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Akt inhibitors reduce glucose uptake independently of their effects on Akt

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The protein kinase Akt is involved in various cellular processes, including cell proliferation, growth and metabolism. Hyperactivation of Akt is commonly observed in human tumours and so this pathway has been the focus of targeted drug discovery. However, Akt also plays an essential role in other physiological processes, such as the insulin-regulated transport of glucose into muscle and fat cells. This process, which is essential for whole-body glucose homoeostasis in mammals, is thought to be mediated via Akt-dependent movement of GLUT4 glucose transporters to the plasma membrane. In the present study, we have investigated the metabolic side effects of non-ATP-competitive allosteric Akt inhibitors. In 3T3-L1 adipocytes, these inhibitors caused a decrease in the Akt signalling pathway concomitant with reduced glucose uptake. Surprisingly, a similar reduction in GLUT4 translocation to the plasma membrane was not observed. Further investigation revealed that the inhibitory effects of these compounds on glucose uptake in 3T3-L1 adipocytes were independent of the Akt signalling pathway. The inhibitors also inhibited glucose transport into other cell types, including human erythrocytes and T-47D breast cancer cells, suggesting that these effects are not specific to GLUT4. We conclude that these drugs may, at least in part, inhibit tumorigenesis through inhibition of tumour cell glucose transport.

Key words: adipocyte, Akt inhibitor, cancer, erythrocyte, glucose transporter 4 (GLUT4), insulin.

INTRODUCTION

The protein kinase Akt plays a key role in many biological processes, including cell survival, proliferation, growth and metabolism [1,2]. Activation of Akt involves binding of an extracellular ligand to its cognate tyrosine kinase receptor, leading to the activation of PI3K (phosphoinositide 3-kinase) and generation of PtdIns(3,4,5)P_3. This facilitates the translocation of Akt to the PM (plasma membrane) where it is phosphorylated at Thr^{308} and Ser^{473} by PDK1 (phosphoinositide-dependent kinase 1) and the mTOR (mammalian target of rapamycin)/rictor (rapamycin-insensitive companion of mTOR) complex respectively [3,4]. Upon activation, Akt phosphorylates a range of substrates that control a variety of key biological processes.

Hyperactivation of Akt is commonly observed in human tumours. This can occur due to mutations that lead to hyperactive PI3K or a reduction in the activity of PTEN (phosphatase and tensin homologue deleted on chromosome 10) [5,6]. Moreover considerable evidence indicates that, at least for cancer cells demonstrating hyperactivation of this pathway, decreased Akt signalling reduces cancer cell proliferation and promotes cell death [7–9]. For these reasons, drugs targeting the PI3K/Akt pathway have been developed in the hope that they will serve as novel cancer therapeutics [7,8,10].

The obvious limitation to this approach is that Akt activity is implicated in many cell types and key biological processes other than cell proliferation. Most notably, Akt has been shown to play an essential role in metabolism. Its activation is important for the translocation of the insulin-responsive glucose transporter GLUT4 (glucose transporter 4) to the PM to mediate glucose uptake [11]. Therefore one potential side effect of drugs that reduce signalling through the PI3K/Akt pathway is possibly decreasing glucose uptake thus increasing the risk of diabetes.

There are several Akt isoforms. Akt1 is expressed in many cells, but its function is more closely linked to growth and proliferation. Mice lacking Akt1 are small throughout their life, but have normal glucose homoeostasis [12]. On the other hand, Akt2 is more involved in metabolism. Akt2-null mice developed insulin resistance and diabetic-like syndromes [13]. Furthermore, loss-of-function mutations in Akt2 have been linked to Type 2 diabetes [14]. This kind of evidence provides the potential for developing isoform-specific inhibitors that might reduce possible off-target effects. A number of pharmacological inhibitors against Akt that demonstrate therapeutic potential have been described in recent years [7,8,10]. In particular, non-ATP-competitive and allosteric compounds that are selective for Akt1 (Akt1i), Akt2 (Akt2i) or for both Akt1 and Akt2 isoforms (Akt1/2i) have been described [8]. These highly selective compounds inhibit the activity of full-length Akt but not PH domain (pleckstrin homology domain)-deleted Akt and they have minimal effects on a comprehensive panel of other kinases [8,15,16]. These inhibitors inhibit insulin-induced Akt phosphorylation at Thr^{308} and Ser^{473} *in vivo*, blocking the phosphorylation of known Akt substrates and preventing glucose uptake in L6 myotubes [17].

In the present study, we make use of these Akt inhibitors to investigate the impact of rapid inactivation of Akt on insulin-induced glucose uptake and the translocation of GLUT4 to the PM in 3T3-L1 adipocytes. We found that the Akt inhibitors potently inhibit Akt phosphorylation and phosphorylation of a range of Akt substrates. Surprisingly, the Akt inhibitors were found to prevent glucose uptake with a minimal effect on GLUT4 translocation to the PM. Further experiments showed that these inhibitors blocked

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FoxO, forkhead box O; GLUT, glucose transporter; GSK3, glycogen synthase kinase 3; HA, haemagglutinin; HRP, horseradish peroxidase; KRP, Krebs–Ringer phosphate; MAPK, mitogen-activated protein kinase; MeAIB, 2-methylaminoisobutyric acid; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PM, plasma membrane; TBS, Tris-buffered saline; TSC2, tuberous sclerosis complex 2.

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glucose transport in 3T3-L1 adipocytes independent of their effect on the Akt signalling pathway. The Akt inhibitors also reduced glucose transport in human erythrocytes that are rich in GLUT1 and in T-47D breast cancer cells. Our data indicate that the Akt inhibitors affect glucose uptake in different cell types and this may have potential implications for using these inhibitors as *in vivo* therapeutics.

