Inhibition of monocarboxylate transporter 1 by AZD3965 as a novel therapeutic approach for the treatment of diffuse large B-cell lymphoma and Burkitt lymphoma


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Inhibition of monocarboxyrate transporter 1 by AZD3965 as a novel therapeutic approach for the treatment of diffuse large B-cell lymphoma and Burkitt lymphoma

Authors

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Abstract

Inhibition of monocarboxylate transporter 1 has been proposed as a therapeutic approach to perturb lactate shuttling in tumor cells that lack monocarboxylate transporter 4. We examined the monocarboxylate transporter 1 inhibitor AZD3965, currently in phase I clinical studies, as a potential therapy for diffuse large B-cell lymphoma and Burkitt lymphoma. Whilst extensive monocarboxylate transporter 1 protein was found in 120 diffuse large B-cell lymphoma and 10 Burkitt lymphoma patient tumors, monocarboxylate transporter 4 protein expression was undetectable in 73% of the diffuse large B-cell lymphoma samples and undetectable or negligible in each Burkitt lymphoma sample. AZD3965 treatment led to a rapid accumulation of intracellular lactate in a panel of lymphoma cell lines with low monocarboxylate transporter 4 protein expression and potently inhibited their proliferation. Metabolic changes induced by AZD3965 in lymphoma cells were consistent with a feedback inhibition on glycolysis. A profound cytostatic response was also observed in vivo: daily oral AZD3965 treatment for 24 days, inhibited CA46 Burkitt lymphoma growth by 99%. Continuous exposure of CA46 cells to AZD3965 for 7 weeks in vitro resulted in a greater dependency upon oxidative phosphorylation. Combining AZD3965 with an inhibitor of mitochondrial Complex I (central to oxidative phosphorylation) induced significant lymphoma cell death in vitro and reduced CA46 disease burden in vivo. These data support clinical examination of AZD3965 in Burkitt lymphoma and diffuse large B-cell lymphoma patients with low tumor monocarboxylate transporter 4 expression and highlight the potential of combination strategies to optimally target the metabolic phenotype of tumors.
Introduction

The increased reliance on glycolytic metabolism under aerobic conditions, termed the “Warburg effect”, is adopted by many tumor types and characterized by an increased utilization of glucose and a corresponding greater efflux of lactate.\textsuperscript{1, 2} Consequently, there has been much interest in targeting this recognized ‘hallmark of cancer’ for therapeutic benefit.\textsuperscript{3, 4} One such approach has been to interfere with lactate transport via inhibition of monocarboxylate transporter (MCT) 1.

MCT1 and MCT4 are cell membrane localized proton-coupled transporters of monocarboxylates such as lactate and pyruvate.\textsuperscript{5} MCT1 is expressed widely and possesses a comparatively high affinity for lactate, allowing it to function as an influx or efflux transporter depending upon the local lactate concentration gradient. In contrast, MCT4 predominantly fulfils an efflux transport role in highly glycolytic tissues. The function of both transporters is dependent upon an association with the transmembrane accessory protein CD147 (basigin; BSG) which ensures their correct orientation at the cell surface.\textsuperscript{6} MCT1 and MCT4 can be differentially over-expressed in cancer,\textsuperscript{7-11} and a subset of tumors express MCT1 in the absence of appreciable MCT4 protein. In such cells MCT1 inhibition can have significant consequences: preventing lactate efflux in highly glycolytic tumor types, and restricting access to lactate in more oxidative cancer types where it may be utilized as a respiratory fuel.\textsuperscript{12, 13}

AZD3965 is an orally bioavailable MCT1 inhibitor, which is currently under Phase I clinical investigation (NCT01791595).\textsuperscript{14} Recent studies have demonstrated that AZD3965 or structurally related MCT1 inhibitors can inhibit the bidirectional
transport of lactate in cancer cells which lack MCT4 protein and this may inhibit their growth.\textsuperscript{7, 12, 14}

We evaluated the metabolic and therapeutic effects of AZD3965 in aggressive forms of non-Hodgkin lymphoma (NHL), namely, diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). We demonstrate in patient samples that these diseases often have high MCT1 and undetectable or negligible MCT4 protein expression. We also show that AZD3965 can inhibit lactate efflux sufficiently in DLBCL and BL cell lines to alter cellular metabolism and exert a profound cytostatic effect on lymphoma cell growth \textit{in vitro} and \textit{in vivo}. Finally, we demonstrate that combining AZD3965 with an inhibitor of oxidative phosphorylation (OXPHOS) can induce significant tumor cell death and reduce lymphoma disease burden \textit{in vivo}.

Collectively these studies define a clear opportunity for the use of AZD3965 in the clinical management of DLBCL and BL.

\section*{Methods}

Information concerning cell origin, authentication, culture conditions, Western blotting and antibody and drug use are detailed in the Supplemental Methods section of the accompanying online supplement.

