The metabolic determinants of the response to ketone bodies in SH-SY5Y neuroblastoma cells

A Thesis Submitted for the Degree of

Master of Science

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August 2020
Declaration

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Signed:

[Signature]

Date: 10/08/2020
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# Table of Contents

1 *Introduction* .......................................................................................................................... 10

1.1 Overview of the ketogenic diet and ketone metabolism ................................................. 10

1.2 Ketone metabolism and the Warburg effect in cancer .............................................. 11

1.3 The β-Hydroxybutyrate Paradox ................................................................................. 13

1.4 Ketone metabolism during brain and foetal development ..................................... 13

1.5 Neuroblastoma ............................................................................................................. 14

1.6 Immortal cell lines ...................................................................................................... 14

1.7 The role of the culture medium in metabolism research .................................. 15

2 *Aims & Hypotheses* ............................................................................................................. 18

2.1 Aims ................................................................................................................................. 18

2.2 Hypotheses ..................................................................................................................... 19

3 *Materials & Methods* ........................................................................................................ 20

3.1 Cell Culture .................................................................................................................... 20

3.2 Experimental Design .................................................................................................. 21

3.3 Cell Viability .................................................................................................................. 22

3.4 Cell Staining & Immunocytochemistry ...................................................................... 22

3.5 Imaging and Analysis .................................................................................................. 22

3.6 A novel method for measuring cell density ............................................................... 25
4 Results & Discussion ........................................................................................................... 28

4.1 Chapter 1: Characterising the effects of glucose deprivation and ketone supplementation ................................................................................................................................. 28
   4.1.1 Introduction ................................................................................................................. 28
   4.1.2 Results ....................................................................................................................... 29
   4.1.3 Discussion ................................................................................................................. 33

4.2 Chapter 2: The interaction of the βOHb response with other substrates in culture ................................................................................................................................. 35
   4.2.1 Introduction ................................................................................................................. 35
   4.2.2 Results ....................................................................................................................... 37
   4.2.3 Discussion ................................................................................................................. 42

4.3 Chapter 3: The therapeutic potential behind the differential responses to βOHb 46
   4.3.1 Introduction ................................................................................................................. 46
   4.3.2 Results ....................................................................................................................... 48
   4.3.3 Discussion ................................................................................................................. 50

5 General Discussion & Conclusions ................................................................................. 51

5.1 Main findings .................................................................................................................. 51

5.2 Limitations ..................................................................................................................... 51

5.3 Conclusions & Future Directions ............................................................................... 52

6 References ...................................................................................................................... 54

7 Appendix ......................................................................................................................... 67
List of Figures

Figure 1.1 The pathways of ketogenesis and ketolysis ........................................... 11
Figure 1.2 The contributions of the main metabolic substrates in culture to the TCA cycle
................................................................................................................................. 17
Figure 3.1 A schematic of the process underlying the cell density quantification in whole-
well images. .................................................................................................................. 27
Figure 4.1 Growth and viability of SH-SY5Y cells in GF, LG and RG conditions at 96 hr.
........................................................................................................................................ 30
Figure 4.2 Increasing concentrations of βOHB do not affect the growth of glucose
deprived SH-SY5Y cells .................................................................................................. 31
Figure 4.3 Increasing concentrations of βOHB do not affect the morphology of glucose
deprived SH-SY5Y cells ................................................................................................. 32
Figure 4.4 βOHB differentially rescues the viability and proliferation of cells in pyruvate
and glucose depleted conditions. .................................................................................... 38
Figure 4.5 The suggested optimization of the control medium results in a maximised
response to βOHB at 96 hr ............................................................................................... 40
Figure 4.6 The differential effect of βOHB on NE-4C cells ........................................... 41
Figure 4.7 A schematic of the potential mechanisms behind the interaction between βOHB
and the fuel substrates of the culture medium ............................................................... 43
Figure 4.8 Metformin modulates the effect of βOHB on SH-SY5Y cell viability .............. 49

List of Tables

Table 1.1 A comparison of the metabolite concentrations in plasma and in culture ....... 16
Table 3.1 A summary of the utilised media groups and their corresponding compositions
........................................................................................................................................ 20
Table 3.2 A summary of the media groups in each experiment ...................................... 21
Table 3.3 The image-based assays ................................................................................... 24
Table 7.1 Cell culture materials ...................................................................................... 67
Table 7.2 Cell fixation and immunocytochemistry reagents ............................................ 68
Table 7.3 Cell viability assay materials ............................................................................ 68
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcAc</td>
<td>Acetoacetate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>βOHB</td>
<td>β-Hydroxybutyrate</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of View</td>
</tr>
<tr>
<td>GF</td>
<td>Glucose Free</td>
</tr>
<tr>
<td>GF-L</td>
<td>Glucose Free without L-glutamine</td>
</tr>
<tr>
<td>GF-P</td>
<td>Glucose Free without pyruvate</td>
</tr>
<tr>
<td>GF-PL</td>
<td>Glucose Free without pyruvate and L-Glutamine</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose Transporter</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LG</td>
<td>Low Glucose</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate Transporter</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NB</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>RG</td>
<td>Regular Glucose</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SC</td>
<td>Suggested Control</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid</td>
</tr>
</tbody>
</table>
Abstract

Neuroblastoma (NB) is a childhood malignancy of the sympathetic nervous system. Its mutual neoplastic and neurodevelopmental underpinnings are characterised by an excessive glucose demand as a hallmark of a high proliferative capacity. This metabolic phenotype is reported as a barrier to ketone utilization and an avenue for dietary therapies designed to alter substrate availability and starve cells from their preferred biosynthetic requirements. However, the effects of ketones in both cancer and foetal development are equally diverse and poorly understood. In pursuit of a transferrable in vitro model of glucose deprivation, ketosis and the potentially confounding interactions with other fuel substrates, we investigate how the primary ketone body Beta-hydroxybutyrate (βOHB) affects the growth, survival and morphology of undifferentiated SH-SY5Y NB cells in the presence or absence of glucose, pyruvate and glutamine from the culture medium. We demonstrate that while glucose deprivation diminishes the growth and viability of cells, they can continue to survive and proliferate, and are initially unaffected by various concentrations of βOHB. Subsequent alteration of the culture conditions reveals that a growth-promoting effect of βOHB is masked by the concentration of glucose and the presence of pyruvate, drawing attention to substrate availability as a determinant of the differential responses reported in models of neoplasia and development. Finally, based on the speculative mechanisms of this differential response, we present findings supporting a rationale for combining ketosis with metformin as a potential anticancer therapy. The implications include a preliminary framework for understanding the interfering mechanisms of other substrates in the metabolism of ketones in terms of reversible enzymatic pathways, the overall energy balance, and the potential for competition at the transporter level. With this understanding of an adjustable response to ketones, variations in how different cells react according to their intrinsic characteristics may allow selective pharmacological inhibition of NB ketone metabolism using metformin for instance, while allowing healthy cells to continue utilising this alternative fuel. In conclusion, the modifiable environment of the cells is a key determinant of their response to βOHB in vitro.
1 Introduction

1.1 Overview of the ketogenic diet and ketone metabolism

The ketogenic diet is a high fat, moderate protein and low carbohydrate diet that aims to induce a state of glucose starvation resulting in shift toward ketone body metabolism. Initially developed as a treatment for epilepsy, its potential therapeutic role has been highlighted in a range of disorders including obesity, diabetes, fatty liver disease, neurological disease and cancer (Puchalska and Crawford, 2017). As such, it is becoming increasingly popular due to its perceived benefits in disease and well-being (Barry et al., 2018). However, it’s effects and safety profile are yet to be fully characterised in the general public (Blagosklonny, 2019) and in pregnant women in particular (Barry et al., 2018). The metabolic shift is achieved by an increase in plasma ketone bodies synthesized by the liver as a by-product of the β-oxidation of fatty acids in hepatic mitochondria (Cotter, Schugar and Crawford, 2013). Released by the liver in response to low-carbohydrate conditions, ketone bodies are the three water-soluble, energy-rich molecules characterised by the presence of a ketone carbonyl group, the most abundant of which is β-Hydroxybutyrate (βOHB), followed by Acetoacetate (AcAc) and Acetone (Laffel, 1999). βOHB is transported into circulation and cells by the monocarboxylate transporters (MCTs) (Tildon, McKenna and Stevenson, 1994). Subsequently, ketolysis takes place in the extrahepatic mitochondria of various tissues with the conversion of the ketones βOHB and AcAc into acetyl-CoA for incorporation into the tricarboxylic acid (TCA) cycle (Cotter, Schugar and Crawford, 2013). The result is the generation of adenosine triphosphate (ATP) via oxidative phosphorylation (Figure 1.1)
1.2 Ketone metabolism and the Warburg effect in cancer

Serving as an alternative energy source to glucose, the primary ketone body βOHB is released by the liver during periods of nutrient deprivation (Grabacka et al., 2016). The presence of ketone bodies in the bloodstream is induced pathologically in diabetic ketoacidosis (Stojanovic and Ihle, 2011) or physiologically through fasting and carbohydrate-restricted dietary regimens including the ketogenic diet described above (Paoli et al., 2013). Such dietary restrictions are frequently purported as a potential cancer therapy in NB and other neoplasms (Morscher et al., 2015; Aminzadeh-Gohari et al., 2017; Weber, Aminazdeh-Gohari and Kofler, 2018). This stems from the observation that increased glucose uptake is a key characteristic of cancer, which also forms the biologic basis of $^{18}$Fluorodeoxyglucose positron emission tomography (FDG-PET) scans for detecting the spread of the disease (Jadvar, 2016).

