Viruses are obligate intracellular pathogens which rely on the cell’s machinery to produce the energy and macromolecules required for replication. Infection is associated with a modified metabolic profile and one pathway which can be modified is glycolysis. In this study, we investigated if the glycolysis pathway is required for alphavirus replication. Pre-treatment of Vero cells with three different glycolysis inhibitors (2-deoxyglucose, lonidamine and oxamate) resulted in a significant reduction (but not abrogation) of Semliki Forest virus and Sindbis virus replication, but not of the unrelated virus, vaccinia virus. Reduced virus yield was not associated with any significant cytotoxic effect and delayed treatment up to 3 h post-infection still resulted in a significant reduction. This suggested that glycolysis is required for optimal replication of alphaviruses by supporting post-entry life cycle steps.

Alphaviruses are zoonotic arboviruses that can infect humans and other vertebrate hosts. Pathogenic members of this group include Venezuelan equine encephalitis virus (VEEV), western equine encephalitis virus, eastern equine encephalitis virus and chikungunya virus (CHIKV). Epizootic strains of VEEV cause sporadic outbreaks of encephalitis in humans in the Americas (Go et al., 2014). In addition, these alphaviruses are listed as Category B agents by the US Centers for Disease Control and Prevention. CHIKV causes severe joint pain and fever in humans, and has historically been present in Asia, Africa, and has recently spread to Europe, the Caribbean, and Central and South America (Morrison, 2014; Rezza et al., 2007). It has been reported that infection by two alphaviruses, Mayaro virus and Sindbis virus (SINV), results in increased glucose uptake and glycolytic flux in cell lines (El-Bacha et al., 2004; Silva da Costa et al., 2012). This suggests that glycolysis may be important in the alphavirus life cycle. If so, this may open up the use of glycolysis inhibitors that have been designed for cancer treatment as potential antivirals for alphavirus infections. In this study, we investigated if the glycolysis pathway is required for alphavirus replication and if disruption of glycolysis could be an antiviral strategy.

Vero cells (African Green monkey kidney cells) were incubated for 16 h in minimal Dulbecco’s modified Eagle’s medium (lacking glutamine and pyruvate, and supplemented with 1 mg glucose ml⁻¹ and 2 % FCS) either in the absence or presence of two concentrations of glycolysis inhibitors. All incubations were performed at 37 °C in a 5 % CO₂, humidified atmosphere. The experiments were performed in the absence of glutamine and pyruvate as these can be used to produce acetyl-CoA, and therefore could overcome the imposed block by glycolysis inhibitors. Three glycolysis inhibitors (Sigma-Aldrich) were used: 2-deoxyglucose (2-DG), an analogue of glucose, which is converted by hexokinase II to an intermediate that cannot be further used in glycolysis, was used at 2 and 10 mM; lonidamine (LON), a hexokinase II inhibitor, which prevents the first step of glycolysis, was used at 62.5 and 312.5 μM; and oxamate (OX), a lactate dehydrogenase (LDH) inhibitor, which prevents the recycling of NADH, was used at 16 and 80 mM. The concentrations of inhibitors used were based
on their reported inhibitory concentrations of glycolysis in cancer cells (Elwood, 1968; Hulleman et al., 2009). After 16 h incubation, the cells were infected with Semliki Forest virus (SFV) at m.o.i. 3 TCID₅₀ per cell. Cultures were incubated for 24 h post-infection (p.i.) to ensure maximum yield of progeny in untreated cultures, after which the released virus was titrated by TCID₅₀ assay (Butcher & Ulaeto, 2005). All three inhibitors resulted in a significant decrease in yield, with 2-DG and OX causing >3 log drop in virus yield, whereas LON caused a 1 log drop (Fig. 1a). To determine if this was specific to SFV, these experiments were repeated using SINV at m.o.i. 3 (Fig. 1b). Treatment of the cells with all three inhibitors significantly reduced SINV yield at the highest concentration, with 2-DG and OX causing a 2 log drop and LON causing a 1 log drop. To determine if this was specific to alphaviruses, the experiment was repeated using 2-DG and an unrelated virus, the orthopoxvirus vaccinia virus (VACV). Treatment of the cells with 10 mM 2-DG did not result in a significant decrease in VACV replication (Fig. 1c). Therefore, a requirement for glycolytic ATP production (to be used for energy generation or as substrate for polynucleotide synthesis) is not a universal feature of virus replicative strategies.

In order to exclude drug cytotoxicity as the mediator of inhibition, viability assays were undertaken with the three compounds. Uninfected cells were incubated with either 10 mM 2-DG, 312.5 µM LON or 80 mM OX for 40 h (the timespan between addition of inhibitor and harvesting of virus) and the culture supernatant tested for LDH activity. Treatment with the glycolysis inhibitors did not result in increased LDH release relative to untreated controls and in fact appeared to reduce LDH release from the cultures (Fig. 2a). However, OX is a LDH inhibitor, which could thus confound the LDH assay for this drug. Consequently the experiment was repeated using the highest concentration of inhibitors, and the cells were analysed for membrane integrity by incubation with SYTOX Orange membrane-impermeable dye according to the manufacturer’s instructions and analysis on a FACS Canto flow cytometer (Fig. 2b). Despite a twofold increase in membrane permeability for cells treated with OX, the inhibitors did not result in a statistically significant increase in the percentage of cells with damaged membranes compared with the control. Therefore, the reduction in virus yield was not due to cellular toxicity of the drugs.