\section*{Determination of intracellular lactate, protein, cell growth and viability}

Lactate concentration was determined by colorimetric assay (Trinity Biotech, Co Wicklow, Ireland) and normalized to protein content. For growth inhibition assays, cells were plated overnight before treatment for 72h and assessed using an XTT assay (Sigma). GI\textsubscript{50} values were determined using GraphPad Prism software (version 6). Cell number and viability were determined concurrently after 72 and 120h of AZD3965 treatment using a hemocytometer and trypan blue exclusion.
Immunohistochemistry

Formalin-fixed paraffin-embedded pre-treatment diagnostic DLBCL and BL tissue samples were obtained from the Newcastle Hematology Biobank (National Research Ethics Service Committee Reference 07/H0906/109+5) and the Children’s Cancer and Leukaemia Tissue Bank (Reference 08/H0405/22+5) respectively. Immunohistochemistry for MCT1 and MCT4 was performed on the Ventana Benchmark automated immunostaining platform using Optiview detection. Staining extent and intensity on tumor cells was evaluated by two hematopathologists (CMB and DT) and a summary H-score (0-300) calculated as previously described. In selected cases, double immunohistochemical staining for PAX5 (SP34 rabbit monoclonal antibody, Ventana) was used to distinguish tumor cell versus non-tumor MCT4 expression. DLBCL cell-of-origin (COO) classification was determined by immunostaining as described in Culpin et al. using the Hans algorithm.

Metabolic assays

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse XF24 analyzer (Agilent, Santa-Clara, CA, USA). CA46 or CA46-R cells (2x10^5) were made to adhere to Seahorse 24-well plates using Cell-Tak tissue adhesive (Corning, Wiesbaden, Germany) 1-2h prior to analysis.

To examine intracellular metabolite concentrations, tumor cells were seeded in media containing dialyzed FCS (10%) and treated with DMSO vehicle or AZD3965 (100 nM) for 2h under normoxic conditions. Following metabolite extraction, LC-MS analysis was performed as previously described.

Extracellular metabolites in RPMI supplemented with dialyzed FCS (10%) were
determined using NMR following incubation with AZD3965 (100 nM) for 24h. The intracellular metabolite composition of tumor xenograft samples was assessed by GC/MS. Additional details on metabolic assays are detailed in Supplementary Information.

**In vivo efficacy of AZD3965**

For *in vivo* studies, luciferase expressing CA46 cells\textsuperscript{18, 19} were injected intravenously, via the tail vein, into NOD/LtSz-scid IL-2R\(\gamma\) null (NSG) mice within a laminar flow hood. Mice were imaged using an IVIS Spectrum pre-clinical imaging system (Perkin Elmer) as previously described.\textsuperscript{20} IVIS spectrum operators were blinded to treatment assignments. Both AZD3965 (100 mg/kg, BID) and BAY 87-2243 (9 mg/kg, QD) or relevant vehicle controls were administered by oral gavage. Animal experiments were approved by Institutional Ethical Review Process Committees and performed under UK Home Office licences.

**Statistical tests**

Statistical significance was examined using a two-tailed Student’s *t*-test, with the exception of group comparisons within *in vivo* experiments which were performed using a two-way ANOVA with a Tukey test, or a Pearson \(\chi^2\) test to examine whether post-treatment tumor volumes had decreased relative to pre-treated volumes. Data comparisons with a *P*-values <0.05 were considered significant.

**Results**

**Diffuse large B-cell lymphoma and Burkitt lymphoma are appropriate diseases for AZD3965 treatment**

We re-analysed MCT1 and MCT4 published gene expression data from tumor
cell lines and revealed DLBCL and BL cell lines to be amongst the lowest expressers of MCT4 (SLC16A3) mRNA, particularly in comparison to those originating from diverse solid tumor types (Supplementary Figure S1). In contrast, MCT1 (SLC16A1) expression was less variable across cancer types. To determine protein expression in clinical lymphoma samples, we stained a cohort of 120 DLBCL patient samples for both MCT1 and MCT4 protein and categorized samples using an immunohistochemical H-score (Figure S2). DLBCL samples were found to be negative for MCT4 (H-Score = 0) in 73% of cases (Figure 1A), despite variable numbers of MCT4 positive stromal cells identified by morphology and the absence of a B-cell marker (PAX5) (data not shown). MCT4 protein staining was absent in both Activated B-cell (ABC) and Germinal Centre B-cell (GCB) COO classifications (Figures 1B and 1C). The majority of samples had significant tumor cell MCT1 protein expression which was not significantly associated with MYC translocation status (data not shown). Ten BL patient specimens were also stained and found to have uniformly strong MCT1 and undetectable MCT4 protein, with the exception of weak tumor cell staining (H-score ≤ 10) in inflamed, ulcerated areas within two intestinal BL tumor samples (Figure 1A).

