

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

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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 patients' 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 of 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 is characterized by an increased utilization of glucose and a corresponding greater efflux of lactate.<sup>1,2</sup> Consequently, there has been much interest in targeting this recognized 'hallmark of cancer' for therapeutic benefit.<sup>3,4</sup> 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



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monocarboxylates such as lactate and pyruvate.<sup>5</sup> 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 fulfills 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.<sup>6</sup> MCT1 and MCT4 can be differentially over-expressed in cancer,<sup>7-11</sup> 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 in which it may be utilized as a respiratory fuel.<sup>12,13</sup>

AZD3965 is an orally bioavailable MCT1 inhibitor, which is currently under phase I clinical investigation (NCT01791595).<sup>14</sup> 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 the cells' growth.<sup>7,12,14</sup>

We evaluated the metabolic and therapeutic effects of AZD3965 in aggressive forms of non-Hodgkin lymphoma, namely, diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL). We demonstrate in patients' 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 *in vitro* and *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 *in vivo*.

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

## Methods

Information concerning cell origin, authentication, culture conditions, western blotting and antibody and drug use are detailed in the *Online Supplementary Information*.

### 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 72 h and assessed using an XTT assay (Sigma, Saint-Louis, MO, USA). GI<sub>50</sub> values were determined using GraphPad Prism software (version 6). Cell number and viability were determined concurrently after 72 h and 120 h of AZD3965 treatment using a hemocytometer and trypan blue exclusion, respectively.

### 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 (Tucson, AZ, USA) automated immunostaining platform using Optiview detection. Staining extent and intensity on tumor cells were evaluated by two hematopathologists (CMB and DT) and a summary H-score (0-300) calculated as previously described.<sup>15</sup> 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 classification was determined by immunostaining, as described in Culpin *et al.*,<sup>16</sup> using the Hans algorithm.<sup>17</sup>

### Metabolic assays

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

To examine intracellular metabolite concentrations, tumor cells were seeded in media containing dialyzed fetal calf serum (10%) and treated with dimethylsulfoxide vehicle or AZD3965 (100 nM) for 2 h under normoxic conditions. Following metabolite extraction, liquid chromatography-mass spectrometry analysis was performed as previously described.<sup>14</sup>

Extracellular metabolites in RPMI supplemented with dialyzed fetal calf serum (10%) were determined using nuclear magnetic resonance following incubation with AZD3965 (100 nM) for 24 h. The intracellular metabolite composition of tumor xenograft samples was assessed by gas chromatography-mass spectrometry. Additional details on metabolic assays are detailed in the *Online Supplementary Information*.

### *In vivo* efficacy of AZD3965

For *in vivo* studies, luciferase-expressing CA46 cells<sup>18,19</sup> 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, Waltham, MA, USA) as previously described.<sup>20</sup> 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 licenses.

### Statistical tests

Statistical significance was examined using a two-tailed Student *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 *P*-values <0.05 were considered statistically significant.

