of ketosis in leukaemia is particularly intriguing because leukemic cells, by nature, are more susceptible to ketosis due to their immediate exposure to the ketogenic environment within the bloodstream. This is in contrast to solid tumours, erratic vasculature and hypoxic regions where the blood cannot reach effectively.

A noteworthy study examining the influence of insulin feedback suppression on drug responses in mouse models of different cancers also included acute myeloid leukaemia (AML) to their study design (12) and found that ketogenic diet enhanced the efficacy of phosphatidylinositol-3 kinase (PI3K) inhibitors. Here we report for the first time that the prolonged ketosis inhibits CML cell viability and enhances ponatinib response *in vitro*. Since physiological ketosis is easily and safely achievable in patients, ketogenic diet or ketone-body induced ketosis could be considered as adjuvant cancer therapy for a spectrum of malignancies including CML. However, it is important to emphasize that while these findings

are compelling, further investigations in clinical settings are imperative to fully elucidate the precise impact of ketosis on patient health and drug response in the context of CML.

Ponatinib is a third-generation kinase inhibitor which can act on BCR-ABL1 kinase and several ABL1 mutations making it an effective treatment for CML patients (8). It is applicable across all phases of the disease and serves as a rescue therapy in cases of resistance or intolerance to dasatinib or nilotinib, as well as in instances where imatinib proves ineffective (13). However, cardiovascular adverse events have emerged as concerningly common complications of ponatinib treatment (14). Since ponatinib-associated cardiovascular toxicity been found to be dose-dependent (15), exploring the combination of ponatinib with other adjuvant therapies, such as a ketogenic diet, to reduce the required therapeutic dose to mitigate the associated side effects is an intriguing and potentially effective strategy. This approach is further supported by the known beneficial effects of the ketogenic diet on cardiovascular risk factors (16).

CONCLUSION

Our study highlights the potential of ketosis as an adjuvant therapy for chronic myeloid leukemia (CML). Nonetheless, it is important to acknowledge that the current research focused on a single cell line and future research should explore cell-line-specific effects of BHB. Finally, more research is warranted to validate these findings in clinical settings.

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Conflict of Interest: Authors declared no conflict of interest.

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