*Figure 1.1 The pathways of ketogenesis and ketolysis* 

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The reliance on excessive glycolysis in neoplastic cells even in the presence of oxygen, coupled with the efflux of lactate was described by Warburg as a hallmark of cancer almost a century ago (Warburg, 1925). For every molecule of glucose, glycolysis produces only 2 molecules of ATP compared with 36 during oxidative phosphorylation (Devic, 2016), and Warburg’s effect is thus perceived by some to be a compensatory mechanism for defective mitochondrial function (Seyfried, 2015). Despite its seemingly inefficient nature however, it is observed in cancer even with normally functioning mitochondria and is thought to be beneficial in rapidly supporting the biosynthetic requirements of uncontrolled proliferation (Liberti and Locasale, 2016) This metabolic profile has been cited as the potential barrier to ketone metabolism (Seyfried et al., 2014) and studies have observed that the lactate efflux associated with this phenomenon correlates with the suppressive effect of ketones on tumour proliferation (Whitaker-Menezes et al., 2011).

These differences from normal cells are the target of the dietary therapeutic proposals, as glucose deprivation achieved through ketosis would be expected to preferentially starve cancer cells reducing their growth and survival (Kansara and Berridge, 2004), while healthy cells can readily adapt their metabolism to use ketone bodies such as βOHB to meet their energy demands (Weber, Aminazdeh-Gohari and Kofler, 2018). In this regard several studies have observed that a reduction in glucose availability as seen in dietary restriction (Longo and Fontana, 2010; O’Flanagan et al., 2017), or pathologically in hypoglycaemia, leads to a preferential damage of cancer cells (Kansara and Berridge, 2004). The mechanisms of the high glycolytic process have also been targeted, such as the upregulated Glucose Transporter 1 (GLUT1) enabling increased glucose uptake (Zambrano et al., 2019), as well as the MCTs which allow the resultant lactate efflux (Pérez-Escuredo, Vincent F Van Hée, et al., 2016; Payen et al., 2020). Inhibitors of these have shown promising results but with the distinct disadvantages of substantial off-target effects (Park et al., 2018), as well as resistance occurring with upregulation of other transporter subtypes (Quanz et al., 2018).
1.3 The β-Hydroxybutyrate Paradox

A non-pharmacological approach may also represent an effective way of circumventing the above issues of drug intervention; however, studies describing the impact of dietary alterations on NB growth and survival have been equivocal largely due to the lack of a mechanistic understanding behind the spectrum of findings. In particular, the “βOHB paradox” describes the conflicting observations of the effect of ketone bodies on cancer growth and progression (Rodrigues et al., 2017). On the one hand, several studies have shown an inhibitory, anti-proliferative effect (Skinner et al., 2009; Poff et al., 2014; Shukla et al., 2014; Martuscello et al., 2016), whereas others have demonstrated contradictory data, that ketone bodies promote the growth and survival of cancer cells (Bonuccelli et al., 2010; Martinez-Outschoorn et al., 2011; Whitaker-Menezes et al., 2011). Other studies have shown that βOHB has no impact on the proliferation rate (Bartmann et al., 2018).

1.4 Ketone metabolism during brain and foetal development

The divergent responses to ketones are similarly implicated in embryological and developmental studies (Barry et al., 2018), and the determinant mechanisms remain poorly understood (Morris, 2005). For example, foetal weight as a result of maternal ketosis was found to be increased (Jones et al., 2009), decreased (Mendes-da-Silva et al., 2014), or variant at different stages of gestation (Sussman, Germann and Henkelman, 2015; Barry et al., 2018). On the potential teratogenic effects of βOHB, Horton (1985) showed that the early embryo is almost wholly-dependent on glycolysis for energy with a minimal capacity for βOHB metabolism, whereas later gestational stages showed a greater rate of utilization with lower rates of glycolysis. These changes and differential effects of ketones may coincide with changes in the availability of other substrates. Postnatal studies have shown the brain’s metabolic pathway activities, substrate transport and availability all change dramatically with maturation, with rates of ketone utilisation exhibiting a 6-fold increase from trough to peak (Cremer, Braun and Oldendorf, 1976; Vannucci and Simpson, 2003). In the initial postnatal period there is transient dependence on lactate, followed by a reliance on both ketones and glucose during breastfeeding (Prins, 2012). Lastly the brain becomes exclusively reliant on glucose after weaning (Prins, 2012). These may be important comparisons with findings of ketone metabolism in cancer.
1.5 Neuroblastoma

NB is a neoplastic proliferation of sympathoadrenal progenitor cells derived from the neural crest (Brodeur, 2003). NBs are the most common extracranial solid tumours in children and the most common cancer diagnosed during the first year of life (Ries et al., 1999), and are responsible for a disproportionately high percentage of cancer related paediatric deaths (Young et al., 1986). Cases can be sporadic or familial, with the most notable genetic aberration being an amplification of the oncogene MYCN in 25% of patients (Matthay et al., 2016). Lesions form predominantly in the adrenal cortex and paraspinal ganglia, presenting anywhere from the neck to the pelvis (Brodeur, 2003). Initial symptoms are dependent on the location of the mass as a swelling or through local compression of surrounding structures, along with the non-specific features of cancer such as weight loss and fatigue. Metastasis is present in more than half of patients at diagnosis, most commonly through hematogenous spread. Confirmation and stratification of risk requires a biopsy, and treatments can be conservative, medical or surgical (Matthay et al., 2016). The prognosis can be extremely varied between the highest rate of spontaneous differentiation and regression amongst cancers (Carlsen, 1990), and in other cases as an aggressive, highly metastatic and treatment-resistant tumour (Davidoff, 2012).

1.6 Immortal cell lines

Immortal cell lines are commonly used in place of primary cells in multiple areas of in vitro research, including drug metabolism, cytotoxicity, vaccine and antibody production, as well as studies of gene function (Kaur and Dufour, 2012). Their unlimited potential for replication, either due to their cancer or stem-cell background, offers several benefits with regards to cost-effectiveness, ease-of-use, and bypassing some of the ethical concerns associated with human or animal tissue. Further, the homogeneous populations of cells aid in the reproducibility of the experiments (Mirabelli, Coppola and Salvatore, 2019). However, they may not always be adequate in representing primary cells due to the inherent mutations and genetic variations associated with serial passaging in culture, which may result in unstable phenotypes, functions and responses (Masters, 2000). With regards to neuronal development, the
use of primary cells is also limited by the fact that neurones cannot be propagated if they are differentiated into mature phenotypes. The use of neuronal cell lines such as SH-SY5Y cells can overcome this limitation, and is commonly used to model neurogenesis as well as neurological pathologies (Xicoy, Wieringa and Martens, 2017). SH-SY5Y cells are derived from the parent SK-N-SH original cell line, which was isolated from a bone marrow biopsy taken in 1970 from a four-year-old female with a malignant NB (Shipley, Mangold and Szpara, 2016).

On the other hand, this study’s secondarily utilised NE-4C cell line originates from murine neuroectodermal stem cells, and is also used in vitro to model neuronal development (Schlett, Herberth and Madarász, 1997). Although of a non-cancerous origin, NE-4C cells were initially isolated from mice deficient in p53 (a key tumour suppressor gene), and were presumably immortalized due to the reduced p53 functionality (Jády et al., 2016). This protein regulates cell cycle progression, DNA repair and apoptosis as well as cellular energy production, and as a result p53-deficient cells including NE-4Cs display similar characteristics to neoplastic cells (Schlett, Herberth and Madarász, 1997).