That the majority of DLBCL and BL patient specimens examined had little or no evidence of MCT4 protein expression but appreciable MCT1, suggests that these are potentially appropriate malignancies in which to examine MCT1 inhibitor treatment.

**AZD3965 induces rapid accumulation of lactate in human lymphoma cell lines and significantly inhibits their growth in vitro**

We assembled a panel of DLBCL and BL cell lines and determined the expression of MCT1 and MCT4 protein by Western blotting (Figure 2A), with confirmatory
immunohistochemistry on a subset (Supplementary Figure S3). The DLBCL cell lines selected included Farage, OCILY18, Pfeiffer and Toledo which are representatives of the germinal center B-cell (GCB) subtype and RIVA, an example of the activated B-cell (ABC) subtype.\textsuperscript{22, 23} A selection of these cells have also been characterized according to the Consensus Cluster Classification (CCC), with Farage and OCILY18 being of the B-cell receptor subtype (BCR), and Pfeiffer and Toledo the oxidative phosphorylation (OXPHOS) subtype.\textsuperscript{24, 25} The BL cell lines examined comprised the Epstein Barr virus (EBV) positive Raji and Daudi cells and EBV negative BJAB, BL41, Ramos and CA46 cells. MCT1 protein expression was detectable in each lymphoma cell line, all BL cell lines being high expressers, but more variation being evident in the DLBCL cell lines, with RIVA being a high expresser, Toledo, Pfeiffer and OCILY18 being intermediate expressers and Farage a comparatively low expresser (Figure 2A). With the exception of BJAB, all cell lines were found to be negative for MCT4 and, consistent with such cells having a reliance on MCT1 for lactate transport, they accumulated intracellular lactate (>25 µg/mg protein, \(P<0.05\)) following AZD3965 treatment (Figure 2B). Notably, neither the absolute concentration of lactate attained following AZD3965 treatment nor the magnitude of accumulation relative to basal control conditions (i.e. fold change) correlated with MCT1 protein expression. In contrast, the MCT4 positive BJAB cell line did not show a significant change in intracellular lactate following MCT1 inhibition (\(P=0.16\)). An examination of the time-dependency of lactate accumulation in CA46 cells, revealed a rapid increase in intracellular lactate within the first 30 minutes following treatment with 100 or 1000 nM of AZD3965 (Figure 2C). Whilst a reduction (of 40-50%) from peak lactate levels was observed by 24 hours, a concentration of approximately 50 µg of lactate per mg of protein was largely maintained between 24 and 72 hours
(Figure 2C) suggesting attainment of an intracellular lactate equilibrium. Although maximal lactate accumulation was not evident until 6 hours following incubation with 10 nM of AZD3965, the lactate concentration at 24 and 72 hours was comparable to that achieved with 100 nM and 1000 nM AZD3965 treatment (Figure 2C). However, a concentration of 100 nM AZD3965 was chosen for all further single concentration experiments, given the more rapid onset of lactate accumulation.

AZD3965 potently inhibited the proliferation of DLBCL and BL cell lines in which lactate accumulation was observed (Figure 2D-F; 72h GI50 values ranged from 3 to 39 nM). However, proliferation of the MCT4 expressing cell line BJAB was not markedly reduced (Figure 2D), <10% growth inhibition being evident following exposure to 10μM AZD3965 (n=3; data not shown). Whilst AZD3965 induced a profound cytostatic effect in DLBCL and BL cells without MCT4, limited effects on cell viability were detected over a 72h period (Figures 2G, H). Furthermore, when incubations with a >GI95 (72h data) concentration of AZD3965 were extended to 120h, only a modest loss of cell viability in Raji (23%) and a <5% change in CA46 viability (Figure 2I) were detectable.

Consequences of AZD3965 treatment on tumor cell metabolism and efficacy in a BL model in vivo

We examined the consequences of AZD3965 treatment (2-hour incubation) on cellular metabolism in three DLBCL and two BL cell lines in vitro. MCT1 inhibition increased the intracellular levels of TCA cycle intermediates across a number of the AZD3965-sensitive cell lines (Figure 3A; Supplementary Figure S4), potentially reflecting increased TCA cycle activity. We also observed changes in the glycolytic pathway including lactate accumulation and increased levels of early glycolytic intermediates, in particular glucose-6-phosphate, consistent with lactate inducing
feedback inhibition on phosphofructokinase. The reduction in fructose-bisphosphate observed in CA46 and Daudi, would also be predicted to reduce pyruvate kinase activity and contribute to reduced glycolytic flux. To determine whether these effects could be reproduced in vivo, we grew CA46 BL cells subcutaneously in NSG mice and harvested tumors 2 hours after mice had been given a single oral dose of AZD3965 (100 mg/kg). Tumor lactate accumulation was evident following either biochemical assay (Figure 3B) or GC-MS analysis (Figure 3C). Reductions in glutamate and succinate were also observed in tumors (Figure 3C).