It has been reported that inhibition of glycolysis with 2-DG can prevent influenza A virus entry by a mechanism involving prevention of endosomal acidification (Kohio & Adamson, 2013). To examine if disruption of glycolysis was affecting the entry of SFV and SINV, Vero cells were either: pre-treated with 2-DG for 16 h and then infected by SFV or SINV; treated with 2-DG at the same time as infection (T₀); or infected and then treated with 2-DG at 3 h p.i. (T₃). At each treatment point, there was a significant reduction in both SFV and SINV yield compared with controls (Fig. 3). Although no SFV was detected in these experiments, the assay used had a limit of detection of 1 × 10² TCID₅₀ ml⁻¹. Consequently progeny virus may have been produced at <1 × 10² TCID₅₀ ml⁻¹ in these samples. Nonetheless, addition of 2-DG 3 h after infection clearly resulted in a significant reduction, indicating that 2-DG-mediated inhibition of replication appears to operate after the phase entry for SFV and SINV. In these experiments, the inhibitor was added to wells without prior removal of the virus inoculum. Although it is possible that the inhibitor was acting to prevent binding or entry of virus after the 3 h incubation period, the published kinetics of SFV and SINV

![Fig. 1. Glycolysis inhibitors reduce alphavirus but not orthopoxvirus yield. Vero cells pre-treated with 2-DG, LON and OX were then infected with (a) SFV, (b) SINV or (c) VACV. At 24 h p.i., the released virus was titrated by TCID₅₀. The data were analysed using a paired t-test (with Bonferroni’s post-test correction where appropriate). *P<0.05; **P<0.01; ***P<0.001.](image-url)
binding and entry indicate that the majority of virus is expected to have entered the cell after 3 h incubation at 37 °C at the m.o.i. used in this study (Byrnes & Griffin, 1998; Helenius et al., 1980; Jan et al., 2000).

Viruses are dependent on cell metabolism for replication and infection by a range of viruses, including alphaviruses, is associated with an increase in glycolysis/glycolytic flux. Pre-treatment of Vero cells with three different glycolysis inhibitors, targeting different steps/enzymes in the glycolysis pathway, resulted in a significant reduction of released SFV and SINV (Fig. 1). Reduced virus yield was not associated with any significant cytotoxic effect (Fig. 2) and delayed treatment up to 3 h p.i. still resulted in a significant reduction that was, however, not significantly different from pre-treatment regimens (Fig. 3). This suggests that glycolysis is required for a post-entry step, unlike the situation with influenza A (Kohio & Adamson, 2013). Treatment with the inhibitors reduced, but did not completely abrogate, replication of SFV and SINV. Therefore, glycolysis appears to be required for optimal SFV and SINV replication. Whilst it is not clear precisely what step(s) in virus replication the glycolysis inhibitors is disrupting, it is likely to be one with a significant energy requirement. However, treatment with 2-DG did not reduce VACV replication (Fig. 1c). This supports a recent finding that glycolysis was not induced in VACV-infected cells and that the removal of glutamine, but not glucose, from culture medium resulted in reduced replication (Fontaine et al., 2014). As the same cell line was used for each virus, this could suggest that the inhibitors are affecting biosynthetic and/or bioenergetic pathways that are required for alphaviruses, but not for orthopoxviruses. Further work is required to examine this issue.

Interestingly, 2-DG and OX had a more profound effect than LON on alphaviruses (Fig. 1). As these inhibitors all target the glycolysis pathway, either LON is less efficient at inhibition of glycolysis than the other two or 2-DG and OX are exhibiting ‘off-target’ effects. LON targets hexokinase II, an enzyme which has been shown to be upregulated by several viruses, including dengue virus (Fontaine et al., 2015). Upregulation could offset the effect of LON. Furthermore, 2-DG, as well as being a glycolysis inhibitor, can affect protein glycosylation and activate the unfolded protein response (Kurtoglu et al., 2007; Xi et al., 2011). As alphaviruses rely on glycoproteins for entry and the endoplasmic reticulum stress response can inhibit translation, these off-target activities could explain the increased antiviral activity of 2-DG. As a LDH inhibitor, OX leads to the accumulation of lactate, which is known to cause a detrimental reduction in intracellular pH and reduced cell metabolism (Patel et al., 2000). Therefore, OX could also have a secondary effect that might potentiate direct glycolysis-associated inhibition of virus replication. Whilst the glycolysis inhibitors affected both SFV and SINV, SINV was less sensitive to these inhibitors than SFV (Fig. 1). It seems likely that this difference must have a genetic basis, e.g. a species-specific
interaction with a cellular pathway. In addition, phylogenetic studies divide the alphaviruses into three clades, with SINV in a separate group to SFV (Levinson et al., 1990). Therefore, further studies using a range of alphaviruses would be required to determine if the differential inhibition is based on the phylogenetic distinction between the different clades of alphaviruses. Such analysis, coupled with mutation studies, could provide additional insight into the strategies employed by viruses to meet their metabolic requirements in host cells.

References