EXPERIMENTAL

Materials and antibodies

Polyclonal rabbit antibodies raised against total Akt, phospho-Thr^{308} Akt, phospho-Thr^{1462} TSC2 (tuberous sclerosis complex 2), phosph-Ser^{256} FoxO (forkhead box O), phospho-Ser$^{21/9}$ GSK3α/β (glycogen synthase kinase 3α/β) and phospho-Thr^{202}/Tyr^{204} MAPK (mitogen-activated protein kinase), and a monoclonal mouse antibody raised against phosho-Ser^{473} Akt were purchased from Cell Signaling Technologies. A polyclonal rabbit antibody raised against 14-3-3β was purchased from Santa Cruz Biotechnology. HRP (horseradish peroxidase)-conjugated secondary antibodies were from Amersham Biosciences, and IRDye® 700- or IRDye® 800-conjugated secondary antibodies were from Rockland Immunochemicals. Paraformaldehyde was from ProSciTech. DMEM (Dulbecco's modified Eagle's medium), RPMI 1640 medium and newborn calf serum were from Invitrogen. FBS (fetal bovine serum) was from Trace Scientific, and antibiotics were from Invitrogen. BSA was from Bovogen. Bicinchoninic acid reagent and SuperSignal West Pico chemiluminescent substrate were from Pierce. Protease inhibitor mixture tablets were from Roche Applied Science. Other materials were obtained from Sigma. The Akt1-specific and Akt2-specific inhibitors have been described previously [8] and were obtained from Merck. The Akt1/2 inhibitor described previously [8] was obtained from Symansis (Auckland, New Zealand).

Cell culture

3T3-L1 fibroblasts (A.T.C.C.) were cultured and differentiated to adipocytes as described previously [18]. To generate 3T3-L1 adipocytes stably expressing HA (haemagglutinin)–GLUT4, fibroblasts were infected with pBabepuro-HA-GLUT4 retrovirus. After a 24-h recovery period, infected cells were selected in DMEM containing 10 % (v/v) FBS and 2 μg/ml puromycin. Polyclonal pools of puromycin-resistant 3T3-L1 fibroblasts were then grown to confluence and subsequently differentiated into adipocytes as described above. T-47D human breast cancer cells were cultured in RPMI 1640 medium containing 10 % (v/v) FBS, 2 μM insulin, 100 units/l penicillin, 100 μg/l streptomycin and 2 mM L-glutamine.

Western blot analysis

Cells were washed twice with ice-cold PBS and solubilized in 2 % (w/v) SDS in PBS containing phosphatase inhibitors (1 mM sodium pyrophosphate, 2 mM sodium vanadate and 10 mM sodium fluoride) and complete protease inhibitor mixture. Insoluble material was removed by centrifugation at 18000 ***g*** for 10 min. The protein concentration was measured using the bicinchoninic acid method. Proteins were separated by SDS/PAGE for immunoblot analysis. After transferring proteins on to PVDF membranes, membranes were incubated in blocking buffer containing 5 % (w/v) skimmed milk powder in TBS (Tris-buffered saline) and immunoblotted with the relevant antibodies overnight at 4 °C in blocking buffer containing 5 % (w/v) BSA and 0.1 % Tween 20 in TBS. After incubation, membranes were washed and incubated with HRP-labelled secondary antibodies and then detected by SuperSignal West Pico chemiluminescent substrate. In some cases, IRDye® 700- or IRDye® 800-conjugated secondary antibodies were used and then scanned at the 700 and 800 nm channel using the Odyssey IR imager. Quantification of the protein levels was performed using Odyssey IR imaging system software or the Wright Cell Imaging Facility ImageJ software.

Quantitative GLUT4 translocation assay

HA–GLUT4 translocation to the PM was measured as described previously [19]. Briefly, 3T3-L1 adipocytes stably expressing HA–GLUT4 in 96-well plates were serum-starved with KRP (Krebs–Ringer phosphate) buffer [0.6 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 120 mM NaCl, 6 mM KCl, 1mM $CaCl_2$, 1.2 mM $MgSO_4$ and 12.5 mM Hepes (pH 7.4)] supplemented with 0.2 % BSA for 2 h. Cells were then treated with DMSO or the indicated compound for 30 min prior to 20 min of insulin stimulation. After stimulation, cells were fixed and immunolabelled with a monoclonal anti-HA antibody, followed by an Alexa Fluor® 488-labelled secondary antibody in the absence or presence of saponin, to analyse the amount of HA–GLUT4 at the PM or the total HA–GLUT4 content respectively.

