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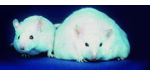

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Next-generation Akt inhibitors provide greater specificity: effects on glucose metabolism in adipocytes

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Many human tumours exhibit activation of the PI3K (phosphoinositide 3-kinase)/Akt pathway, and inhibition of this pathway slows tumour growth. This led to the development of specific Akt inhibitors for *in vivo* use. However, activation of Akt is also necessary for processes including glucose metabolism. Therefore a potential complication of such anticancer drugs is insulin resistance and/or diabetes. In the process of characterizing the metabolic effects of early-phase Akt inhibitors, we discovered an off-target inhibitory effect on mammalian facilitative glucose transporters. In view of the crucial role of glucose transport for all mammalian cells, such an off-target effect would have major implications for further development of this family of compounds. In the present study, we have characterized a next-generation Akt inhibitor, MK-2206. MK-2206 is an orally active allosteric Akt inhibitor under development for treating solid tumours. We report that MK-2206 potently inhibits Thr^{308}Akt and Ser^{473}Akt phosphorylation in 3T3-L1 adipocytes (IC_{50} 0.11 and 0.18 μM respectively) as well as downstream effects of insulin on GLUT4 (glucose transporter 4) translocation (IC_{50} 0.47 μM) and glucose transport (IC_{50} 0.14 μM). Notably, the potency of MK-2206 is approximately 1 log higher than previous inhibitors and its specificity is significantly improved with modest inhibitory effects on glucose transport in GLUT4-expressing adipocytes and GLUT1-rich human erythrocytes, independently of Akt. Nevertheless, MK-2206 clearly has potent effects on Akt2, the principal isoform involved in peripheral insulin action, in which case insulin resistance will probably be a major complication following *in vivo* administration. We conclude that MK-2206 provides an optimal tool for studying the effects of Akt *in vitro*.

Key words: adipocyte, Akt inhibitor, cancer, insulin, MK-2206, signalling.

INTRODUCTION

Akt (also known as protein kinase B) is a serine/threonine kinase activated by growth factors or hormones such as insulin. PI3K (phosphoinositide 3-kinase), through Akt, promotes cell growth and survival, among other cellular processes. Hyperactivation of the PI3K/Akt pathway is commonly associated with tumour growth [1,2]. In some instances, activation or overexpression of Akt in tumour cells is linked to resistance to various therapies such as radiotherapy or chemotherapy [3,4]. Therefore inhibitors of Akt were postulated to have great potential in cancer treatment [5–7].

Inhibiting Akt activity may have various biological effects besides preventing tumour growth and proliferation. Akt activity is involved in various cell types and participates in many biological processes. One critical role of Akt is in metabolism. Akt is a major regulator of many of insulin's metabolic actions in muscle, fat and liver [8]. Indeed, in some contexts, it has been shown that activation of Akt is both necessary and sufficient for certain actions of insulin such as the translocation of the insulin-responsive GLUT (glucose transporter) 4 to the PM (plasma membrane) in adipocytes [9,10]. Therefore one potential side effect of drugs that inhibit Akt is insulin resistance, which may lead to an increased risk of diabetes.

Previously, we, and others, have examined the metabolic effects of early-generation Akt inhibitors [11–13]. The inhibitory effects of these inhibitors were thought to be dependent on the PH (pleckstrin homology) domain of Akt and are thought to inhibit the association of Akt with the PM, thus blocking its activation [6]. Several versions of these were described, including two isoform-specific inhibitors referred to as Akt1i and Akt2i, and an inhibitor that inhibits both isoforms referred to as Akt1/2i. These compounds displayed reasonable efficacy, but their potency was somewhat limited in large part by the need to comprehensively inhibit Akt phosphorylation to limit its *in vivo* activity [11,12]. Unexpectedly, we also observed a potent off-target effect of these compounds. All three inhibitors were shown to inhibit the activity of the facilitative GLUT1 and GLUT4, and these effects were independent of the ability of these compounds to inhibit Akt [12]. Hence this posed some limitations for the *in vivo* specificity of these reagents.