The use of SH-SY5Y and NE-4C cells in this study, given their mutual neoplastic and neuronal features may help to bridge some of the observations in cancer and development. Importantly, a key parallel in the metabolism of both cancer and developing cells is their high proliferation rates and reliance on aerobic glycolysis (DeBerardinis et al., 2008). As such, the findings of this study may be of particular relevance to NB, which is described as a neurodevelopmental malignancy (Johnsen, Dyberg and Wickström, 2019).

1.7 The role of the culture medium in metabolism research

The discrepancies highlighted above on the response to ketones between studies of diverse methodologies come with a background of an increasingly scrutinised role of experimental culture conditions and medium compositions in influencing outcomes (Cantor et al., 2017), especially in the context of cancer metabolism (Ackermann and Tardito, 2019). The choice between the two most commonly used types of media, Dulbecco’s Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute
Medium (RPMI) for instance (McKee and Komarova, 2017), is known to alter the metabolome of various cell lines (Wu et al., 2009; Huang et al., 2015). Further, the choice of at least 20 formulations of DMEM as advertised by a single supplier (Thermo Fisher Scientific, Rockford, IL, USA), formulated with or without pyruvate for instance, plays a determining role when assessing the in vitro cytotoxicity of various chemicals (Babich et al., 2009). A systematic review of the literature on the SH-SY5Y NB cell line in Parkinson’s disease has identified the use of at least 8 different media formulations which are further distinguished by the addition of various supplements (Xicoy, Wieringa and Martens, 2017). Moreover, studies reporting the use of DMEM for example commonly do not specify whether the utilised formulation includes or excludes specific supplements (Babich et al., 2009), perhaps with an expectation that the components of culture media act in isolation (Yao and Asayama, 2017). These factors have been cited as a contributor to irreproducibility and a low translation rate into clinical success (Begley and Ellis, 2012), especially in the area of cancer research (Hutchinson and Kirk, 2011). In the study of the deprivational state of ketosis (Paoli et al., 2015), these variations of methodology and the unstandardised presence of other major metabolic substrates may be important. As such, we speculate that these wide-ranging outcomes are at least partly mediated by the chosen culture conditions, in particular, the presence of other fuels and metabolites such as pyruvate and glutamine which are commonly supplemented at supraphysiological concentrations when compared to human plasma, as further detailed in Table 1.1 below (Ackermann and Tardito, 2019).

Table 1.1 A comparison of the metabolite concentrations in plasma and in culture

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Human Plasma (mM)</th>
<th>DMEM (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.5–5.3</td>
<td>25</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.4–0.7</td>
<td>2–4</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.009–0.059</td>
<td>1–2</td>
</tr>
</tbody>
</table>
Furthermore, presented in (Figure 1.2) below are the separate and overlapping means by which the main fuel substrates in culture enter the cells and the TCA cycle, contributing to the process of anaplerosis and the generation of ATP.

![Figure 1.2 The contributions of the main metabolic substrates in culture to the TCA cycle](image)

The metabolites in culture aid in replenishing the intermediates of the TCA cycle by a process known as anaplerosis (Gibala, Young and Taegtmeyer, 2000). Glycolysis generates pyruvate from glucose which is converted to acetyl-CoA by PDH. Pyruvate can also be converted to lactate by LDH in highly glycolytic cells which exits cells via the MCT. Glutamine is converted to glutamate and subsequently α-KG by glutaminase and GDH respectively. βOHB is converted to acetyl-CoA by a series of ketolytic enzymes which can enter the TCA cycle to generate ATP. α-KG, alpha-ketoglutarate; GDH, glutamate dehydrogenase; GLUT, glucose transporter; LDH, lactate dehydrogenase; OAA, oxaloacetate; PDH, pyruvate dehydrogenase. Created with BioRender.com
2 Aims & Hypotheses

Considering the paucity of consistent research underlying the potential effects of ketosis on tumour growth in vitro, we firstly interrogated the effects of glucose deprivation on the growth, viability, proliferation and morphology of SH-SY5Y NB cells. Secondly, we aimed to see if various concentrations of ketones had any rescuing effect on glucose deprived cell growth and health. Finally, we examined whether proliferative responses were affected by different medium compositions and incubation periods, with the aim of contributing to a standardised protocol which adequately recognises the extracellular environment as a primary determinant of experimental outcome (Yao and Asayama, 2017), and characterises the possible interactions of metabolic substrates with the βOHB variable. This may then provide a framework for understanding some of the determinants of variation in response to ketosis in vitro and point towards potential mechanisms of action. The aims and hypotheses in this study were arrived at in a stepwise consequential manner, such that the results of one investigation informed the basis of the succeeding aims:

2.1 Aims

1. To investigate the effects of glucose deprivation on SH-SY5Y cell growth and morphology
2. To investigate whether ketone supplementation can rescue or exacerbate the effects of glucose deprived cells
3. To examine the roles and interactions of other culture substrates such as pyruvate and glutamine on the effects of ketone supplementation
4. To search for and test the optimal conditions in the culture medium composition for a maximal response to the ketone
5. To replicate any potential substrate interactions in the NE-4C cell line
6. To assess whether the potential conditions preventing the effects of ketones can be simulated exogenously, such that the same effects can be achieved with the aid of a pharmacological agent
2.2 Hypotheses

1- There will be a gradual and detrimental effect of glucose withdrawal on the tested parameters of growth and morphology.

2- Higher concentrations of ketones will increasingly rescue the effects of glucose deprivation.

3- The presence of culture components such as pyruvate and glutamine, as well as the drug metformin may interfere to reduce the maximal cellular response to ketones.
3 Materials & Methods

3.1 Cell Culture

The SH-SY5Y human NB cells were recovered from -80°C and cultured in a T75 flask with DMEM (Sigma, UK), supplemented at baseline with 10% foetal bovine serum (FBS), 5 mM glucose, 2 mM L-glutamine, 1mM sodium pyruvate and 100 U/ml penicillin/streptomycin at 37°C in a 5% CO₂ incubator (inCu safe, MCO-5AC). Sub-culturing or experimental seeding were carried out when the confluency of cells reached approximately 70%. Trypsin (3 ml) was used in this process to detach the adherent cells and allow a proportion to be sub-cultured in fresh medium. For the experiments, a 1 ml suspension of centrifuged cells was created to allow counting using a haemocytometer, and the necessary volume of the cell solution was subsequently calculated for cells to be cultured at a density of 5 x 10³ cells per well. The calculated seeding density of NE-4C cells was 2 x 10³ cells per well. Cells were seeded in poly-L-lysine coated 96-well plates with the freshly prepared medium compositions summarised in Table 3.1 below. All procedures were carried out using aseptic technique in a sterile laminar flow hood (Telstar Bio IIA Class II cabinet) to reduce the risk of contamination. This involved handwashing with anti-bacterial soap, using nitrile-free gloves disinfected with 70% ethanol, wearing cuffed laboratory coats, and disinfecting materials and reagents before use. The cabinet is emptied, cleaned and disinfected after use and ultraviolet light switched on for 30 minutes for sterilisation.

Table 3.1 A summary of the utilised media groups and their corresponding compositions

<table>
<thead>
<tr>
<th>Medium Group</th>
<th>Composition: (Baseline of DMEM and 10% FBS) +/- βOHB (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG</td>
<td>Glucose (5 mM), Sodium Pyruvate (1 mM), L-Glutamine (2 mM)</td>
</tr>
<tr>
<td>LG</td>
<td>Glucose (1 mM), Sodium Pyruvate (1 mM), L-Glutamine (2 mM)</td>
</tr>
<tr>
<td>GF</td>
<td>Sodium Pyruvate (1 mM), L-Glutamine (2 mM)</td>
</tr>
<tr>
<td>GF-P</td>
<td>L-Glutamine (2 mM)</td>
</tr>
<tr>
<td>GF-L</td>
<td>Sodium Pyruvate (1 mM),</td>
</tr>
<tr>
<td>GF-PL</td>
<td>-</td>
</tr>
<tr>
<td>SC</td>
<td>Glucose (5 mM) +/- (Metformin 1 mM)</td>
</tr>
</tbody>
</table>
3.2 Experimental Design

In order to assess the effects of glucose deprivation in the first experiments, cells were grown in regular glucose (5 mM) (RG), low glucose (1 mM) (LG), glucose free (0 mM) (GF) conditions. The effects of ketone supplementation were initially investigated in the second set of experiments with the addition of 1, 5, 10, and 50 mM concentrations of βOHB to the GF medium. The subsequent third set of experiments probing the effect of media composition on the response to ketone bodies employed the RG, LG, GF groups, followed by the removal of pyruvate (GF-P), L-glutamine (GF-L) or both (GF-PL), resulting in six medium groups of sequentially deprived fuel sources, paralleled with another six (10 mM) βOHB-containing equivalents, as shown in Table 3.2. In the final experiment, a suggested control (SC) medium is tested with and without 10 mM of βOHB. The SC medium composition was informed by the results of the preceding experiments, with the aim of isolating and maximising the effect of βOHB while maintaining resemblance to physiological conditions. As such, it is suggested as an optimised control medium for future investigations of βOHB in vitro. The ingredients of each media formulation are summarised in Table 3.1 above. In order to fulfil a $2^2$ full factorial design eliciting the potential interaction between βOHB and metformin, each of the previous two groups also received 1 mM of Metformin (M). The experimental media conditions are summarised in Table 3.2 below.