2-Deoxyglucose uptake assay

Uptake of 2-deoxyglucose into different cell types was performed with slight modifications to standard published methods [20–22]. 3T3-L1 fibroblasts were seeded and differentiated into adipocytes in 24-well plates. Cells were washed twice and incubated with KRP buffer supplemented with 0.2 % BSA for 2 h before treatment of cells with inhibitors prior to insulin stimulation for the indicated times. Glucose transport was initiated by addition of 2-[^{3}H]deoxyglucose (PerkinElmer) (0.25 μCi, 50 μM) for 5 min. To determine non-specific glucose uptake, 25 μM cytochalasin B was added into the wells before addition of 2-[^{3}H]deoxyglucose. Uptake was terminated with three rapid washes in ice-cold PBS, after which the cells were solubilized in 1 % (v/v) Triton X-100 in PBS. Samples were assessed for radioactivity by scintillation counting using a β-scintillation counter. The non-specific uptake was subtracted and results were normalized for protein content using bicinchoninic acid analysis. For T-47D cells, 2-deoxyglucose uptake was measured as described for 3T3-L1 adipocytes without the addition of insulin. Uptake rates for 3T3-L1 adipocytes and T-47D cells were expressed as nmol of glucose/min per mg of protein. For measurements in erythrocytes, human blood samples were obtained from two individuals following consent. Erythrocytes were purified from 10 ml of blood. Cells were washed three times with 5 vol. of PBS (pH 7.4) by centrifugation at 4000 ***g*** for 5 min at 4 °C. The pellet was resuspended in isotonic buffered solution [125 mM NaCl, 5 mM KCl, 3.8 mM $CaCl_2$, 2.5 mM $MgCl_2$ and 5 mM Tris/HCl (pH 7.4)] containing 0.5 mM 2-deoxyglucose. Uptake of 2-[^{3}H]deoxyglucose was performed using 200 μl of cells ($\sim 2 \times 10^8$ cells). 2-[^{3}H]deoxyglucose (10.1 μl; 0.5 μCi, 0.5 mM) was added to the cells for 10 s. Assays were stopped by adding 8 vol. of isotonic buffer containing 2 mM mercuric chloride. Cells were centrifuged at 14000 ***g*** for 30 s at 4 °C. The pellet was washed once with isotonic buffer containing 2 mM mercuric chloride and lysed in 500 μl of water. A portion (400 μl) of the lysates was added to 4 ml of scintillation fluid and counted for radioactivity.

Glucose uptake rate for erythrocytes was expressed as nmol of glucose/min per 10^8 cells.

Amino acid uptake

Amino acid uptake experiments were performed as described previously [23] with minor modifications. 3T3-L1 fibroblasts were seeded and differentiated into adipocytes in 24-well plates. To determine the sodium-dependent uptake of the alanine analogue MeAIB (2-methylaminoisobutyric acid), cells were washed three times with sodium-containing buffer [135 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 10 mM Tris/HCl (pH 7.5) with 0.2 % BSA] and incubated at 37 °C for 2 h. Cells were treated with the indicated inhibitors for 30 min prior to initiation of MeAIB uptake. Uptake of MeAIB was initiated by addition of ^{14}C-MeAIB (PerkinElmer) (0.025 μCi, 100 μM) for 5 min. Uptake was stopped by rapidly washing the cells three times with ice-cold PBS, after which the cells were solubilized in 1 % (w/v) SDS in PBS. To isolate the sodium-independent amino acid uptake system, NaCl was substituted with choline chloride in the buffer. Sodium-independent uptake of leucine was initiated by addition of L-[^{3}H]leucine (PerkinElmer) (0.25 μCi, 100 μM) for 30 s. Uptake was terminated with three rapid washes in ice-cold choline buffer, after which the cells were solubilized in 1 % (w/v) SDS in PBS. Samples were assessed for radioactivity by scintillation counting using a β-scintillation counter. Uptake rates were expressed as nmol of glucose/min per mg of protein

RESULTS

Effects of Akt inhibitors on Akt phosphorylation and activity in 3T3-L1 adipocytes

Insulin stimulation of 3T3-L1 adipocytes resulted in robust phosphorylation of Akt at Thr^{308} and Ser^{473} (Figure 1). Phosphorylation of Akt on Thr^{308} and Ser^{473} was inhibited to a different extent when using either Akt1i or Akt2i (Figure 1). Akt2i inhibited insulin-dependent activation of Akt to a greater extent than Akt1i (Figures 1A and 1B). Akt1i inhibited Akt phosphorylation at Thr^{308} and Ser^{473} by 20 and 50 % respectively, whereas Akt2i inhibited the phosphorylation of the same residues by 40 and 80 % respectively (Figures 1A and 1B). One explanation for the greater inhibition by Akt2i is because Akt2 is the predominant isoform in 3T3-L1 adipocytes [24]. Treatment of 3T3-L1 adipocytes with both Akt1i and Akt2i led to a further reduction in Akt phosphorylation. Akt1/2i, which targets both Akt1 and Akt2 isoforms, resulted in almost complete inhibition of Akt activation, similar to that observed with the PI3K inhibitor wortmannin (Figures 1A and 1B). The difference in the degree of inhibition between each inhibitor may also be attributed to the relative potency of each inhibitor [8]. Consistent with inhibition of Akt activity, phosphorylation of a range of Akt substrates, such as FoxO and GSK3α/β, was also reduced by the inhibitors to different extent (Figures 1A and 1C). These data are in line with previous studies showing that Akt inhibitors potently inhibit Akt phosphorylation and reduce the phosphorylation of downstream signalling components [8,15–17].