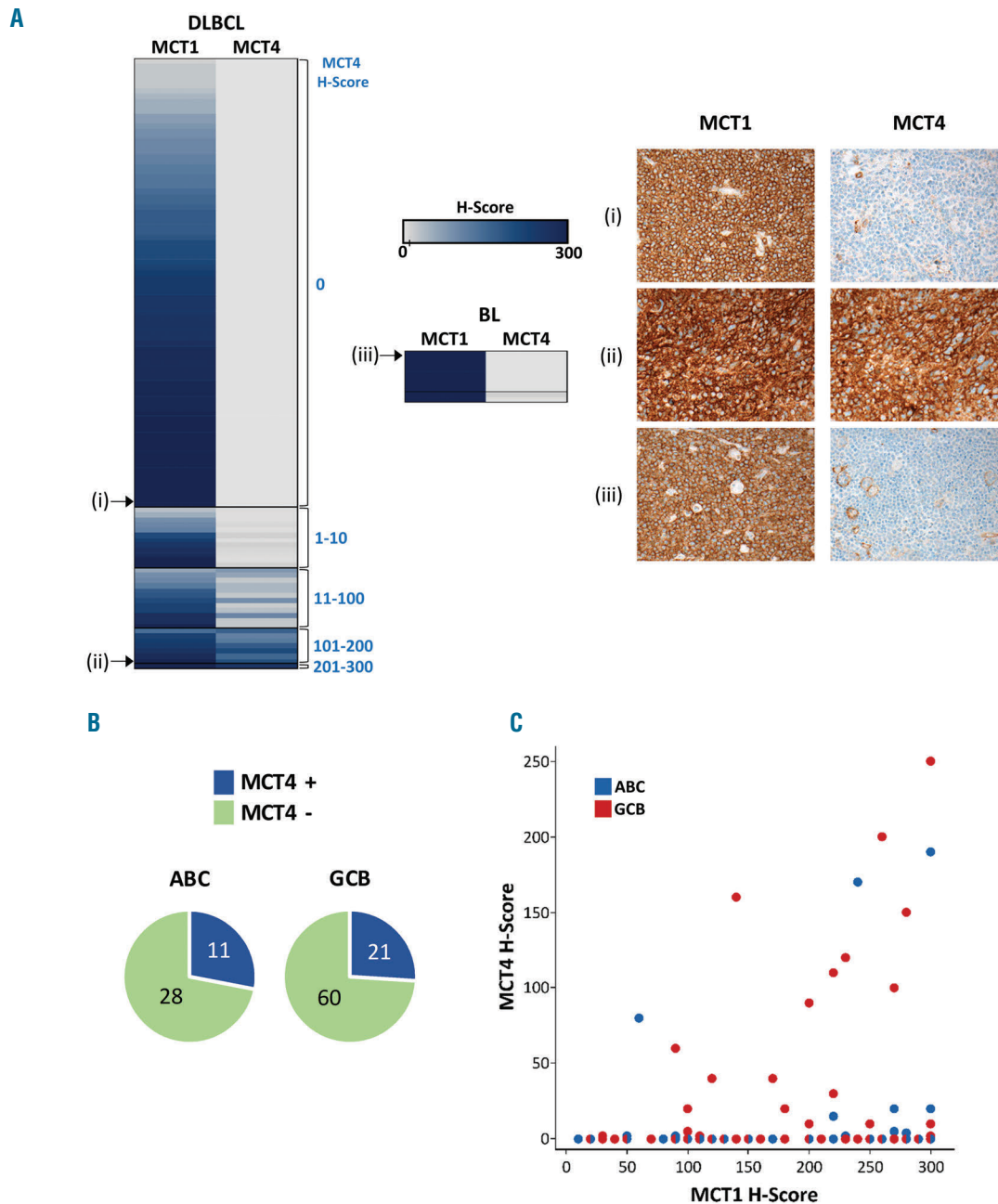
## Results

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

We re-analyzed MCT1 and MCT4 published gene expression data from tumor cell lines and found DLBCL and BL cell lines to be among the lowest expressers of

MCT4 (*SLC16A3*) mRNA, particularly in comparison to those originating from diverse solid tumor types (*Online Supplementary Figure S1*).<sup>21</sup> 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 patients' samples for both MCT1 and MCT4 protein and categorized samples using an immunohistochemical H-score (*Online Supplementary 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 and germinal center B-cell cell-of-origin classifications (Figure 1B,C). The majority of samples had significant tumor cell MCT1 protein expression which was not significantly associated with *MYC* translocation status (*data not shown*). Specimens from ten patients



**Figure 1. Diffuse large B-cell lymphoma and Burkitt lymphoma are appropriate diseases for AZD3965 treatment.** (A) H-score analysis of MCT1 and MCT4 tumor cell protein expression in 120 samples from 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 immunohistochemical staining from two DLBCL samples (i and ii) and one BL sample (iii) 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 activated B-cell (ABC) and germinal center B-cell (GCB) subsets. (C) MCT4 vs. MCT1 H-score plot for the DLBCL samples in relation to ABC/GCB classification.

were also stained and found to have uniformly strong MCT1 and undetectable MCT4 protein, with the exception of weak tumor cell staining (H-score  $\leq 10$ ) in inflamed, ulcerated areas within two intestinal BL tumor samples (Figure 1A).

That the majority of DLBCL and BL patients' 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 their expression of MCT1 and MCT4 protein by western blotting (Figure 2A), with confirmatory immunohistochemistry on a subset (*Online Supplementary Figure S3*). The DLBCL cell lines selected included Farage, OCILY18, Pfeiffer and Toledo, which are representatives of the germinal center B-cell subtype, and RIVA, an example of the activated B-cell subtype.<sup>22,23</sup> A selection of these cells have also been characterized according to the Consensus Cluster Classification, with Farage and OCILY18 being of the B-cell receptor subtype, and Pfeiffer and Toledo the OXPHOS subtype.<sup>24,25</sup> The BL cell lines examined comprised the Epstein-Barr virus-positive Raji and Daudi cells and Epstein-Barr virus-negative BJAB, BL41, Ramos and CA46 lines. 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 being reliant on MCT1 for lactate transport, they accumulated lactate intracellularly ( $>25$   $\mu\text{g}/\text{mg}$  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 min following treatment with 100 or 1000 nM of AZD3965 (Figure 2C). While a reduction (of 40-50%) from peak lactate levels was observed by 24 h, a concentration of approximately 50  $\mu\text{g}$  of lactate per mg of protein was largely maintained between 24 and 72 h (Figure 2C) suggesting attainment of an intracellular lactate equilibrium. Although maximal lactate accumulation was not evident until 6 h following incubation with 10 nM of AZD3965, the lactate concentration at 24 and 72 h 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; 72 h  $\text{GI}_{50}$  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 10  $\mu\text{M}$  AZD3965 ( $n=3$ ; *data not shown*). Although AZD3965 induced a profound cytostatic effect in DLBCL and BL cells without MCT4, limited effects on cell viability were detected over a 72 h period (Figure 2G,H). Furthermore, when incubation with a  $>\text{GI}_{95}$  (72 h data) concentration of AZD3965 was prolonged to 120 h, only a modest loss of cell viability in Raji (23%) and a  $<5\%$  change in CA46 viability (Figure 2I) were detectable.