Given that intracellular tumor lactate was elevated by AZD3965 treatment, we next sought to determine the consequences of this on CA46 growth in vivo. CA46 tumor cells, engineered to ectopically express firefly luciferase, were inoculated intravenously and their growth determined by bioluminescence in vivo imaging. Cell engraftment was confirmed 6 days after inoculation, prior to commencing oral treatment with AZD3965 or vehicle. AZD3965 treatment for 24 days inhibited tumor growth by 99% (Figures 3 D, E). Reduced CA46 cell engraftment in AZD3965 treated animals was also evident from a lack of human CD20 staining in spleen (Figures 3 F, G) and retention of normal spleen weight. Evidence of CD20 staining was found in only 8% (1/13) of femurs recovered from AZD3965 treated mice, whereas engraftment was observed in 86% (12/14) of vehicle treated mice (Figure 3G and data not shown).

**Adaptive resistance to AZD3965 in vitro involves a greater dependency on OXPHOS**

To determine whether an adaptive resistance to AZD3965 could be induced in vitro, CA46 cells were cultured continuously in the presence of 10 nM compound for
four weeks followed by 100 nM for three weeks. This resulted in cells with reduced sensitivity to the anti-proliferative effects of the compound (termed CA46-R cells) (Figure 4A). Significant AZD3965-induced intracellular lactate accumulation was observed in both CA46 and CA46-R cells, and although the concentration of lactate in CA46-R was 28% less than in the parental cell line, the level attained was comparable or greater than that achieved in other AZD3965-sensitive lines following drug treatment, including Daudi, Toledo, and Pfeiffer cells. There was no evidence of MCT4 being expressed as a potential compensatory mechanism to mediate lactate efflux (Figure 4B). In contrast, the levels of MCT1 and its co-chaperone CD147 were lower in CA46-R cells, suggesting that the level of functional MCT1 may be reduced (Figure 4B). CA46 and CA46-R demonstrated comparable doubling-times (Supplementary Figure S5A) and resistance was maintained following culture in drug-free medium for 2 weeks (data not shown). To explore an altered metabolic phenotype, the relative consumption and release of metabolic substrates was assessed following 24h of AZD3965 treatment. CA46-R showed increased glutamine uptake, decreased lactate release and increased pyruvate export (Supplementary Figure S5B); changes consistent with reduced glycolytic lactate production and increased glucose and glutamine oxidation with which to fuel TCA cycle activity.

We also examined the respective contributions of glycolysis and OXPHOS in CA46 and CA46-R cells. Acute exposure to AZD3965 triggered a rapid decrease in ECAR in CA46 cells but not in CA46-R cells which demonstrated a lower basal ECAR (Figure 4C). CA46 and CA46-R differed markedly in their basal OCR, with CA46-R utilizing more oxygen (Figure 4C). Collectively, these measurements are indicative of CA46-R cells having a more oxidative metabolic phenotype (additional details available in Supplementary Information).
Combining AZD3965 with inhibitors of Complex I triggers cell death

Since an inhibition of glycolysis will generate a greater reliance on OXPHOS for ATP generation, simultaneous inhibition of mitochondrial Complex I may trigger cell death\cite{12}, a phenomenon demonstrated in Raji by combining AZD3965 with metformin (Figure 5A). However, the concentrations of metformin required to demonstrate this effect are significantly in excess of those that can be achieved in mice following oral dosing.\cite{27} We therefore examined the potent Complex I inhibitor BAY 87-2243, which would subsequently permit inhibition of OXPHOS to be studied in mice.\cite{28} Whilst the BL cell lines, Raji and CA46 were insensitive to BAY 87-2243 monotherapy in vitro, the combination of AZD3965 with BAY 87-2243 induced profound cell death in both cell lines (Figures 5 B, C). CA46-R cells were more sensitive to the growth inhibitory effect of BAY 87-2243 than parental CA46 cells, but a combination of BAY 87-2243 and AZD3965 was similarly required to induce cell death (Figure 5D). In contrast, cell death was not evident when AZD3965 was combined with BAY 87-2243 in MCT4 protein expressing BJAB cells (data not shown).