Effects of Akt inhibitors on glucose transport and GLUT4 translocation in 3T3-L1 adipocytes

Rapid activation of Akt2 alone is sufficient to stimulate GLUT4 translocation to the PM in 3T3-L1 adipocytes [11]. Therefore

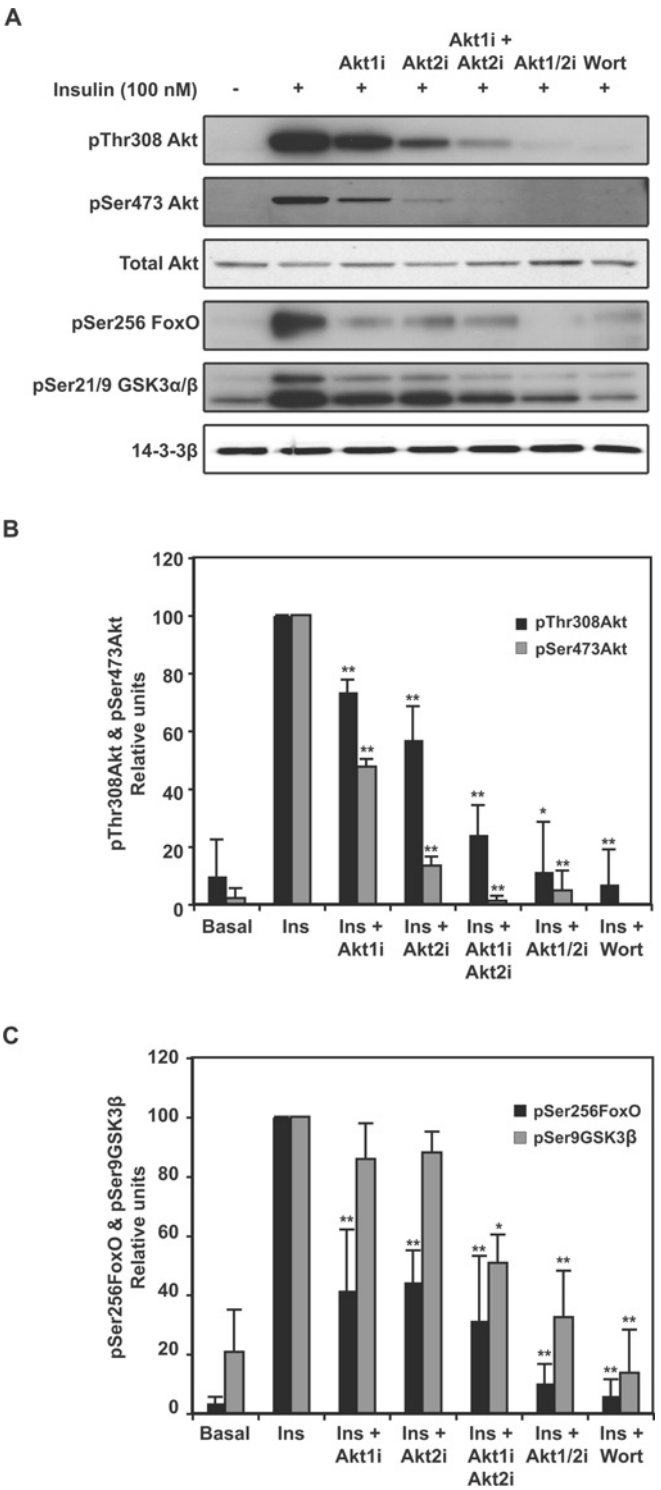

Figure 1 Effects of Akt inhibitors on Akt signalling in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were serum-starved in KRP buffer for 2 h. Cells were treated with 0.1 % DMSO, 10 μM of the Akt inhibitors (Akt1i, Akt2i, Akt1i + Akt2i or Akt1/2i) or 100 nM wortmannin (Wort) for 30 min prior to exposure to 100 nM insulin (Ins) for 20 min. (**A**) Total cell lysates were immunoblotted with phospho-Thr^{308} Akt, phospho-Ser^{473} Akt, total Akt, phospho-Ser^{256} FoxO, phospho-Ser$^{21/9}$ GSK3α/β and 14-3-3 antibodies. (**B**) Quantification of phospho-Ser^{473} Akt and phospho-Thr^{308} Akt. Data were normalized to total Akt as the loading control and are expressed as relative units to the insulin response. (**C**) Quantification of phospho-Ser^{256} FoxO and phospho-Ser^{9} GSK3β. Data were normalized to 14-3-3 as the loading control and are expressed as relative units to the insulin response. Results are means ± S.D, $n = 3$. $^*P < 0.05$ and $^{**}P < 0.01$ compared with insulin treatment alone.

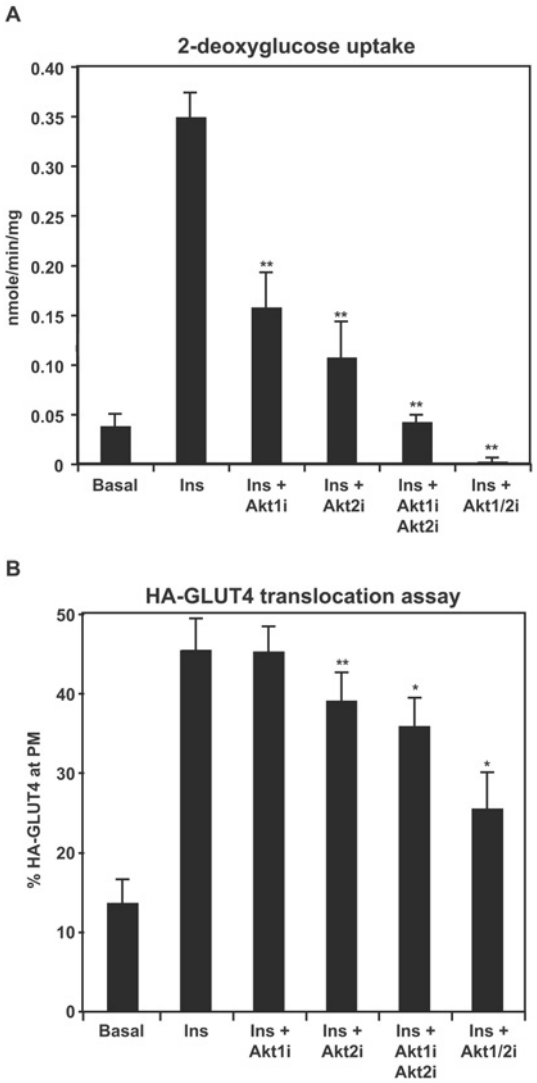

Figure 2 Discrepancy between glucose transport and HA–GLUT4 translocation to the PM in 3T3-L1 adipocytes treated with Akt inhibitors