A next-generation Akt inhibitor known as MK-2206, an orally active allosteric Akt inhibitor that is under development for the treatment of solid tumours, was described recently [14]. In the present study, we characterized the specificity and potency of this compound on metabolism in adipocytes. Our studies show that MK-2206 demonstrates improved potency and specificity as defined by its reduced Akt-independent inhibitory action on cellular glucose transport in 3T3-L1 adipocytes and human erythrocytes. However, this compound will probably cause insulin resistance *in vivo*. We suggest that the development of Akt isoform-specific inhibitors is still warranted.

Abbreviations used: AS160, Akt substrate of 160 kDa; FoxO1, forkhead box O1; GLUT, glucose transporter; GSK3, glycogen synthase kinase 3; PI3K, phosphoinositide 3-kinase; PM, plasma membrane; PRAS40, proline-rich Akt substrate of 40 kDa; TSC2, tuberous sclerosis complex 2.

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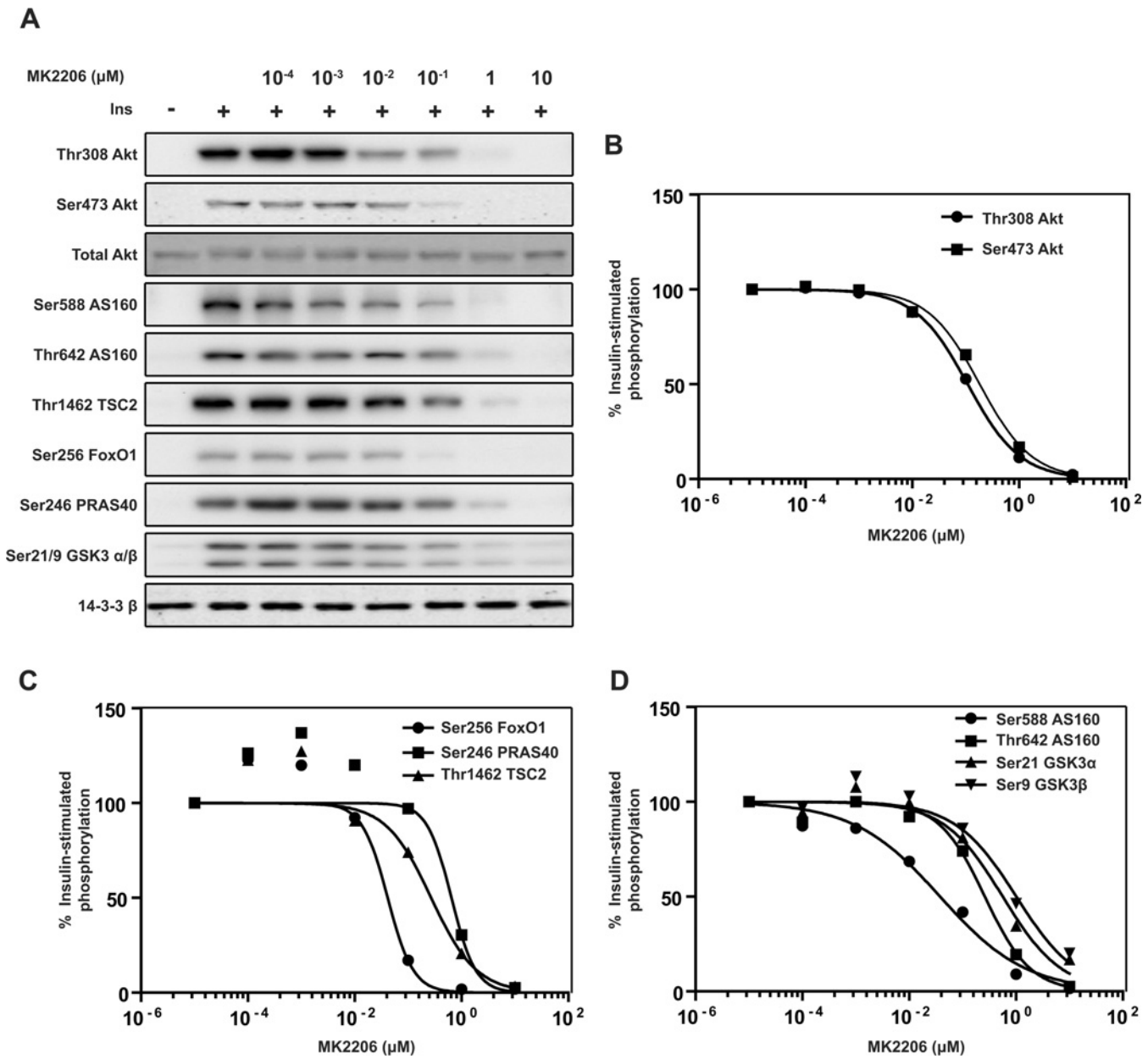