**Table 3.2 A summary of the media groups in each experiment.**

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG</td>
<td>GF</td>
<td>βOHB</td>
<td>-βOHB</td>
</tr>
<tr>
<td>LG</td>
<td>+ 1 mM βOHB</td>
<td>βOHB</td>
<td>+βOHB</td>
</tr>
<tr>
<td>GF</td>
<td>+ 5 mM βOHB</td>
<td>βOHB</td>
<td>+βOHB</td>
</tr>
<tr>
<td></td>
<td>+ 10 mM βOHB</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 50 mM βOHB</td>
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</table>
3.3 Cell Viability

Thiazolyl Blue Tetrazolium Bromide (MTT) (ThermoFisher, ROI) assays were performed as an indicator of metabolic activity and cell viability. MTT was added to cells in each well at a concentration of 0.5 mg/ml in the culture medium and incubated for 4 hr. The culture medium was removed, and the cells were lysed in DMSO and repeatedly aspirated to solubilise the precipitated formazan crystals. Colorimetric absorbance was measured at a wavelength 562 nm using an Epoch microplate reader.

3.4 Cell Staining & Immunocytochemistry

Cultures were fixed in 4% paraformaldehyde in Phosphate-Buffered Saline (PBS) for 15 min at room temperature (RT). Cells were permeabilized in 0.05% triton X-100 (PBS-T in in 1X PBS) and counterstained for 1 hour (hr) at RT with 4’-6-diamidino-2-phenylindole (DAPI) 1:5000 to visualise the nuclei, and actin for other cellular morphology parameters (ActinRed ThermoFisher, RoI) (2 drops per mL) in 0.05% triton X-100 (PBS-T in in 1X PBS). The actin staining solution comprises of phalloidin, an actin-binding peptide conjugated to the fluorescent dye TRITC. Following this, cells underwent 3 x 5 min PBS washes. Other labelling in accordance with manufacturer’s protocols involved Bromodeoxyuridine (BrdU) (Biolegend, CA) to assess proliferative activity, and FITC Annexin V, Ethidium Homodimer III as part of the Apoptosis and Necrosis Quantification Kit (Biotium, UK) investigating the mode of cellular death. A detailed description of the fluorescent imaging-based parameters and their method of quantification in this study is shown in Table 3.3 below.

3.5 Imaging and Analysis

Cells were imaged using an Olympus IX81 motorized epifluorescent microscope utilizing Cellsens Dimension software (version 1.12). ImageJ Fiji (Schindelin et al., 2012) was subsequently used for processing and analysis. In each experimental run, measurements of cell parameters were obtained in triplicates from three wells or three images representing fields of view. The average of these triplicates was then taken as a single datapoint. The following independent repetitions of these experimental runs to
obtain other datapoints constituted the $n$ number provided in the figure descriptions. There were at least three repetitions of each experiment, and the degrees of freedom in the statistical tests we carried out was therefore based on this number. Data are presented in charts as the mean of these datapoints $\pm$ the standard error of the mean (SEM). Datasets of individual measurements were normally distributed and there were no violations of parametric assumptions. Statistical analysis and graphs were generated using GraphPad Prism 8 (GraphPad Software, CA USA) or Minitab 19 (Minitab LLC, PA USA). Statistical differences in the data were analysed using one-way or two-way ANOVA as appropriate with a post hoc Bonferroni test to reveal the differences between specific groups. $P \leq 0.05$ was considered to be statistically significant.
Table 3.3 The image-based assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Method</th>
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<tbody>
<tr>
<td>Cell density</td>
<td>Whole-well images were taken of the DAPI counterstained nuclei and density was measured as the percentage of the well covered by the nuclei (Detailed in section 3.6 below)</td>
</tr>
<tr>
<td>Cell proliferation</td>
<td>As above for cell density in independent plates cultured over four timepoints: 48, 72, 96 and 120 hr to obtain a time-pattern.</td>
</tr>
<tr>
<td>Bromodeoxyuridine (BrdU) assay</td>
<td>BrdU as an indicator of proliferating cells was added to the cultured plates for 4 hr prior to fixation and labelling. Cells were washed in PBS and non-specific binding was prevented by incubation in normal goat serum for 40 min. Primary mouse anti-BrdU IgG antibody (Sigma, UK) was then added to the cells for 2 hr before incubating in the FITC secondary (a goat anti-mouse IgG) overnight at 4°C. Proliferative activity here was taken as the percentage of cells expressing green fluorescence.</td>
</tr>
<tr>
<td>Process length</td>
<td>Neuronal processes of the Actin labelled cytoskeleton of cells were manually traced using ImageJ and the average length of processes in an image was obtained.</td>
</tr>
<tr>
<td>Cell size</td>
<td>The total area covered by Actin fluorescence measured using the Analyse Particles function in ImageJ, divided by the number of cells in an Image, to obtain the average size.</td>
</tr>
<tr>
<td>Nuclear size</td>
<td>The average size of the DAPI stained nuclei in a field of view was measured with ImageJ’s Analyse Particles function following binary conversion and watershedding to separate adjacent nuclei with a line.</td>
</tr>
<tr>
<td>Apoptosis/Necrosis assay</td>
<td>This was in accordance with manufacturer’s protocol of the Apoptosis and Necrosis Quantification Kit (Biotium, UK). The staining solution comprised of the Annexin V binding the apoptosis marker phosphatidylserine, and the associated dye FITC, which emits green fluorescence indicating apoptotic cells. Additionally, Ethidium Homodimer III is an intercalating molecule binding to DNA, which emits red fluorescence on ultraviolet exposure indicating necrotic cells. The two parameters were quantified as a percentage of the total number of cells in an image.</td>
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</table>
3.6 A novel method for measuring cell density

For the image-based variables, three fields of view imaged systematically for each well were considered sufficient for the quantification of most parameters such as the process length, cell size, nuclear size, and the apoptosis/necrosis assay, as there was no cause to assume that these parameters might differ if images were taken elsewhere in the well. However, this approach posed several problems for the quantification of the cell density: 1) Cells appeared to aggregate where they become inconsistently dense in certain areas and sparse in other areas in a single well. 2) Depending on the experimental conditions, some fields of view contained hundreds of cells which had to be manually and inefficiently counted. 3) As such, this process was subject to substantial human error, particularly in images of high counts. 4) Later technical problems were observed such as the presence of pipette marks on the surface of the well meant a complete absence of cells in some corners while a high density of cells was found in other areas. 5) The task of randomising the fields of view was not straightforward or sufficient in addressing many of the problems.

Therefore, it was deemed that this approach was unlikely to be adequate in accurately characterising the cell density. Instead, along with addressing some of the technical issues such as the pipette marks, a new method was developed to measure the cell density of the entire well in a manner that avoids image sampling and its associated issues. This was partly based on a method by (Guzmán et al., 2014) for quantifying colony formation in 6-24 well plates stained with crystal violet, where image acquisition of large wells was relatively direct. However, due to the smaller wells and the fluorescent labelling, an image of high resolution, contrast and magnification had to be taken for each whole-well, with the surface coverage of DAPI labelled nuclei as the variable of interest. To that purpose, the Well Navigator tool within the CellSens microscopy software was used to calibrate the edges of the plate and demarcate circles around the wells of interest. The Multiple Image Alignment (MIA) function was used so that the motorised microscope can automatically capture 30-40 sequential images taken at 10x to be stitched into a single large image representing the entire well. One disadvantage for this method is the large image sizes of up to 0.5 Gigabytes, which required substantial storage and computer processing requirements to analyse. This
method also relies on the assumption of a constant nuclear size, which appears satisfied in our analysis shown in (Figure 4.3).