(**A**) 3T3-L1 adipocytes were serum-starved in KRP for 2 h prior to exposure to either 0.1 % DMSO or 10 μM of the Akt inhibitors (Akt1i, Akt2i, Akt1i + Akt2i or Akt1/2i) for 30 min. Cells were then treated with 100 nM insulin (Ins) for 20 min. Glucose uptake assays were performed as indicated in the Experimental section. Results are means $\pm$ S.D, $n = 3$. $^{**}P < 0.01$ compared with insulin alone. (**B**) 3T3-L1 fibroblasts retrovirally infected with HA–GLUT4 were differentiated into adipocytes and treated as above. The amount of HA–GLUT4 at the PM was determined by anti-HA fluorescence immunolabelling of non-permeabilized cells, and is expressed as a percentage of total cellular HA–GLUT4, determined by labelling of permeabilized cells. Results are means $\pm$ S.D, $n = 3$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with insulin alone.

we next determined the effects of the isoform-specific Akt inhibitors on GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. Treatment of 3T3-L1 adipocytes with either Akt1i or Akt2i resulted in a significant reduction ($\sim$50–70 %) in glucose uptake (Figure 2A). An almost complete inhibition of glucose uptake was observed when cells were treated with both Akt1i and Akt2i or Akt1/2i (Figure 2A). To verify that these effects were due to inhibition of Akt, we next examined the effects of these inhibitors on GLUT4 translocation since this process underpins insulin-stimulated glucose transport in these cells [25] and GLUT4 is the major insulin-responsive glucose transporter in adipocytes [26]. Surprisingly, treatment of 3T3-L1 adipocytes with the Akt inhibitors had a relatively minor effect on GLUT4 translocation compared with the effect on glucose transport (Figure 2).

Akt inhibitors prevent glucose uptake independently of their effects on Akt phosphorylation

The discrepancy between the effect of the Akt inhibitors on glucose uptake and GLUT4 translocation raised the possibility that these compounds may directly inhibit the glucose transport activity of the transporter at the membrane independently of Akt activity. Two approaches were employed to test this possibility. In the first approach, cells were first treated with insulin at 37 °C to activate Akt and facilitate GLUT4 translocation to the PM. Following insulin treatment, cells were rapidly chilled to 4 °C on ice. This treatment 'freezes' the signalling process and prevents endocytosis of GLUT4. Insulin treatment of cells resulted in a 7-fold increase in glucose uptake as compared with basal cells when glucose uptake was performed at 4 °C (Figure 3A). Treatment of cells with the inhibitors for 5 min at 4 °C had no effect on phosphorylation of Akt at Ser^{473} or phosphorylation of its substrates FoxO and GSK3α/β (Figure 3A), whereas glucose transport was significantly inhibited by all of the Akt inhibitors. The PI3K inhibitor wortmannin had no significant effect on insulin-stimulated glucose transport under these conditions, indicating that the inhibitory effect is unlikely to be due to the effect of the Akt inhibitors inhibiting a PM pool of Akt (Figure 3A).

The second approach relies on the observation that sucrose-induced osmotic shock prevents GLUT4 endocytosis, resulting in its accumulation at the PM in an Akt-independent manner [27,28]. Consistent with previous studies, osmotic shock resulted in activation of MAPK [29], but not Akt [28,30] (Figure 3B). Osmotic shock increased glucose uptake by approx. 10-fold (Figure 3B). Treatment of cells with the Akt inhibitors significantly reduced osmotic-shock-induced glucose uptake (Figure 3B). Wortmannin did not exert any effect on glucose uptake under these conditions. Collectively, the data obtained from these two separate approaches indicate that the Akt inhibitors reduce glucose uptake into 3T3-L1 adipocytes independently of their effects on Akt activity.

To determine whether the Akt inhibitors could modulate alternate transport systems, we next examined their effect on amino acid uptake in 3T3-L1 adipocytes. The Akt inhibitors did not effect the sodium-dependent uptake of the alanine analogue MeAIB nor the sodium-independent uptake of leucine (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/432/bj4320191add.htm), indicating that the inhibitory effects of these compounds is likely to be confined to the facilitative glucose transporter family.

Reduction in glucose uptake by the Akt inhibitors in human erythrocytes and T-47D breast cancer cells

It is unclear from the studies described above whether the effects of these inhibitors to block glucose transport are specific to cells expressing the GLUT4 transporter. Thus we next embarked on a series of studies to examine the effects of these inhibitors in cell types that predominantly express alternate glucose transporter isoforms. Human erythrocytes have long been used to study glucose transport kinetics because they are highly enriched in GLUT1 [31] and they do not possess any intracellular organelles that may potentially complicate the effect of the Akt inhibitors. Human erythrocytes treated with cytochalasin B, a potent inhibitor of the glucose transporter, displayed an 85 % reduction in glucose