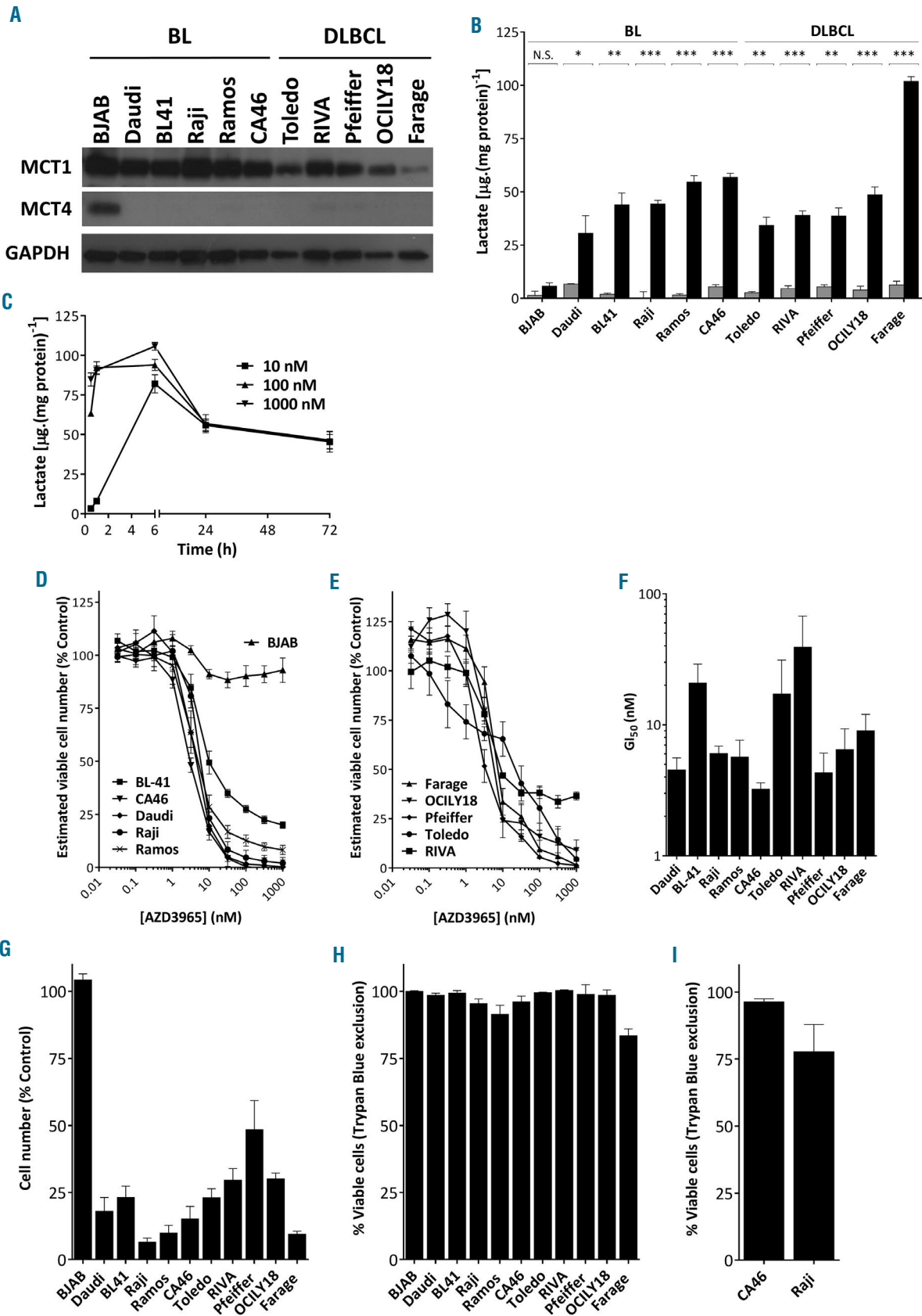
### **Consequences of AZD3965 treatment on tumor cell metabolism and efficacy in an *in vivo* Burkitt lymphoma model**