Combining AZD3965 with BAY 87-2243 in vivo

To examine MCT1 inhibition combined with Complex I inhibition in vivo, mice were inoculated intravenously with luciferase expressing CA46 cells (Figure 6A). Mean tumor engraftment was equivalent 12-days post-inoculation (\(P>0.05\) for all group comparisons by two-way ANOVA; Figure 6B), prior to dosing. Following treatment (72h after the last dose) mice were re-imaged and AZD3965 monotherapy treatment again resulted in significant control of tumor growth, mean tumor volume being not significantly different from pre-treatment values (Figure 6B; \(P>0.05\) by two-tailed \(t\)-test). Whilst tumor burden in control or BAY 87-2243 treated animals had
increased markedly from each pre-treatment value, the combination of AZD3965 with BAY 87-2243 led to a reduction in mean tumor burden compared with the pre-treated value in 4/5 mice, with signal intensities being reduced by between 10 to 267-fold (Figure 6B; \( P=0.01 \) by Pearson \( \chi^2 \) test). This is consistent with the induction of lymphoma cell death \textit{in vivo}. The combination treatment regimen was well tolerated with no significant differences in body weight when compared to the control vehicle treated group throughout treatment (data not shown). All tumor inhibitory effects were lost upon cessation of treatment as engraftment progressed in each drug treated group, indicating that targeting of metabolism is likely to require continuous therapy (Figure 6C).

**Discussion**

This study aimed to evaluate DLBCL and BL as potential tumor types appropriate for the clinical development of AZD3965, a novel MCT1 inhibitor currently undergoing Phase I evaluation.

Whilst a glycolytic phenotype and increased generation of lactate is implicated in the pathogenesis of both DLBCL and BL, the relative expression of MCT1 versus MCT4 in DLBCL has been less clear. A previous report examining clinical gene expression data confirmed high expression of MCT1 mRNA and low expression of MCT4 mRNA in BL but suggested that the converse was true in a cohort of non-Hodgkin lymphoma that will have contained predominantly DLBCL samples.\textsuperscript{12} Our examination of MCT1 and MCT4 protein using immunohistochemistry showed uniformly strong MCT1 staining in BL with a corresponding lack of MCT4. However, our analysis also indicated that the majority of DLBCL does not stain positive for MCT4 protein. DLBCL samples without MCT4 protein expression were observed
amongst both COO subgroups, and with and without any MYC aberration. This suggests all major DLBCL subgroups contain patients with an MCT1 positive/MCT4 negative protein expression profile, who may be appropriate candidates to receive AZD3965 treatment.

Additional experiments examining neuroblastoma cell lines (IMR-32 and SH-SY5Y; Supplementary Figure S6) and a previous report in SCLC cells demonstrate that only partial sensitivity to AZD3965 can be observed in solid tumor cell lines lacking MCT4 expression, despite significant lactate accumulation. Solid tumor cell lines also have a much broader spectrum of MCT4 mRNA expression in comparison to those of hematological origin (Supplementary Figure S1). Whilst these data do not exclude AZD3965 as being suitable for the treatment of a subset of solid tumors, the more potent GI50 and uniform response to AZD3965 treatment observed in DLBCL and BL cell lines suggest that these B-cell malignancies are better disease indications in which to initially examine AZD3965 clinical activity.

Importantly, MCT1 protein expression per se does not clearly correlate with the extent of lactate accumulation or growth inhibition observed in vitro following AZD3965 treatment. Therefore, prospective patient stratification for AZD3965 treatment should prioritize the treatment of patients whose tumors do not stain positive for MCT4 protein, rather than treatment based upon the magnitude of MCT1 protein expression. The lack of an effect of AZD3965 on either the intracellular lactate concentration or growth of the MCT4 expressing BJAB lymphoma cell line, is consistent with previous data showing that MCT4 overexpression in a breast cancer cell line or RAS-transformed fibroblasts confers resistance to MCT1 inhibitor treatment.12, 29

The effect of inhibiting MCT1 in the DLBCL and BL cell lines in vitro was
predominantly cytostatic. Encouragingly however, we also observed a striking cytostatic response in vivo with AZD3965 treatment, which halted progressive splenic engraftment of the lymphoma. The incidence of BL is increased in elderly patients and the median age of diagnosis for DLBCL is around 70 years.\textsuperscript{30, 31} Given that not all of these patients will be fit enough to tolerate multi-agent chemo-immunotherapy, a well-tolerated oral cytostatic therapy could have significant clinical utility in this patient group or those that have relapsed following current standard of care treatment.

The compensatory metabolic alterations observed following AZD3965 treatment in vitro, either following acute or chronic exposure, support an adaptive metabolic response that causes a greater reliance on OXPHOS and increased TCA activity. When targeting the glycolytic tumor phenotype, to elicit tumor cell death it may be necessary to inhibit multiple metabolic pathways, or nodes in a given pathway, ensuring that there is a basis for tumor selectivity with at least one of the approaches used. Our data confirm that a combination of AZD3965 with different Complex I inhibitors induces rapid cell death in BL (Figure 5 A-D) and DLBCL (data not shown) cell lines in vitro. We also verified that such a combination can reduce CA46 disease burden in vivo, in contrast to the cytostatic effect induced by administration of AZD3965 alone. Approaches to induce tumor cell death and impart curative activity would be particularly desirable in the treatment of children, where BL accounts for the majority of non-Hodgkin lymphoma.\textsuperscript{32}