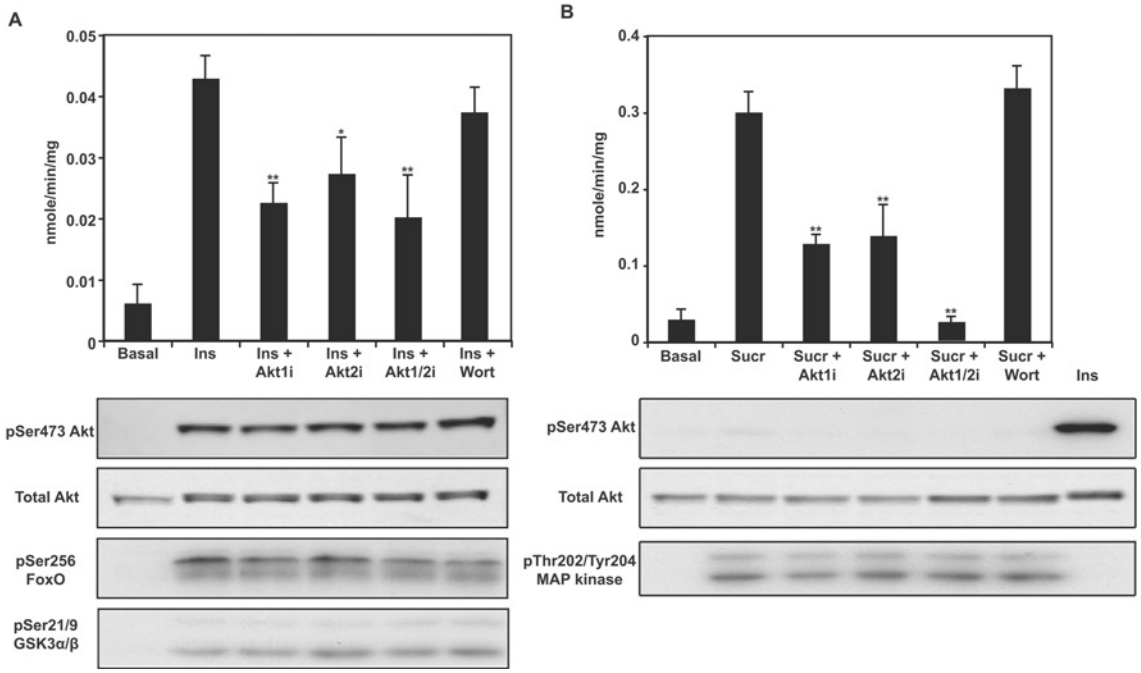

Figure 3 Akt inhibitors inhibit glucose uptake independently of Akt signalling in 3T3-L1 adipocytes

(**A**) 3T3-L1 adipocytes were serum-starved for 2 h in KRP buffer. Cells were treated with 100 nM insulin (Ins) for 20 min at 37 °C and immediately chilled on ice for 2 min prior to exposure to 0.1 % DMSO, 10 μM of the Akt inhibitors (Akt1i, Akt2i or Akt1/2i) or 100 nM wortmannin (Wort) for 5 min. Glucose uptake assays were then performed while cells were incubated on ice. Total cell lysates were immunoblotted with phospho-Ser^{473} Akt, total Akt, phospho-Ser^{256} FoxO or phospho-Ser$^{21/9}$ GSK3α/β antibodies. Results are means $\pm$ S.D, $n = 4$. *$P < 0.05$ and **$P < 0.01$ compared with insulin alone. (**B**) 3T3-L1 adipocytes were treated with 0.45 M sucrose (Sucr) for 30 min prior to 5 min treatment with 0.1 % DMSO, 10 μM of the Akt inhibitors (Akt1i, Akt2i or Akt1/2i) or 100 nM wortmannin (Wort). Glucose uptake was performed as described in the Experimental section. Total cell lysates were immunoblotted with phospho-Ser^{473} Akt, total Akt or phospho-Thr^{202}/Tyr^{204} MAPK antibodies. Results are means $\pm$ S.D, $n = 3$. *$P < 0.05$ and **$P < 0.01$ compared with sucrose alone.

uptake compared with untreated cells (Figure 4A). Although the Akt inhibitors were not as potent as cytochalasin B in inhibiting glucose uptake, all three inhibitors significantly ($P < 0.01$) inhibited uptake, whereas wortmannin had no significant effect (Figure 4A). It was observed that the inhibitory effect of Akt1i on glucose transport in erythrocytes was less than that with Akt2i or Akt1/2i (Figure 4A). This difference was not observed in 3T3-L1 adipocytes (Figure 3) and may indicate that Akt1i preferentially inhibits uptake by GLUT4 rather than GLUT1.

The Akt inhibitors have been shown to inhibit Akt phosphorylation in cancer cells [8]. We next determined whether Akt inhibitors could inhibit glucose uptake in T-47D breast cancer cells. This cell line harbours a mutation that results in the hyperactivation of PI3K, leading to constitutive activation of Akt in the absence of growth factors [5] (Figure 4B). Treatment of T-47D cells with the Akt inhibitors prevented Akt phosphorylation at Thr^{308} and Ser^{473} and also resulted in reduced phosphorylation of a range of Akt substrates, such as TSC2 and GSK3α/β (Figure 4B). The Akt inhibitors also significantly inhibited glucose uptake into T-47D cells. Intriguingly, whereas wortmannin markedly inhibited Akt, TSC2 and GSK3α/β phosphorylation (Figure 4B), it had no significant effect on glucose uptake (Figure 4C). These data clearly indicate that these Akt inhibitors specifically inhibit glucose uptake into a range of different cell types in a manner that does not rely on modified Akt activity.