In order to facilitate an automated, streamlined analysis which avoids human input, a Macro code was written encompassing the necessary steps utilised in ImageJ for each stack of images. These steps involved blocking the background, converting the image into a binary mode, applying the Watershed function to distinguish the separate nuclei, and finally using the Analyse Particles function to obtain a measurement for the total area covered by the DAPI stained nuclei against the black background, which is expressed as a percentage of the surface of the whole well. This process is displayed in (Figure 3.1) below.
Figure 3.1 A schematic of the process underlying the cell density quantification in whole-well images.

A The sequence of steps utilised in ImageJ in analysing the surface area of the well covered by the nuclei, shown for the entire wells in the upper panel and at 150% magnification in the lower panel. B The code of the custom macro created to automate the steps for stacks of images.
4 Results & Discussion

4.1 Chapter 1: Characterising the effects of glucose deprivation and ketone supplementation

4.1.1 Introduction

In light of the increased consumption of glucose in cancer and developing cells and the excessive glycolysis characterizing the Warburg effect, as well as the requirement of glucose deprivation in achieving a state of ketosis, we firstly sought to determine the effects of withdrawing glucose from the cell culture medium. For this purpose, three concentrations of glucose were tested: a physiological 5 mM (RG), a reduced 1 mM (LG) and a deprived 0 mM (GF), with the hypothesis that key parameters of cellular health and morphology will be negatively impacted. The GF group will then serve as a baseline for subsequent ketone supplementation.

Although limited in number, studies examining the effect of βOHB on SH-SY5Y cells specifically have shown beneficial outcomes, with an increased mitochondrial activity in a model of epilepsy (Hughes et al., 2014), reduced toxicity in a model of Parkinson’s disease (Imamura et al., 2006), and recovery of the deficit in glucose oxidation in an Alzheimer’s disease model (Findlay, Hamilton and Ashford, 2015). In a cancer model of NB however, βOHB is reported as detrimental to growth and viability (Skinner et al., 2009). Therefore, we next wished to determine if the addition of the ketone body βOHB can rescue or further exacerbate any detrimental effects of glucose deprivation in our culture conditions. Furthermore, given that one of the unique features of NB is its occasional propensity for spontaneous resolution through differentiation (Brodeur and Bagatell, 2014), we assessed the effect of ketone supplementation on morphological parameters such as process length. This may increase if the action of ketones in their proposed inhibition of tumour growth is through inducing differentiation into a benign subtype, a feature of which is longer neuronal processes (Kwiatkowski et al., 1998).
4.1.2 Results

Glucose deprivation is detrimental to SH-SY5Y cell density and viability

An MTT assay at 96 hr showed a reduction in cell viability when the culture medium was glucose free (GF), compared to regular glucose (RG) and low glucose (LG) conditions (Figure 4.1 A). To determine whether the decrease in viability could be attributed to a decline in cell numbers or health, we examined cell size/area (Figure 4.1 B, E), process length (Figure 4.1 C, E), cell death (Figure 4.1 D, E), and cell density (Figure 4.1 E, F). There were no significant differences in morphology characteristics between GF, LG and RG groups (Figure 4.1 B, C); however, a significant reduction was observed in cell density in LG and GF conditions when compared to the RG condition (Figure 4.1 F). Next, the modes of cell death in response to glucose deprivation were investigated. There were no significant differences in the percentages of apoptotic and necrotic cell death in RG, LG and GF conditions at 96 hr (Figure 4.1 D, E),
Figure 4.1 Growth and viability of SH-SY5Y cells in GF, LG and RG conditions at 96 hr.

A MTT viability assay of LG and GF conditions expressed as a percentage of RG (F=21.6). B Cell area in RG, LG and GF conditions (F=0.61). C Process length of cells in RG, LG and GF conditions (F=0.79). D Percentage of apoptotic and necrotic cells in RG, LG and GF conditions (F=0.11, 0.84). E Upper panel: Actin (red) labelled cytoskeleton and DAPI (blue) labelled nuclei of cells in RG, LG and GF conditions. Middle panel: EthD-III (red) and Annexin V-FITC (green) assessing necrosis and apoptosis respectively. Lower panel: DAPI labelled nuclei showing cell density in GF, LG and RG conditions. F Percentage cell density in RG, LG and GF conditions (F=11.6). (n = 3 independently for each of the experiments) Scale bar = 20 µms. One-way ANOVA, *P<0.05. Data are represented as mean ± SEM.
βOHB does not impact the viability or proliferation of glucose deprived cells

We next wished to determine if the addition of the βOHB reversed or exacerbated the effects of glucose deprivation. The presence of 1 mM, 5 mM, 10 mM 25 mM or 50 mM βOHB had no effect on the cell viability of glucose deprived (GF) SH-SY5Y cells (Figure 4.2 A). Cell growth as determined by measuring cell density was not significantly increased when compared to GF conditions (Figure 4.2 B). A uniform rate of proliferation in the GF and βOHB-containing conditions was confirmed with consistent BrdU expression (Figure 4.2 C, D).

Figure 4.2 Increasing concentrations of βOHB do not affect the growth of glucose deprived SH-SY5Y cells

A MTT viability assay of cell growth in RG, GF and βOHB conditions expressed as a percentage of RG (F=0.51). B Cell density number / mm2 in RG, LG and βOHB conditions (F=1.7) C Percentage of in RG, GF and βOHB cells expressing BrdU (F=0.35). D Representative images of BrdU (green) labelled proliferative cells and DAPI (blue) labelled nuclei in RG, GF and βOHB conditions. Scale bar = 40 µm. (n = 3 independently for each of the experiments) One-way ANOVA comparing GF and βOHB. RG illustrated for visual comparison. No significant p-values observed. Data represented as mean±SEM.
**βOHB does not affect the morphology of glucose deprived cells**

The addition of up to 50 mM of βOHB to the culture medium did not affect any of the morphological parameters investigated in this experiment. Cell size, nuclear size and neuronal process length remained unchanged when cells were cultured in βOHB containing glucose free conditions (Figure 4.3 A, B, C, D)

![Images of cell size, nuclear size, process length, and DAPI/Actin](images)

**Figure 4.3 Increasing concentrations of βOHB do not affect the morphology of glucose deprived SH-SY5Y cells**

A Cell size in RG, GF and βOHB conditions (F=0.61). B Nuclear size of cells in RG, GF and βOHB conditions (F=0.12). C Process length of cells in RG, GF and βOHB expressed as a percentage of RG (F=0.66). D Actin (red) labelled cytoskeleton and DAPI (blue) labelled nuclei of cells in RG, GF and βOHB conditions. Scale bar = 20 µm. (n = 3 independently for each of the experiments) One-way ANOVA comparing GF and βOHB. RG illustrated for visual comparison. No significant p-values observed. Data represented as mean±SEM.
4.1.3 Discussion

With regards to the impact of glucose concentrations on cell death, the lack of effect we observed contradicts a previous study which showed that glucose deprivation induces increased apoptosis in SH-SY5Y cells over the first 24 hr (Lamichhane et al., 2017). The two findings may be reconciliated by an initial period of increased cell death followed by an acclimatisation to other fuels. Our subsequent measurements of cell density at 48, 72, 96 and 120 hr in Chapter 2 (Figure 4.4) are a further indication that the unchanged morphological features and death rates along with the lower cell density in response to glucose deprivation are perhaps due to changes primarily on the proliferative activity. This correlates with findings showing that cells reduce their proliferation rates in response to glucose withdrawal in favour of maintaining the health of existing cells, which is facilitated in some cases by the presence and consumption of glutamine (Le et al., 2012; Kaplon, van Dam and Peeper, 2015; Bajpai et al., 2016). Given the diminishing density and viability with the reductions in glucose concentration, our results are also consistent with the established role of the Warburg effect and a reliance on glycolysis in maintaining the biosynthetic requirements of a high proliferative activity (Heiden, Cantley and Thompson, 2009).