We examined the consequences of AZD3965 treatment (2 h incubation) on cellular metabolism in three DLBCL and two BL cell lines *in vitro*. MCT1 inhibition increased the intracellular levels of tricarboxylic acid cycle (TCA) intermediates across a number of the AZD3965-sensitive cell lines (Figure 3A; *Online Supplementary Figure S4*), potentially reflecting increased activity of the TCA cycle. 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 of phosphofructokinase.<sup>26</sup> The reduction in fructose-bisphosphate observed in CA46 and Daudi cells, 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 h after mice had been given a single oral dose of AZD3965 (100 mg/kg). Tumor lactate accumulation was evidenced by both biochemical assay (Figure 3B) and gas chromatography – mass spectrometry analysis (Figure 3C). Reductions in glutamate and succinate were also observed in tumors (Figure 3C).

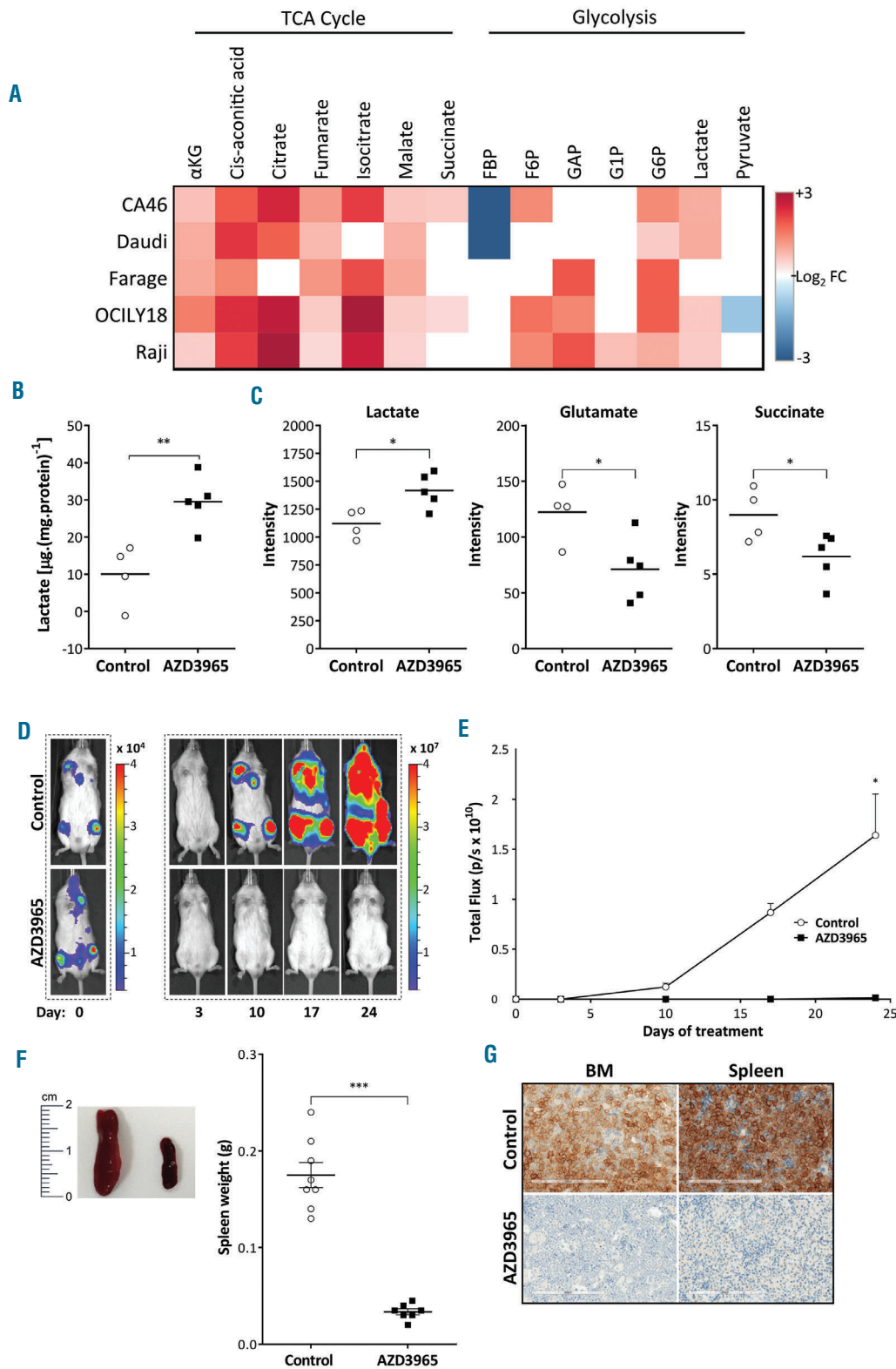
Given that intracellular tumor lactate was elevated by AZD3965 treatment, we next sought to determine the consequences of this on CA46 growth *in vivo*. CA46 tumor cells, engineered to ectopically express firefly luciferase, were inoculated intravenously and their growth determined by bioluminescence *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% (Figure 3D,E). Reduced CA46 cell engraftment in AZD3965-treated animals was also evident from a lack of human CD20 staining in spleen (Figure 3F,G) and preservation of normal spleen weight. Evidence of CD20 staining was found in only 8% (1/13) of femora 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 oxidative phosphorylation**