DISCUSSION

Hyperactivation of Akt is one of the most frequent mutations detected in human cancer [5,6], whereas down-regulation of Akt activity is linked to Type 2 diabetes [14]. Therefore understanding the activation or down-regulation of the Akt signalling pathway is of interest in cancer and Type 2 diabetes research. A promising approach in inhibiting Akt activation has recently emerged with the development of a series of allosteric compounds that specifically target Akt isoforms [8].

In the present study we confirm that these inhibitors reduce Akt phosphorylation, which in turn leads to the reduction in phosphorylation of the Akt substrates FoxO and GSK3 in 3T3-L1 adipocytes. Given the importance of Akt in GLUT4 translocation [11], it is not surprising that these inhibitors resulted in a robust reduction in glucose uptake in 3T3-L1 adipocytes. It was therefore unexpected that 3T3-L1 adipocytes treated with these Akt inhibitors had only a minimal reduction in GLUT4 translocation to the PM upon insulin stimulation. This was surprising since it is believed that GLUT4 translocation is both necessary and sufficient for the increase in glucose transport observed with insulin in these cells [11,32].

We have shown that a minimal amount of Akt phosphorylation is required for robust GLUT4 translocation [33–35]. Hence a marked reduction in Akt phosphorylation may not necessarily translate into a similar reduction in GLUT4 translocation to the PM. It should be noted that a small inhibition of GLUT4 translocation was observed using Akt inhibitors, although this was relatively minor compared with the inhibition of glucose transport. Moreover, we have observed that use of larger doses of the Akt inhibitors was able to more robustly inhibit GLUT4 translocation (results not shown). Hence these data are not necessarily inconsistent with a role of Akt in insulin-stimulated glucose transport or GLUT4 translocation.

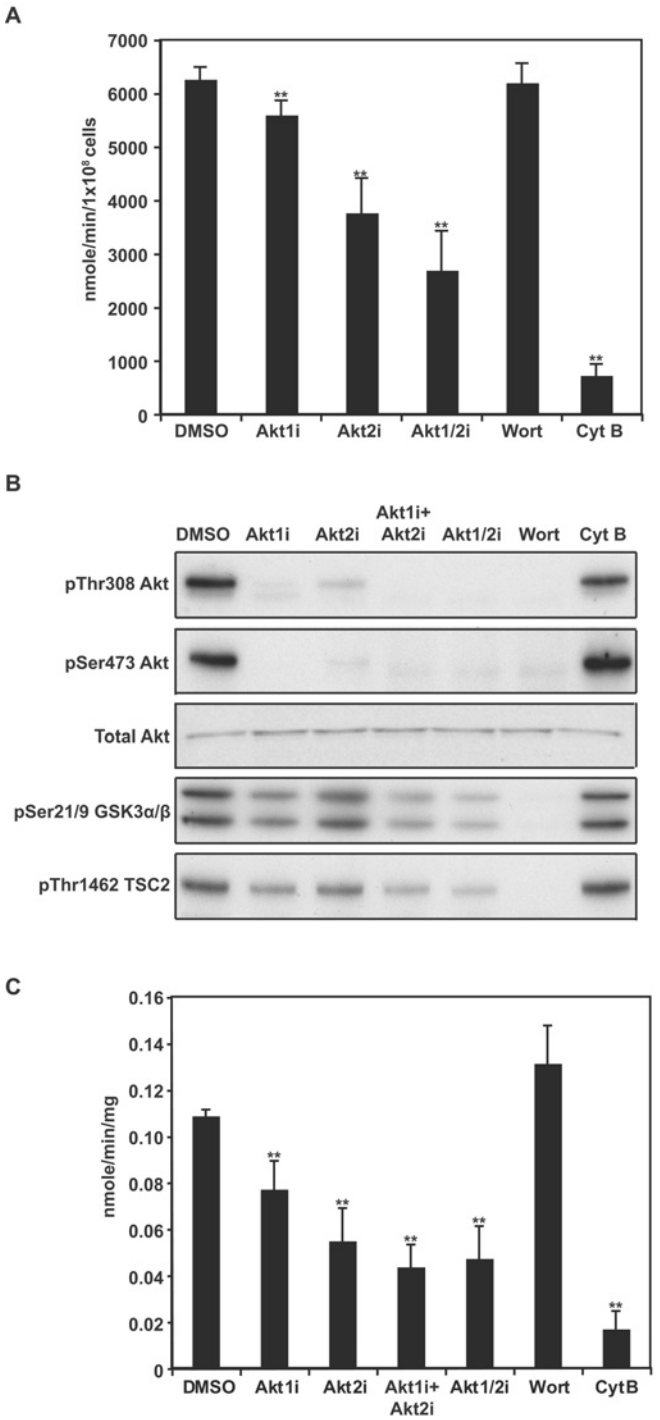

Figure 4 Akt inhibitors prevent glucose uptake in human erythrocytes and in T-47D breast cancer cells

(**A**) Purified human erythrocytes were treated with 0.1% DMSO, 10 μM of the Akt inhibitors (Akt1i, Akt2i or Akt1/2i), 100 nM wortmannin (Wort) or 25 μM cytochalasin B (CytB) for 5 min. Glucose uptake assays were performed as described in the Experimental section. Results are means $\pm$ S.D., $n = 3$. $^{**}P < 0.01$ compared with DMSO treatment. (**B**) T-47D cells were serum-starved for 16 h and treated with 0.1% DMSO, 10 μM of the Akt inhibitors (Akt1i, Akt2i, Akt1i + Akt2i or Akt1/2i), 100 nM wortmannin (Wort) or 25 μM cytochalasin B (CytB) for 30 min. Total cell lysates were immunoblotted with phospho-Thr^{308} Akt, phospho-Ser^{473} Akt, total Akt, phospho-Ser$^{21/9}$ GSK3α/β and phospho-Thr^{1462} TCS2 antibodies. (**C**) T47D cells were treated as described in (**B**). Glucose uptake assays were performed for a duration of 5 min. Results are means $\pm$ S.D., $n = 5$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with DMSO treatment.