The inertia of various concentrations of βOHB on the tested parameters conforms with previous research on another NB cell line (SK-N-AS) which had showed that ketone supplementation was ineffective in reversing cell viability imposed by glucose deprivation (Skinner et al., 2009). However, we fall short of concluding at this stage that NB cells lack the ability to use ketone bodies as an energy source, or that ketones inhibit their viability. The comparison in MTT viability which contributed to these conclusions in the aforementioned study was made against a glucose-supplemented control, indicating that the perceived effects of the two independent factor levels, namely glucose deprivation and ketone supplementation could not be adequately disentangled. When compared to the glucose deprived conditions instead, βOHB did not have any effect. Therefore, these data sets are potentially more easily interpreted as the inability of βOHB to metabolically compensate the absence of glucose in these conditions, as opposed to an inherent ability to inhibit the growth of cells.
Of importance in these results is the non-significance observed in any of the parameters when βOHB concentrations of up to 50 mM are supplemented to the cells, as shown by the failure to either ‘rescue’ or further exacerbate the effects of glucose withdrawal. In parallel to our null findings, it is notable that despite the heterogeneous energy requirements and utilisation of non-oxidative and oxidative energy-producing pathways observed in various breast cancer cell lines (Martin and McGee, 2019), a separate study by (Bartmann et al., 2018) showed a uniform lack of proliferative response to ketone bodies in seven genetically diverse breast cancer cell lines with varying ketolytic enzyme expressions. Taken together, these results may imply a constant and likely extrinsic factor which could be preventing the considerable variety of cells in the same investigation from showing any response. Notably, while efforts were made towards a physiologically determined media composition, the aforementioned study utilised a 1mM concentration of pyruvate which is ten-fold higher than the physiologic concentration.
4.2 Chapter 2: The interaction of the βOHB response with other substrates in culture

4.2.1 Introduction

The inertia we observed with all ketone concentrations represents the midpoint in the wide spectrum of findings in the literature, which range between detrimental and beneficial effects. Along with the continued viability and considerable density of cells after 96 hr of complete glucose deprivation, this may suggest the contribution of an extrinsic methodological factor which might interfere with the response to βOHB. This also comes with the previously highlighted role of the culture medium composition, in which the addition or omission of the various commonly supplemented metabolic substrates has been shown to alter experimental outcomes.

Since the extent of nutrient deprivation has been shown to be a determinant of ketolytic enzyme activity, the excess variety of fuel sources in the culture medium presented the potential candidates which could preferentially be utilised as an alternative source of energy. This instigated the rationale for the following investigation: The baseline medium described was sequentially adjusted to lower glucose concentrations as in the first experiment, followed by withdrawal of pyruvate, L-glutamine, or both. The resultant six groups of media composition (RG, LG, GF, GF-P, GF-L, GF-P/L) as schematised in (Figure 4.4 A) were paralleled with the same six groups with the addition of 10mM βOHB. We tested this stepwise deprivation of fuel sources, firstly to find out if any of these are dispensable for survival, and to see whether cells can utilise ketones if starved enough. Since there were no differences observed in the various concentrations of ketones previously tested, 10 mM of βOHB was used in these experiments as a midpoint taking into account the range found in various physiological and pathological states. This was also to ensure that its availability in the medium does not become the rate limiting factor over the observed timepoints. βOHB in circulation can reach up to 25 mM in poorly controlled diabetes, and up to 7.5 mM in prolonged fasting, while it remains below 0.5 mM in normal circumstances (Kanikarla-Marie and Jain, 2016).
Subsequently, a suggested control (SC) for testing the response to βOHB is proposed with the aim of eliminating any confounding interactions, on balance with several considerations which must be taken into account for maintaining physiological resemblance of the culture medium. To test this proposition, we suggest a control medium for future investigations of βOHB, from which pyruvate was firstly removed due to its potentially masking effect and supraphysiological presence. Secondly, a physiological 5 mM concentration of glucose was maintained due to its presence in vivo at approximately in this concentration even during ketosis. Lastly, given our observation that cells continue to survive even in the complete absence of glucose, its presence at a 5 mM concentration could alone be sufficient in maintaining health. Hence, we removed glutamine which could have an effect on the response to βOHB according to previous studies (Huang et al., 2016). We therefore tested the effect of βOHB on the SC group of 5 mM glucose containing medium without glutamine or pyruvate. These conditions were also tested with the MTT viability of NE-4C cells.
4.2.2 Results

The proliferative effect of βOHB on SH-SY5Y cells is influenced by culture substrates

There is a gradual reduction in both density and viability with successive media groups as fuel substrates are sequentially removed (Figure 4.4 A, B). The cell viability as measured at 96 hr revealed that βOHB promotes viability with two instances in a conditional manner: 1) when glucose is reduced (LG), 2) when pyruvate is removed (GF-P) (Figure 4.4 B). The cell density was measured for each of the various media groups at 24, 72, 96 and 120 hr timepoints to get an indication of the proliferation rates and patterns. Our findings confirm that by 120 hr, the only significant differences observed are in the two conditions mentioned previously (Figure 4.4 C). Further, differences between control and ketone in the significant groups appear to emerge after 96 hr and are greatest at 120 hr in vitro. Finally, cells continue to show evidence of proliferation and survival unless all three fuels (glucose, pyruvate, l-glutamine) are removed from the medium.
Figure 4.4 βOHB differentially rescues the viability and proliferation of cells in pyruvate and glucose depleted conditions.

A Representative images of DAPI labelled nuclei in whole-wells showing the effect of βOHB on cell density at 96 hr and their schematised compositions. B MTT viability of cells in glucose containing and GF conditions supplemented with βOHB at 96 hr. βOHB effect (F=30.4), Medium effect (F=28.4), βOHB*Medium Interaction (F=5.7). C The temporal changes in cell density in RG, LG, GF, GF-P and GF-L at 24, 72, 96 and 120 hr in the presence (black line) and absence (purple line) of βOHB. βOHB effect(F=20.1), Medium effect(F=60.6), βOHB*Medium Interaction(F=4.0). (n=3 for all group for the four time points independently) Two-way ANOVA, *P<0.05. Data represented as mean ± SEM.
Modifying the control medium results in a maximal response to βOHB.

The addition of βOHB results in a highly significant increase in viability in comparison with SC, not previously seen in any of the media groups, indicating a beneficial effect of βOHB in these conditions (Figure 4.5 A, B). This positive effect of βOHB on cellular viability is contrary to our initial findings on the inertia of the ketone body on SH-SY5Y growth and health.
Figure 4.5 The suggested optimization of the control medium results in a maximised response to βOHB at 96 hr.

A MTT viability expressed as a percentage of the control. B Representative whole-well images of DAPI stained nuclei in the two groups. (n = 4) Student’s t-test, **P<0.01. Data represented as mean ± SEM.
NE4C viability in response to βOHB is affected by the medium composition.

In an attempt to replicate the findings in a different cell line, NE-4C cells showed a gradual decline in viability to substrate deprivation, with a significant overall effect of the group variable, while a significant increase of viability in response to βOHB was observed only in the GF-P group (Figure 4.6 A). The subsequent addition of βOHB to the SC medium resulted in a highly significant increase in viability (Figure 4.6 B). Lastly in the limiting absence of formal quantification, βOHB appeared to increase the density of NE-4C cells but there was no visible change in morphology under brightfield microscopy (Figure 4.6 C).

Figure 4.6 The differential effect of βOHB on NE-4C cells

A MTT viability of NE-4C cells in glucose containing and GF conditions supplemented with βOHB at 96 hr. B The effect of βOHB on cell viability of the SC group as a percentage of the control. C Representative brightfield images of NE-4C cells in the presence and absence of βOHB, Scale bar = 50 µm. (n = 4) Two-way ANOVA and student’s T-test for A and B respectively, *P<0.05, **P<0.01. Data represented as mean ± SEM.
4.2.3 Discussion

These findings shed light on two potential determinants of the reported variations on the effect of βOHB on the proliferation of cells. Firstly, with regards to time in culture it appears that it takes at least 96 hr for cells to begin to show a divergent response in the significant groups. Secondly, the presence of pyruvate and concentration of glucose in the culture medium appear to influence the perceived effect of the ketone. This may be relevant to the findings of Bartmann et al. (2018) discussed previously, which showed a lack of βOHB effect in the presence of pyruvate across seven different cell lines. As such, it may be helpful to view results of this nature in light of the chosen culture conditions. Further, this result may provide a route for explaining the variations of the effect of ketosis on the developing brain and foetus in terms of interactions with other circulating metabolites during gestation or in the postnatal period.