Collectively, the striking monotherapy activity of AZD3965 observed in DLBCL and BL cell lines, and its potential for use in combination, provides a rationale for examining the efficacy of this agent against these malignancies.
References


27. Dowling RJ, Lam S, Bassi C, et al. Metformin Pharmacokinetics in Mouse


Figures

**Figure 1.** DLBCL and BL are appropriate diseases for AZD3965 treatment. (A) H-score analysis of MCT1 and MCT4 tumor cell protein expression in 120 DLBCL and 10 BL patient samples. A sample was considered negative (H-score of 0) when no staining was evident on tumor cells, staining on stromal cells or inflammatory infiltrate being excluded from the analysis. Representative MCT1 and MCT4 IHC staining from two DLBCL (i and ii) samples and one BL (iii) sample are shown. (B) Pie charts indicate that the majority of DLBCL samples are MCT4 negative and the relative proportion of MCT4 negative samples is similar in both ABC and GCB subsets. (C) MCT4 versus MCT1 H-score plot for the DLBCL samples in relation to ABC/GCB classification.

**Figure 2.** MCT1 inhibition induces rapid accumulation of lactate and significant anti-proliferative activity in DLBCL and BL cell lines. (A) MCT1 and MCT4 protein expression in cell lines using GAPDH as a loading control. (B) Intracellular lactate in cell lines following 24h incubation with AZD3965 (1 µM) or vehicle. (C) Concentration and time dependency of intracellular lactate accumulation in CA46 cells following treatment with AZD3965 or vehicle. (D-F) Sensitivity of BL or DLBCL cells treated with AZD3965 for 72h assessed by XTT assay. (G and H) Cell number and viability following AZD3965 (100 nM) treatment for 72h. (I) Cell viability following an extended 120h exposure to AZD3965 (100 nM). Graphs represent the mean of ≥3 independent experiments ± SEM. *P<0.05, ** P<0.01, ***P<0.001 by unpaired two-tailed t-test.

**Figure 3.** AZD3965 alters cellular metabolism *in vitro* and *in vivo* causing growth inhibition. (A) Levels of TCA cycle and glycolytic intermediates in cell lines following
a 2h exposure to AZD3965 (100 nM) determined by LC-MS. Significantly altered metabolites (p <0.05) are expressed as log₂ fold-change relative to vehicle treated control. Metabolite abbreviations used; αKG (alpha-ketoglutarate), FBP (Fructose-bisphosphate), F1P (Fructose-1-phosphate), F6P (Fructose-6-phosphate), GAP (Glyceraldehyde-3-phosphate), G1P (Glucose-1-phosphate), G6P (Glucose-6-phosphate). (B) NSG mice with subcutaneous CA46 xenografts were treated with AZD3965 (100 mg/kg) or vehicle and tumors collected after 2h. Lactate concentrations were normalized to protein. (C) Significantly altered (unpaired two-tailed t-test) intra-tumoral metabolite levels determined by GC-MS. (D) NSG mice were inoculated intravenously with luciferase-expressing CA46 cells and 6 days later (treatment day 0) treated with AZD3965 (100 mg/kg, BID) or vehicle for 24-days. Representative images from two mice within the AZD3965 and vehicle treated groups using different radiance scales (p/sec/cm²/sr) for mice prior to treatment and during treatment to avoid image saturation. (E) Mean total flux from AZD3965 and vehicle treated mice (n=8 per group). (F) Spleen weights from AZD3965 and vehicle treated mice. Reference historical spleen weights from NSG mice were 0.02–0.05g.¹⁹ (G) Immunohistochemical analysis of CA46 infiltration via anti-CD20 staining of bone marrow and spleen sections from mice treated with AZD3965 or vehicle. Statistical significance assessed by unpaired two-tailed t-test *P<0.05, ***P<0.001.

**Figure 4.** Acquired resistance to AZD3965 *in vitro* is associated with increased oxidative metabolism. (A) The sensitivity of CA46 and CA46-R cells to AZD3965 (72h treatment) determined by XTT assay and cell counting. (B) Intracellular accumulation of lactate determined after 24h exposure to AZD3965 (1 µM). MCT1, MCT4 and CD147 protein levels assessed by Western blotting. (C) ECAR in CA46
and CA46-R with and without treatment with AZD3965 (100 nM) or vehicle. OCR in CA46 and CA46-R cells, indicating the effects following addition of oligomycin, FCCP and antimycin. ECAR and OCR values (mean ± SEM) are normalized to protein expression and representative of 3 independent experiments.

**Figure 5.** Combining AZD3965 with inhibitors of mitochondrial Complex I induces Burkitt lymphoma cell death. Viable cell numbers were determined by cell counting with trypan blue exclusion over a 72h period, following treatment with AZD3965, a Complex I inhibitor, or the combination. (A) Raji cells treated with vehicle, AZD3965 (100 nM), metformin (1 mM) or the combination. (B) Raji cells treated with vehicle, AZD3965 (5 nM), BAY 87-2243 (100 nM) or the combination. (C) CA46 and (D) CA46-R cells treated with vehicle, AZD3965 (10 nM), BAY 87-2243 (10 nM) or the combination. All graphs represent the mean of ≥3 independent experiments ± SEM.