To determine whether an adaptive resistance to AZD3965 could be induced *in vitro*, CA46 cells were cultured continuously in the presence of 10 nM of the compound for 4 weeks followed by 100 nM for 3 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-



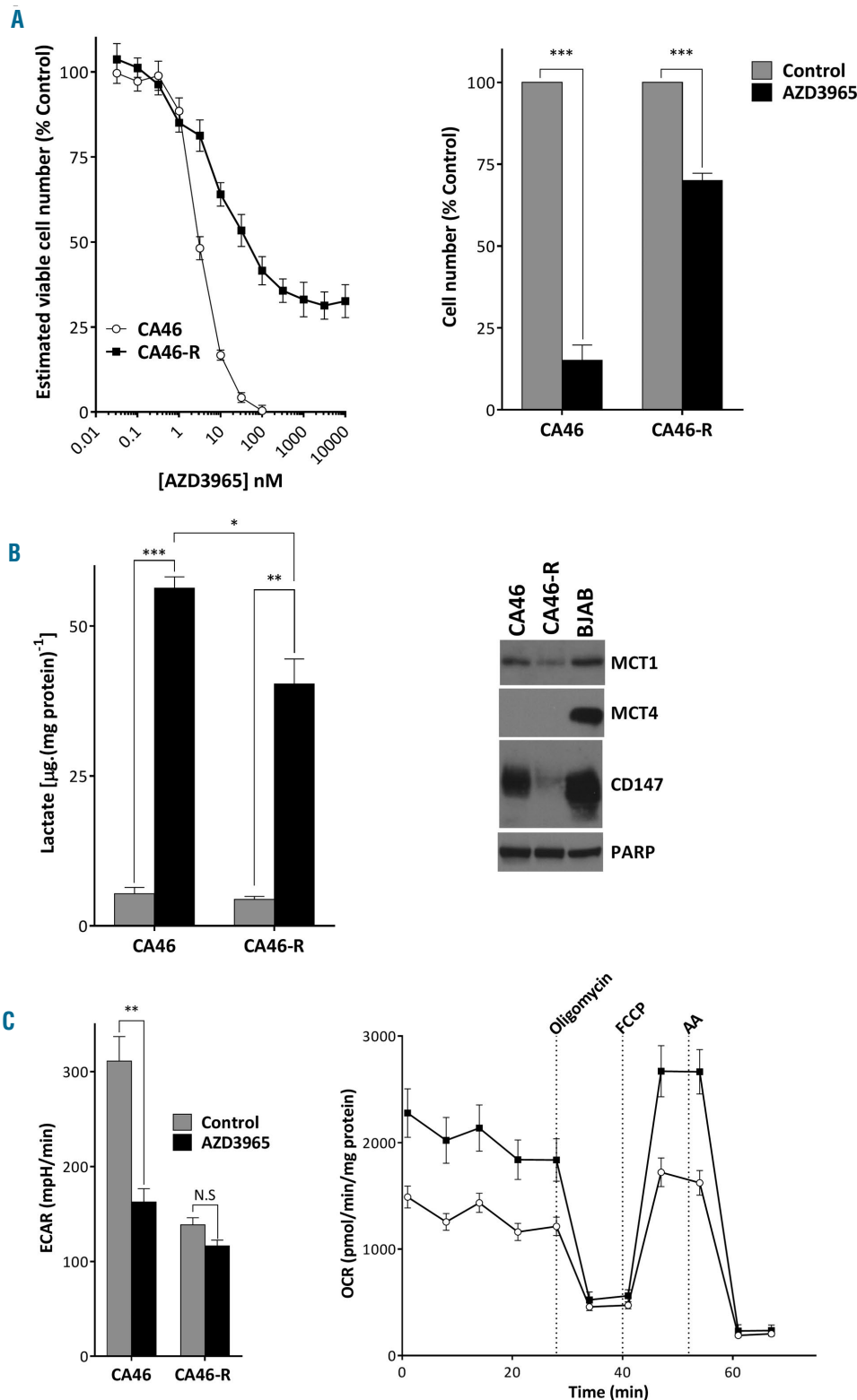
**Figure 2. MCT1 inhibition induces rapid accumulation of lactate and significant anti-proliferative activity in diffuse large B-cell lymphoma and Burkitt lymphoma cell lines.** (A) MCT1 and MCT4 protein expression in cell lines using GAPDH as a loading control. (B) Intracellular lactate in cell lines following 24 h incubation with AZD3965 (1  $\mu\text{M}$ ) 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 72 h assessed by XTT assay. (G and H) Cell number and viability following AZD3965 (100 nM) treatment for 72 h. (I) Cell viability following an extended 120 h exposure to AZD3965 (100 nM). Graphs show the means of  $\geq 3$  independent experiments  $\pm$  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 tricarboxylic acid (TCA) cycle and glycolytic intermediates in cell lines following 2 h exposure to AZD3965 (100 nM) determined by liquid chromatography-mass spectrometry. Significantly altered metabolites ( $P < 0.05$ ) are expressed as  $\log_2$  fold-change relative to vehicle-treated control.  $\alpha$ KG: 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 2 h. Lactate concentrations were normalized to protein. (C) Significantly altered (unpaired two-tailed t-test) intra-tumoral metabolite levels determined by gas chromatography-mass spectrometry. (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 in the AZD3965 and vehicle-treated groups using different radiance scales ( $\text{p/s/cm}^2/\text{sr}$ ) 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 0.02–0.05 g.<sup>19</sup> (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 was assessed by an unpaired two-tailed t-test \* $P < 0.05$ , \*\*\* $P < 0.001$ .