Despite this, the discrepancy between glucose transport and GLUT4 translocation observed with the Akt inhibitors suggested that these drugs may directly inhibit the transporter itself. Using two independent approaches, we demonstrated that the Akt inhibitors reduce glucose uptake independently of its effect on the Akt signalling pathway in 3T3-L1 adipocytes. First, rapid cooling of insulin-treated adipocytes on ice, followed by treatment with the Akt inhibitors, did not lead to a reduction in Akt phosphorylation, but glucose uptake was inhibited to a similar extent as observed at 37 °C. Secondly, the Akt inhibitors also prevented glucose uptake under conditions where glucose transport was activated in an Akt-independent manner. In contrast wortmannin, a PI3K inhibitor, had no effect on transport in both approaches. These two approaches strongly indicate that the inhibitors block glucose uptake independently of Akt. In fact, the experiment where the Akt inhibitors were added at 4 °C suggests that the inhibitory effect is manifest after the transporter has been inserted into the PM.

The Akt inhibitors also blocked glucose transport into human erythrocytes. This observation suggests that the inhibitory effect is not specific to GLUT4 since erythrocytes express mainly the GLUT1 isoform. Moreover, in view of the absence of intracellular organelles or any obvious regulation of glucose transport in these cells, this is consistent with the conclusion that these compounds directly influence the transport activity of facilitative glucose transporters. This observation is of interest because cancer cells rely heavily upon aerobic glycolysis for energy and most cancer cells express high levels of GLUT1 at the PM [36,37]. Strategies that reduce GLUT1 expression have been shown to reduce tumour growth, and GLUT1 is considered a potential target for cancer therapy [37–39]. On the basis of these observations, combined with the present findings, it is tempting to speculate that the ability of Akt inhibitors to inhibit growth of cancer cells might be related to their ability to inhibit glucose uptake into the cells [8]. This might also account for the reported hyperglycaemic effects of Akt inhibitors [7], which may simply reflect systemic inhibition of glucose uptake into many cells throughout the body.

In conclusion, our present study indicates that these Akt inhibitors potently inhibit Akt activation but have a minimal effect on GLUT4 translocation to the PM in 3T3-L1 adipocytes. These inhibitors also have a secondary effect in affecting glucose uptake in cells. This off-target effect may have potential implication on the development of these and related Akt inhibitors for therapeutic use.

AUTHOR CONTRIBUTION

Shi-Xiong Tan and Yvonne Ng performed the research and wrote the paper. David James supervised, reviewed and edited the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Akt inhibitors reduce glucose uptake independently of their effects on Akt

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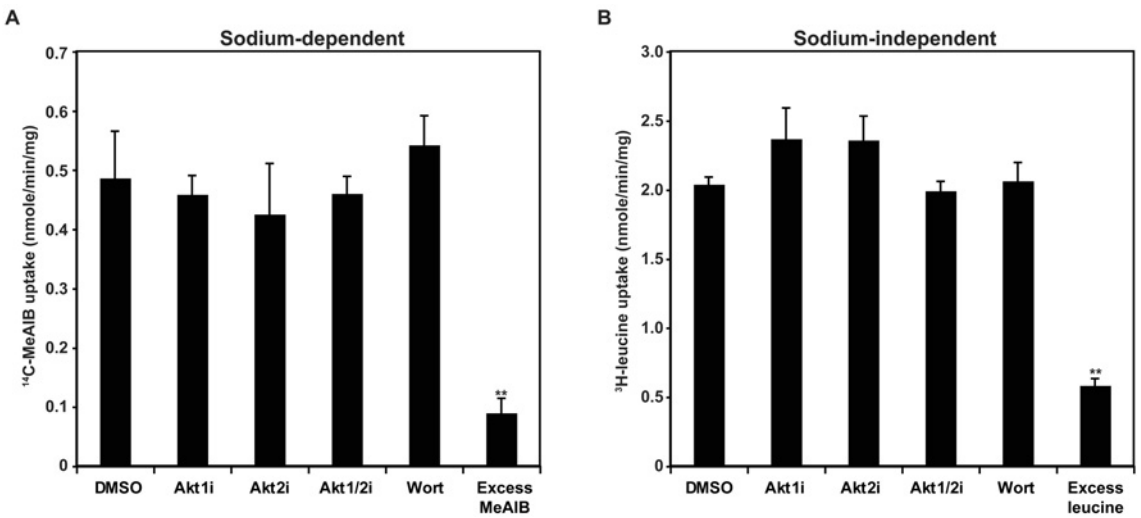

Figure S1 Akt inhibitors have no effect on amino acid uptake in 3T3-L1 adipocytes

(**A**) Sodium-dependent [^{14}C]-MeAIB uptake and (**B**) sodium-independent L-[^{3}H]leucine uptake were examined in 3T3-L1 adipocytes. Cells were treated with 0.1 % DMSO, 10 μM of Akt inhibitors (Akt1i, Akt2i or Akt1/2i) or 100 nM wortmanin for 30 min prior to the amino acid uptake assay. Unlabelled MeAIB or L-leucine (10 mM) was added as a competitive inhibitor. Results are means $\pm$ S.D, $n = 3$. $^{**}P < 0.01$ compared with DMSO treatment.

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