As opposed to SH-SY5Y cells in which both the LG and GF-P groups revealed an increase in viability with βOHB supplementation, NE-4C cells responded only to the removal of pyruvate (GF-P). Nevertheless, in both cell types βOHB significantly improved the viability when added to the SC group. This difference in response between cell types may either be reflective of the reduced power in the NE-4C experiment compared to SH-SY5Y (n=3 and n=6 respectively), or due to the contribution of differences in the intrinsic metabolic phenotype of the cells. For example, variations in the cells’ baseline glucose demand or reliance on aerobic glycolysis may modulate the metabolism of βOHB. No studies thus far have employed both SH-SY5Y and NE-4C cell lines to enable comparisons of their metabolic characteristics. However, if the proliferation rate is taken as a proxy for the extent of the Warburg effect and its role in supporting proliferative requirements (Heiden, Cantley and Thompson, 2009), differences in glycolytic capacities might be inferred from the doubling times of NE-4C and SH-SY5Y cells. These are 16 hr (Jády et al., 2016) and 27 hr (Kovalevich and Langford, 2013) respectively, which may imply a greater glycolytic activity in the NE-4C cells.

Our results demonstrate that both SH-SY5Y NB and NE-4C cells have a high tolerance for nutrient deprivation and are versatile in using different fuels including βOHB to maintain survival or proliferation unless all three major fuel sources in the culture
medium (glucose, pyruvate, glutamine) are removed. Further, the increased proliferative response to βOHB was only observed in some conditions, leading us to speculate on the potential mechanisms which are summarised in the schematic in (Figure 4.7) below and considered in detail subsequently.

![Figure 4.7](image)

**Figure 4.7** A schematic of the potential mechanisms behind the interaction between βOHB and the fuel substrates of the culture medium.

Highlighted in red are some of the processes and barriers preventing βOHB metabolism. Created with BioRender.com

**The overall energy balance and the NAD+/NADH ratio.**

In viewing ketolysis as a process in which cells resist a deteriorating environment, where gradual nutrient deprivation activates dormant ketolytic enzymes (Huang et al., 2016), we assumed that there may be an equally gradual increasing response to ketones upon the removal of energy sources. While this was partly reflected by the responses in the LG medium and GF-P medium, for this to be the sole determinant we would expect a proportional response to each stepwise removal of fuel substrates, but this is not the
case. Instead, the complete lack of response in some of the successive groups beyond the initial rate limiting factor point to other potential explanations, such as the interplay of MCT’s, the expression of GLUT1, and the NAD+/NADH ratio. One possible mediator is the reversible process of ketolysis in which some of the enzymatic steps are in an equilibrium reliant on a relative shortage of NAD+ in comparison to NADH for forward metabolism (Puchalska and Crawford, 2017). In relation to our observation of an effect the LG group in, high extracellular glucose levels lead to increased NADH (Kelly et al., 2018) In addition, pyruvate as a metabolic substrate is a potent generator of NADH. (Zhang and Xie, 2017) Therefore, their presence might result in a net increase in NADH, and a correspondingly decreased NAD+/NADH ratio(Kelly et al., 2018), which would favour a shift in equilibrium towards preventing the oxidation of βOHB into AcAc. Further, the proliferation of cancer cells has been shown to depend less on mitochondrial respiration when cultured with excessive concentrations of pyruvate, (Gui et al., 2016), which could be an additional preventive factor in pyruvate-containing media.

The roles of glucose in influencing ketone metabolism

Glucose is further implicated in mediating the metabolism of ketone bodies through its by-products: Pyruvate as previously discussed and also lactate, the efflux of which has been observed as a factor by Whitaker-Menezes et al. (2011) in whether ketones can fuel the proliferation of cancer cells. While reducing glucose levels appears beneficial to ketone metabolism in cancer cells as also shown by (Huang et al., 2016), in order to explain the paradoxical lack of response to βOHB in the GF group compared to the LG group, a switch in the determining step may be seen to occur with the complete withdrawal of glucose, as determined by the interaction of GLUT1 and the ketolytic enzyme 3-oxoacid CoA-transferase 1 (OXCT1). Overexpression of GLUT1 which becomes upregulated due to glucose deprivation (Boado and Pardridge, 2002) has been shown to inhibit OXCT1 gene expression and downregulates ketones terminal oxidation (Yan et al., 2009). As such, despite the benefit in reducing glucose, some baseline level of glucose may be necessary to prevent it from becoming a rate limiting factor.
MCT substrate competition as a basis for the differing responses to βOHB

MCT subtypes 1-4 are a group of passive bi-directional transporters allowing βOHB across membranes (Halestrap and Wilson, 2012). They are also responsible for the passage of other substrates such as pyruvate and lactate to which they have varying affinities (Pérez-Escuredo, Vincent F. Van Hée, et al., 2016). As a common denominator behind the observed significances in the LG and GF-P groups, there is some evidence to suggest a degree of competition at the MCT level between substrates such that the entry of βOHB into the cell becomes limited, and therefore partly explaining our findings in relation to pyruvate and reduced glucose contributing to less lactate production. Other MCT1 substrates such as phenylpyruvate have been shown to result in slow rates of net transport across MCT1 acting as competitive inhibitors of the transport of other monocarboxylates (Carpenter and Halestrap, 1994; Halestrap and Wilson, 2012) Further, in examining transport of βOHB in rat brain astrocytes (Tildon, McKenna and Stevenson, 1994) showed that Another MCT substrate, alpha-ketoisocaproate had a considerable effect on the rate of βOHB transport, decreasing it by more than 90%. Phenylpyruvate, although less effective has been shown to decrease the rate of transport by about 50%, while lactate resulted in a 20% decrease (McKenna, 2012). Furthermore, one of the earliest characterizations of MCT1 and 2 examined 14C-pyruvate uptake in the presence of various unlabelled monocarboxylates showing that it can be inhibited up to 100% by various concentrations of lactate or βOHB (Lin et al., 1998). It is therefore conceivable that the levels of other MCT substrates in the cellular environment can interfere with the cellular impact of βOHB through competition at the transport level.
4.3 Chapter 3: The therapeutic potential behind the differential responses to βOHB

4.3.1 Introduction

In light of the highlighted differential response to ketone bodies in vitro, we speculated that some of the discussed elements at play may be exogenously simulated to limit the proliferative effect of ketone bodies in cells that would have otherwise benefited. We draw parallels from the biguanide metformin and its known effects on the metabolic profiles and enzymatic pathways of cancer cells. Metformin’s anticancer activity was initially recognized in epidemiological studies which showed a lower incidence and improved outcomes of cancer amongst those who were prescribed the medication (Kasznicki, Sliwinska and Drzewoski, 2014). This observation must be qualified with the nature of the cohorts in these studies involving those with Type 2 Diabetes Mellitus, who are prescribed this drug as a first line hypoglycaemic agent. Incidentally, those with diabetes are at an increased risk of several malignancies, and this risk is ameliorated with the use of metformin in comparison with other hypoglycaemic drugs (Smith and Gale, 2010). In the non-diabetic population however, the anti-cancer association is controversial with limited evidence (Chen et al., 2020). In pre-clinical studies, while its effect as an anti-proliferative agent has been established in vitro including on SH-SY5Y cells (Costa et al., 2014), the concentrations used are often up to 100 times higher than the therapeutic concentrations which frequently fail to illicit any effect, adding to the uncertainties behind the potential mechanism of this drug (Erices et al., 2013). A possible reason for this discrepancy is that metformin’s activity at low levels as an activator of the AMPK enzyme, which serves to stimulate increased glucose uptake and ketogenesis, becomes superseded by a complete inhibition of mitochondrial complex 1 when given in high concentrations as seen in vitro (He and Wondisford, 2015).

With this background, metformin’s primary indication as an anti-diabetic drug mandates a consideration of the metabolic characteristics of the group in which it operates and hence it’s interplay with the pathological changes observed in diabetes.
Diabetes is a state of relative glucose insufficiency with a resultant increase in circulating ketones (Kanikarla-Marie and Jain, 2016), argued to be a compensatory energy mechanism (Kruljac et al., 2016). This is facilitated by an increase in the hepatic synthesis of ketone bodies as a consequence of the relative insulin deficiency, sometimes resulting in diabetic ketoacidosis, a lethal complication (Laffel, 1999).