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 to 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). The doubling times of CA46 and CA46-R were comparable (*Online Supplementary Figure S5A*) and resistance was main-



**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 (72 h treatment) determined by an XTT assay and cell counting. (B) Intracellular accumulation of lactate determined after 24 h exposure to AZD3965 (1  $\mu\text{M}$ ). MCT1, MCT4 and CD147 protein levels assessed by western blotting. (C) Extracellular acidification rate (ECAR) in CA46 and CA46-R with and without treatment with AZD3965 (100 nM) or vehicle. Oxygen consumption rate (OCR) in CA46 and CA46-R cells, indicating the effects following addition of oligomycin, FCCP and antimycin. ECAR and OCR values (mean  $\pm$  SEM) are normalized to protein expression and representative of three independent experiments.

tained 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 24 h of AZD3965 treatment. CA46-R showed increased glutamine uptake, decreased lactate release and increased pyruvate export (*Online 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 extracellular acidification rate in CA46 cells but not in CA46-R cells which demonstrated a lower basal extracellular acidification rate (Figure 4C). CA46 and CA46-R differed markedly in their basal oxygen consumption rate, 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 are available in the *Online Supplementary Information*).

### Combining AZD3965 with inhibitors of complex I triggers cell death

Since inhibition of glycolysis will generate greater reliance on OXPHOS for ATP generation, simultaneous inhibition of mitochondrial complex I may trigger cell death,<sup>12</sup> a phenomenon demonstrated in Raji cells by combining AZD3965 with metformin (Figure 5A). However, the concentrations of metformin required to demonstrate this effect were significantly in excess of those that can be achieved in mice following oral dosing.<sup>27</sup> We therefore examined the potent complex I inhibitor BAY 87-2243, which would subsequently permit inhibition of OXPHOS to be studied in mice.<sup>28</sup> While 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 (Figure 5B,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 after inoculation ( $P > 0.05$  for all group comparisons by two-way ANOVA; Figure 6B), prior to dosing. Following treatment (72 h 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 the two-tailed *t*-test). While 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 four of five mice, with signal intensities being reduced by between 10- to 267-fold (Figure 6B;  $P = 0.01$  by the Pearson  $\chi^2$  test). This is consistent with the

induction of lymphoma cell death *in vivo*. The combination treatment regimen was well tolerated with no significant differences in body weight when compared to that in 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.

Although a glycolytic phenotype and increased generation of lactate are implicated in the pathogenesis of both DLBCL and BL, the relative expression of MCT1 *versus* MCT4 in DLBCL has been less clear. A previous study 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 lymphomas that would have contained predominantly DLBCL samples.<sup>12</sup> 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 among both cell-of-origin subgroups, and in groups with and without any *MYC* aberration. This suggests that all major DLBCL subgroups contain patients with an MCT1-positive/MCT4-negative protein expression profile, who may be appropriate candidates to receive AZD3965 treatment.