**Figure 6.** Combining AZD3965 with an inhibitor of mitochondrial Complex I *in vivo*. (A) Schema indicating treatment duration and scan intervals. (B) Pre- and post-treatment bioluminescent signals for mice within each group with a representative image from one of the mice receiving the combination (inset). (C) Change in signal intensity subsequent to treatment. Graph shows the mean ± SD total flux (n ≥ 5 per group).
Figure 1
Figure 2

A. Western blot analysis of MCT1, MCT4, and GAPDH in BL and DLBCL cell lines.

B. Bar graph showing lactate production in BL and DLBCL cell lines at different concentrations of AZD3965.

C. Graph showing lactate production over time with different concentrations of AZD3965.

D. Estimated viable cell number in response to different concentrations of AZD3965.

E. Estimated viable cell number at different cell lines.

F. Graph showing G50 for different cell lines.

G. Cell number (% control) for different cell lines.

H. % Viable cells (Trypan Blue exclusion) for different cell lines.

I. % Viable cells (Trypan Blue exclusion) for different cell lines.
Figure 3
**Treatments:**
1. Vehicle A (BID) + Vehicle B (QD)
2. AZD3965 (100mg/kg BID) + Vehicle B (QD)
3. Vehicle A (BID) + BAY 87-2243 (9mg/kg QD)
4. AZD3965 (100mg/kg BID) + BAY 87-2243 (9mg/kg QD)

**Figure 6**
Supplementary Information

Inhibition of monocarboxylate transporter 1 by AZD3965 as a novel therapeutic approach for the treatment of diffuse large B-cell lymphoma and Burkitt lymphoma

Authors

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Supplemental Methods

Cell culture, drug and antibody preparation
Farage, Pfeiffer and Toledo were obtained from ATCC (Manassas, VA, USA) and BL-41, BJAB, CA46, Daudi, OCILY18, Raji and Ramos from DSMZ (Braunschweig, Germany). RIVA were obtained from Prof Alison Banham, (University of Oxford). All cell lines were re-authenticated at the end of the study by short tandem repeat (STR) analysis (New Gene, Newcastle upon Tyne). All cell lines were cultured in RPMI-1640 media (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco).

For in vitro experiments, AZD3965 (AstraZeneca, Cambridge, UK) was prepared as a 10mM stock solution in dimethyl sulfoxide (DMSO) and BAY 87-2243 (SelleckChem, Houston, TX, USA) was prepared at 1 mM in ethanol. All in vitro experiments were vehicle-controlled with a final concentration of 0.01% DMSO or 0.01% ethanol. For in vivo studies, AZD3965 and BAY 87-2243 were formulated as previously described.1, 2

Antibodies used for Western blotting were GAPDH (FL-335), MCT4 (H-90) and PARP (H-250) (Santa Cruz, Dallas, TX, USA) and CD147 (UM-8D6) (Ancell, Bayport, MN, USA). MCT1 antibodies used were (C-20) Santa Cruz) and (20139-1-AP) (Proteintech, Manchester, UK). For immunohistochemical analysis, monoclonal MCT1 and MCT4 antibodies were used (AstraZeneca, Cambridge, UK).
Determination of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

Cells were maintained in cell Seahorse Base Media without FCS, or bicarbonate, and incubated for 45 min in a non-CO$_2$ incubator at 37°C. The wells were mixed for 2 min and the pH and oxygen concentration measured every 22 seconds for 3 min. Compounds were injected into the wells via reagent delivery ports to give predetermined final concentrations; AZD3965 (100 nM), BAY 87-2243 (100 nM), oligomycin (Sigma, 1 µg/mL), carbonyl cyanide 4(trifluoromethoxy)phenylhyrazone (FCCP) (Sigma, 300 nM) and antimycin A (Sigma, 2.5 µM). After each addition, two data points of 3 min were taken to determine the OCR (pM O$_2$/min) and ECAR (mpH/min), with data being normalised to protein concentration.