The biochemical profile characteristic of diabetes therefore has parallels with the changes induced through a ketogenic diet, namely the reduced glucose utilisation and the high circulating ketone levels. At a molecular level, some of the potential barriers to ketone metabolism discussed previously are also known effects of metformin on cancer cells, namely: 1) the reduced expression of OXCT-1, a rate limiting ketolytic enzyme (Udhane et al., 2017), and 2) the increased aerobic glycolysis and lactate efflux (Andrzejewski et al., 2014). These factors may combine to block the action of ketone bodies. As such, metformin’s observed anticancer efficacy may be complementary to the metabolic changes associated with the ketosis that occurs in patients with diabetes, perhaps due to limiting the metabolic versatility of cancer cells, and in particular the ability to utilize ketone bodies towards a higher proliferative capacity. In turn, ketosis achieved through calorie-restricted ketogenic diets may also mirror those conditions, providing some rationale for combined metformin and dietary therapy in cancer. This forms the basis for the hypothesis that even at a low concentration (1 mM) which is closer to that in therapeutic doses, metformin will interact to limit the gains in viability made by the addition of βOHB to our suggested control. To illicit the presence of any effect, a full factorial experimental design was utilized with two factor levels for each of the independent variables.
4.3.2 Results

**Metformin inhibits the viability response to βOHB**

The main effects plot (Figure 4.8 A) shows an overall positive effect of βOHB and an overall negative effect of metformin on the viability of SH-SY5Y cells. However, the subsequent interaction plot (Figure 4.8 B) shows that the negative effect of metformin appears to be restricted to the presence of βOHB, while a null effect can be seen in its absence. The main effects of the two variables as well as their interaction are confirmed as significant at P < 0.05, supporting our hypothesis that the addition of metformin may limit the positive effect of βOHB on cell viability. Further, inspection of these cells (Figure 4.8 D) shows corresponding variations in the appearance of cell density but no clear morphological changes, which might imply an effect primarily on the proliferative activity. However, these parameters require formal quantification and testing.
Figure 4.8 Metformin modulates the effect of βOHB on SH-SY5Y cell viability

A Main effects plot showing the mean response in viability for metformin and βOHB. 
B Interaction plot showing the effect of metformin in the presence and absence of βOHB 
C Bar chart of the differences in viability in each of the experimental conditions. D Representative brightfield images of SH-SY5Y cells in their respective conditions. Two-way ANOVA, *P<0.05. βOHB effect (F=32.93), Metformin effect (F=29.26), βOHB*Metformin Interaction (F=17.30) Data represented as mean±SEM.
4.3.3 Discussion

The above is a preliminary attempt at a therapeutic exploitation of the differential response to ketone bodies using a pharmacological agent which simulates many of the proposed conditions leading to the inertia of βOHB. While not directly addressing the interaction of ketones and metformin, a number of studies have arrived at a similar result. An in vivo study of mice fed with the ketogenic diet with the goal of reducing blood glucose levels found an increased efficacy of metformin in inhibiting tumour growth (Zhuang et al., 2014). Similarly, a study which combined caloric restriction with metformin found a lower incidence of metastases and reduced tumour growth amongst cancer patients (Dilman et al., 1988; Berstein, 2010) We propose that these effects could be due to metformin’s ability to prevent cancer cells from using βOHB as an alternative fuel.

This proposal must meet a few assumptions for its mechanistic feasibility as a therapeutic option. Firstly, the metabolic effects of metformin must be selective to cancer cells, such that healthy cells can continue to metabolise βOHB in maintaining viability. Evidence for this can be derived from PET studies, where the administration of metformin results in a selective increase of FDG uptake in the tumour mass (Lord et al., 2018), such that the increase reliance on glucose and the Warburg effect may be limited to the transformed neoplastic cells. Cultures of primary cells may shed further light on this issue, as we suspect the effect of βOHB on non-neoplastic cells to be contrastingly unchanged with the addition of metformin. Secondly, the issue of safety becomes paramount in the non-diabetic population when considering metformin’s glucose lowering action. However, studies have shown that it does not result in hypoglycaemia when circulating glucose levels are within the normal range (Goodwin et al., 2008; Berstein, 2010). In addition, metformin is also used in conditions beyond diabetes with appropriate safety, such as in polycystic kidney disease and antipsychotic-induced wight gain (Stoica et al., 2020). It’s potential efficacy against cancer in non-diabetics has also been demonstrated (Lerner et al., 2017). However, the combined approach of metformin and ketosis in non-diabetics, which must be induced through fasting or the ketogenic diet, is yet to be assessed in human observations.
5 General Discussion & Conclusions

5.1 Main findings

In line with the first hypothesis presented in this study, the data acquired has expectedly shown that glucose withdrawal is indeed detrimental to SH-SY5Y proliferation and viability. Next, in trying to ascertain the effect of βOHB on the glucose deprived cells, we initially rejected the second hypothesis of a rescuing effect of the ketone. However, the persisting growth of cells without glucose and their irresponsiveness to all concentrations of βOHB led us to speculate on the role of other major substrates in culture such as pyruvate and glutamine. As likely sources of continued nourishment to the glucose-deprived cells and an unaddressed variable in the conflicting literature, the presence or absence of these presented a potential explanation for the reports of differential responses to ketone bodies. With this hypothesis, we showed that the presence of pyruvate and the concentration of glucose can mask the growth promoting effects of βOHB. In addition, what we perceived to be a modifiable inhibitory effect could also be induced using the drug metformin, thereby confirming the final hypothesis.

5.2 Limitations

Our findings are limited with regards to generalized applicability due to the data being mostly on the SH-SY5Y cell line. The effects on the NE-4C cells were inconsistent in the low-glucose conditions, which may be a result of different metabolic characteristics or the reduced power in statistical tests as previously discussed. As such, employing various cell lines of different origins with more repetitions will give greater confidence to our conclusions for in vitro cancer models. In addition, we had no means of assessing the potential explanations we have proposed by measuring direct substrate concentrations. Other limitations include the potential human error in manually measuring some of the morphological characteristics such as the neuronal process length, particularly in the more confluent groups.
5.3 Conclusions & Future Directions

Our results show that given the right conditions, βOHB is able to exert a positive influence on the proliferative capacity of SH-SY5Y cells. As also seen, an unsuitable environment will prevent this possibility. Interestingly, it is in the latter negative observation that the therapeutic potential for ketogenic diets arises, since the proposed utility for ketones in cancer is their lack of metabolism. The adjustable nature of these responses offers a window not only into the mechanisms at play, but an opportunity to manipulate whether cancer cells respond to ketone bodies or not by emulating the proposed inhibitory mechanisms by an exogenous agent or through the specific nature of the neoplasm. However, the largely indirect nature of these findings requires further investigations to illuminate a clear mechanistic understanding.

Future and ongoing studies will firstly attempt to replicate these findings in multiple cell lines, and aim to establish correlations between glucose input, lactate output and βOHB utilization with direct measurement of concentrations, as well as the differential effect of metformin on these parameters. Another route to address the potential mechanisms may involve utilising MCT or GLUT1 inhibitors and assessing changes to the ketone effect in the various media groups. The use of gene expression data for correlations of the involved enzymes and transporters may also be of interest, although this is limited by the reversible and bidirectional nature of the processes described. Hence, these must be complemented with the direct measurements in order to disentangle whether the raised expression profiles are due to an increase in one process or its opposite. The use of animal models of neuroblastoma will provide a particularly valuable insight into our proposal of combining ketogenic dietary therapies with metformin as a therapeutic option.

These findings may have general applicability to in vitro models of βOHB metabolism and may explain the wide variety of findings in the literature according to their respective methodologies. Proposed in this study is a general paradigm which presents the ability to metabolise ketone bodies not as a purely intrinsic property of the cells in question, but as a dynamic function of an interplay between the extrinsic elements as well as the cell’s own metabolic phenotype. Thus, the results of previous and future in
vitro investigation into the proliferative effects of ketone bodies, given the widely varied methodologies will naturally fall on a point in a spectrum which has been predetermined by a choice of the culture conditions. By extension, it is prudent for any in vitro study of the metabolic effects of ketones to consider the contributions of their culture conditions and supplements as suggested by others and as practically shown in this study. We therefore suggest a standard control which should be used if possible while being aware of the background processes at play, with the aim of contributing to a consensus of replicable findings with regard to ketone body metabolism and its therapeutic applications.
6 References


8) Bartmann, C. *et al.* (2018) ‘Beta-hydroxybutyrate (3-OHB) can influence the energetic phenotype of breast cancer cells, but does not impact their


10.1038/nmeth.2019.


106) Yan, J. *et al.* (2009) ‘Increased glucose uptake and oxidation in mouse hearts prevent high fatty acid oxidation but cause cardiac dysfunction in diet-induced


## Appendix

### Table 7.1 Cell culture materials

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<th>Material</th>
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### Table 7.2 Cell fixation and immunocytochemistry reagents

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### Table 7.3 Cell viability assay materials

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<td>Dimethyl Sulfoxide (DMSO)</td>
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