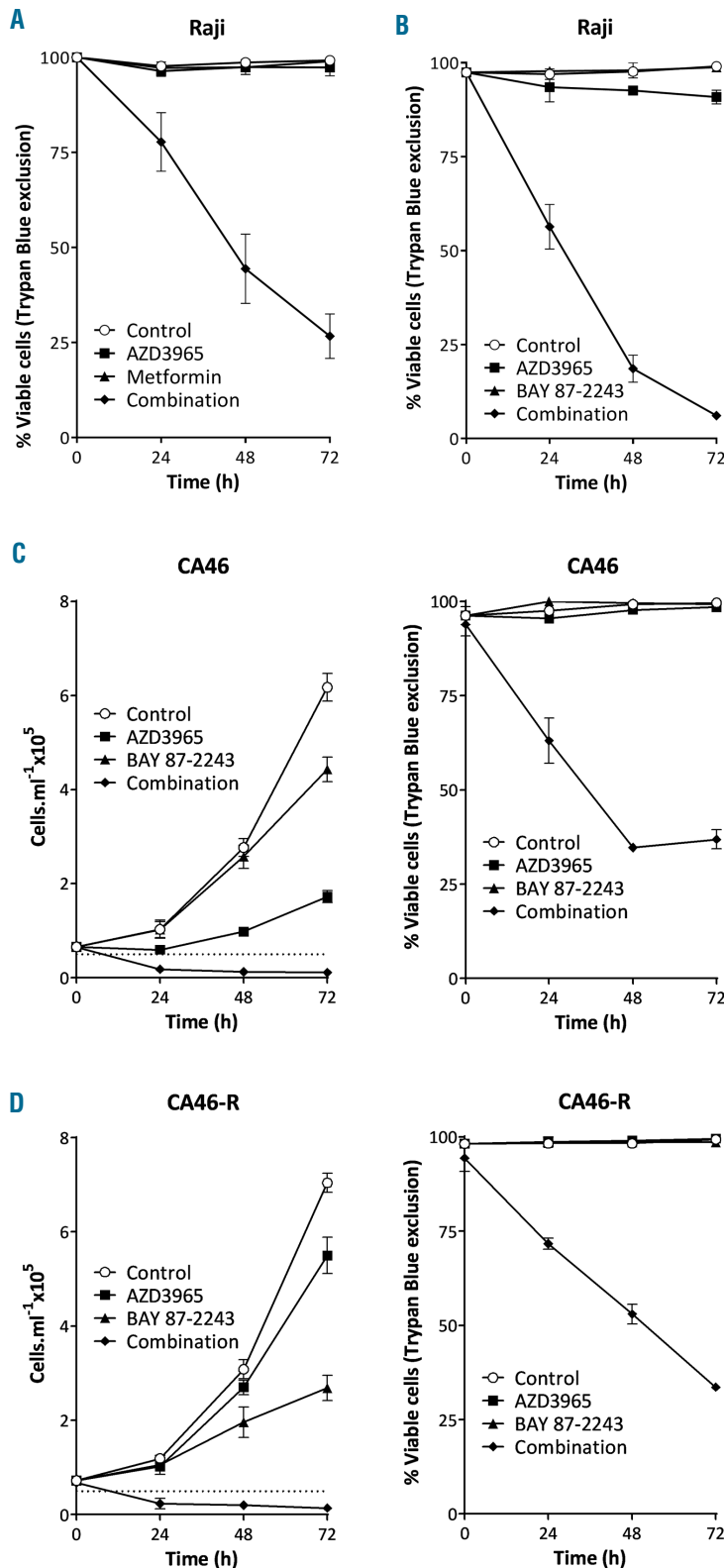
Additional experiments examining neuroblastoma cell lines (IMR-32 and SH-SY5Y; *Online Supplementary Figure S6*) and a previous study on small cell lung cancer cells<sup>7</sup> demonstrated that only partial sensitivity to AZD3965 can be observed in solid tumor cell lines lacking MCT4 expression, despite significant lactate accumulation. Solid tumor cell lines also have a much broader spectrum of MCT4 mRNA expression in comparison to those of hematologic origin (*Online Supplementary Figure S1*). Although these data do not exclude AZD3965 as being suitable for the treatment of a subset of solid tumors, the more potent GI<sub>50</sub> 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 the clinical activity of AZD3965.