Oligomycin treatment, which inhibits ATP generation, revealed that this increased OCR reflects increased oxygen consumption coupled to ATP generation in CA46-R. In addition to an increase in basal OCR, the reserve oxidative capacity of CA46-R greatly exceeded that of parental CA46 cells, as indicated by their response to the protonophore FCCP (Figure 4C). Since the basal respiratory production of CO$_2$ from OCR can also contribute to ECAR, the glycolytic rate of CA46-R cells may be even less than inferred from our measurement of ECAR.$^3$

Metabolite determination using nuclear Magnetic Resonance (NMR)

High Resolution One dimensional (1D) $^1$H NMR spectra were acquired using a Bruker AVANCE spectrometer (Bruker Bio Spin) at 298K, 14.1 T and 400 MHz $^1$H frequency using a 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) internal standard prepared in deuterated water. All spectra were acquired using a single pulse experiment with solvent presaturation, 32 free induction decays (FIDs), a spectral width of 6402.049 Hz, 64K complex data points and a recycle delay of 4 seconds. Spectra were
processed with 0.3 Hz line broadening. Peaks were integrated and resonances assigned to metabolites using spectral comparison with the Human Metabolome Database (HMDB). The metabolite composition of spent and fresh media were compared using CORE profiling with normalisation to cell number as previously described.4

**Metabolite determination using gas chromatography mass spectrometry (GCMS)**

For metabolite extraction samples were kept on dry ice throughout. Tumor samples were weighed and added to screw-cap tubes containing 0.1 mm glass beads. Pre-chilled 80% methanol (800 µL) was added and samples homogenised using a Precellys 24 bead beater (Bertin Technologies). Supernatants were collected after centrifugation (12 000 x g, 5min, at 4°C). The extraction was repeated and the fractions for each sample were pooled. Supernatants were then dried in a vacuum concentrator and subjected to dual-phase extraction. 300 µL of chloroform/methanol (2:1) was added to each sample followed by vortexing. 300 µL of water was then added to each sample followed by vortexing and centrifugation (21 000 x g, 10min at 4°C). The aqueous (upper) layer from each sample was transferred to silanized GC-MS vials. The dual-phase extraction was repeated and fractions were pooled for each sample. Tumor sample extract (20 mg) was dried in a vacuum concentrator and analysed by GC-MS.

GC-MS analysis was performed using in-house protocols (Imperial College London).5 Metabolites intensities were normalised to a myristic acid-d27 internal standard. Metabolite extracts were derivatized using the two-step method of derivatisation: methoxymation and silylation.6 When required, samples were diluted with anhydrous pyridine. Samples were analysed in a splitless mode on an Agilent
7890 GC with a 30m DB-5MS capillary column and a 10m Duraguard column, coupled to an Agilent 5975 MSD. Metabolites were assigned using FiehnLib assisted processing in AMDIS\(^6, 7\) and manually assessed using the Gavin package.\(^5\)

**Supplemental References**


Figure S1: Cancer cell line expression of MCT1 and MCT4

RMA-normalised mRNA expression of MCT1 and MCT4 in hematological □ and solid tumor ■ cell lines. Data from the Cancer Cell Line Encyclopaedia.⁸
Figure S2: MCT1 and MCT4 staining of DLBCL patient samples

Immunohistochemical staining of MCT1 and MCT4 was performed and H-scores determined as detailed in the Methods section of the main manuscript. Panels show representative images from lymphomas with H-scores within each category (0, 1-10, 11-100, 101-200, 201-300). H-scoring considered only scoring on tumor cells. An MCT1 H-score of 0 was not recorded for any of the 120 samples examined. The lymphoma with an MCT1 H-score of 10 shown, comprised small strongly positive lymphocytes and macrophages with only a few weakly positive tumour cells (inset). The representative MCT4 H-score of 0 shows staining on positive macrophages only and the MCT4 H-score of 2 shown is indicative of weak sporadic tumour cell staining (inset).
Figure S3: Cancer cell line expression of MCT1 and MCT4

Immunohistochemical staining of MCT1 and MCT4 in a sub-set of B-NHL cell lines
Figure S4: AZD3965 increases levels of TCA cycle intermediates

Enrichment in TCA cycle intermediates in CA46 cells following 2h exposure to AZD3965 (100 nM), significant alterations relative to vehicle treated control (Log₂FC) mapped to KEGG pathways using Pathview.⁹
Figure S5: AZD3965 resistant CA46-R do not exhibit an altered proliferative rate.

CA46 and CA46-R proliferation rates were assessed by daily cell counts (0-96h). Mean of three independent experiments ± SEM. Analysis of culture media (24h incubation) from untreated CA46 and CA46-R cells. Metabolite concentrations (mM) are shown per 1x10^6 cells.
Figure S6: Low MCT4 expression is not sufficient for MCT1 inhibition to be efficacious.

(A) Expression of MCT1 and MCT4 protein following treatment with AZD3965 (1 µM for 72h) in neuroblastoma (IMR-32 and SH-SY5Y), BL (Raji) and B-ALL (697). HT-29 was used as an MCT4 positive control. β-actin was used as a loading control. (B) Intracellular lactate was measured in cells following exposure to AZD3965 (1 µM) or DMSO vehicle for 72h. (C) Cells were treated with AZD3965 (1 µM) for 72h and sensitivity assessed by XTT assay. Data are expressed relative to vehicle treated control. All data represent mean ± SEM of 3 independent experiments.