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. Prospective stratification of patients 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 effect of AZD3965 on both intracellular lactate concentration and 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.<sup>12,29</sup>

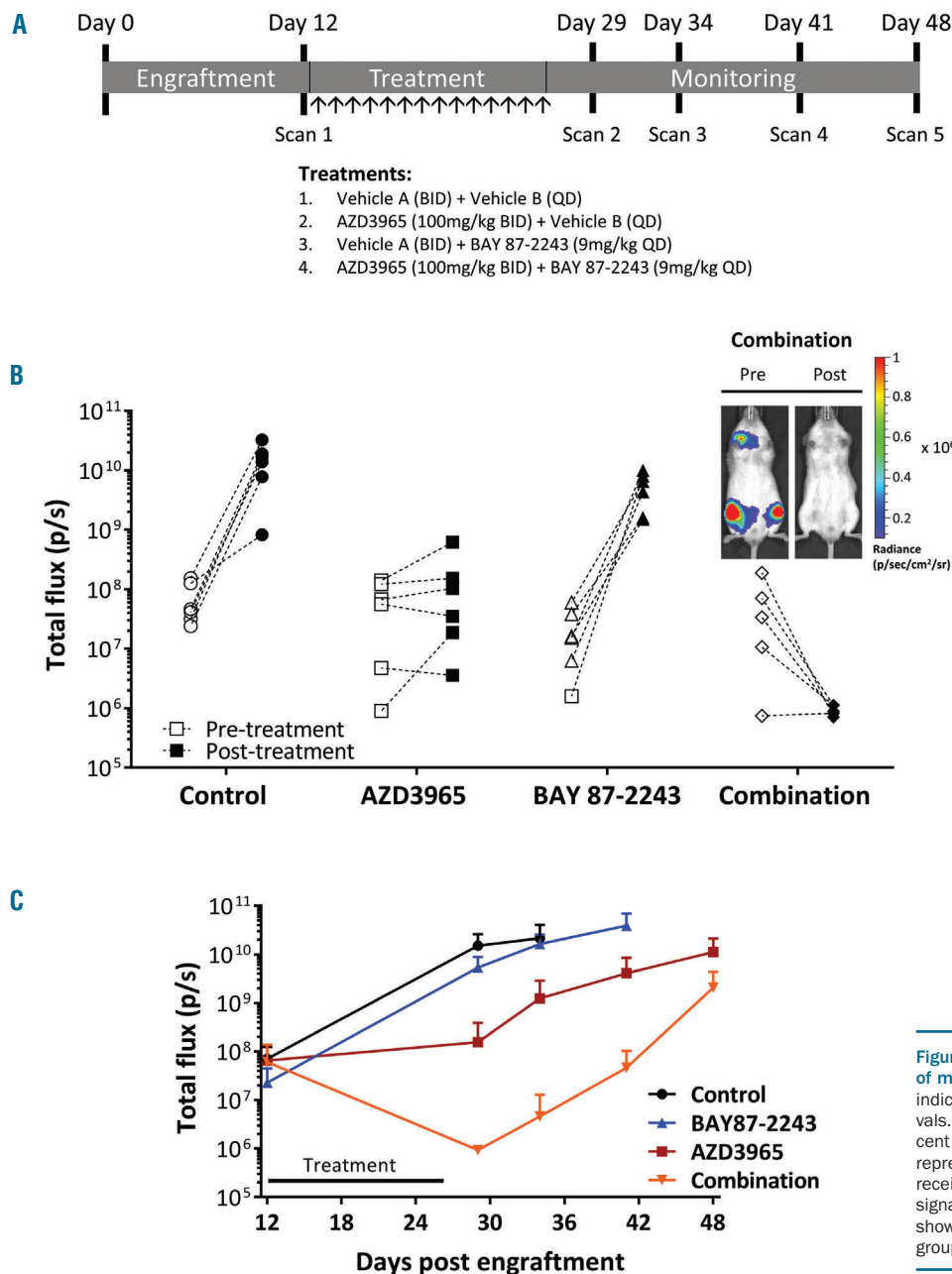


The effect of inhibiting MCT1 in DLBCL and BL cell lines *in vitro* was predominantly cytostatic. Encouragingly, however, we also observed a striking cytostatic response *in vivo* with AZD3965 treatment, which halted progressive splenic engraftment of the lymphoma. The incidence of BL is increased in elderly patients and the median age of

diagnosis for DLBCL is around 70 years.<sup>30,31</sup> 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 group of patients or in individuals who have relapsed following current standard-of-care treatment.



**Figure 5. Combining AZD3965 with inhibitors of mitochondrial complex I induces death of Burkitt lymphoma cells.** Viable cell numbers were determined by cell counting with trypan blue exclusion over a 72 h 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 show the means of  $\geq 3$  independent experiments  $\pm$  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 that received the combination (inset). (C) Change in signal intensity subsequent to treatment. Graph shows the mean + SD total flux (n ≥ 5 per group).

The compensatory metabolic alterations observed after AZD3965 treatment *in vitro*, following either acute or chronic exposure, support an adaptive metabolic response that causes a greater reliance on OXPHOS and increased TCA activity. In order to elicit tumor cell death, when targeting the glycolytic tumor phenotype 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 5A-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 treat-

ment of children, in whom BL accounts for the majority of non-Hodgkin lymphoma.<sup>32</sup>

Collectively, the striking activity of AZD3965 monotherapy 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.

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