Figure 1 Effect of MK-2206 on insulin-stimulated Akt signalling and downstream events in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were serum-starved in KRP (Krebs–Ringer phosphate) buffer for 2 h. (**A**) Cells were treated with either 0.1 % DMSO or MK-2206 at the indicated dose for 30 min before exposure to 100 nM insulin (Ins) for 20 min. Total cell lysates were immunoblotted with antibodies against pThr^{308} Akt, pSer^{473} Akt, total Akt, pSer^{588} AS160, pThr^{642} AS160, pThr^{1462} TSC2, pSer^{256} FoxO1, pSer^{246} PRAS40, pSer^{21}/pSer^{9} GSK3α/β and 14–3-3 protein. (**B**–**D**) Western blots were quantified using densitometry, normalized to 14-3-3 protein and expressed as percentage change of insulin-stimulated phosphorylation of MK-2206-treated cells compared with DMSO-treated control cells. Curves were fitted using non-linear regression.

MATERIALS AND METHODS

MK-2206 was dissolved in DMSO as a 10 mM stock solution and stored at $-20\,^{\circ}C$. All other materials and methods were as described previously [12]. IC_{50} values were calculated using Prism software. The densitometric analysis of Western blots for DMSO-treated cells were indicated as treated with MK-2206 at 10^{-5} μM in Figure 1 for the purpose of curve fitting. Data were fitted using non-linear regression with a variable Hill slope.

RESULTS

The phosphorylation of Akt at Thr^{308} and Ser^{473}, and that of a range of Akt substrates, was substantially increased by insulin in 3T3-L1 adipocytes (Figure 1A). Treatment of cells with MK-2206 led to a dose-dependent inhibition of insulin-stimulated Akt phosphorylation and a concomitant decrease in insulin-stimulated Akt substrate phosphorylation (Figure 1). The IC_{50} values of MK-2206 on insulin-stimulated phosphorylation of Akt and its substrates are summarized in Table 1. MK-2206

Table 1 Effect of MK-2206 on insulin-stimulated phosphorylation of Akt and its substrates, GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes

IC_{50} values and R^2 were calculated using data from Figures 1(B)–1(D). The percentage of inhibition was calculated by quantification of Western blot analysis in Figure 1 for phosphorylation of Akt and its substrates, and Figure 2 for glucose uptake and for GLUT4 assay. Values are expressed as the percentage of inhibition as compared with 1 μM insulin-treated cells.

Substrate	IC_{50} (μM)	R^2	Inhibition (%)
Thr^{308} Akt	0.11	1.00	88.66
Ser^{473} Akt	0.18	1.00	83.23
Ser^{588} AS160	0.04	0.98	90.91
Thr^{642} AS160	0.26	0.99	80.58
Thr^{1462} TSC2	0.27	0.91	79.36
Ser^{256} FoxO1	0.04	0.95	98.20
Ser^{246} PRAS40	0.65	0.84	69.58
Ser^{21} GSK3α	0.56	0.98	65.44
Ser^{9} GSK3β	1.01	0.96	53.66
PM GLUT4	0.47	0.99	54.30
Glucose uptake	0.14	0.98	63.69

had profound inhibitory effects on Thr^{308} Akt phosphorylation and Ser^{473} Akt phosphorylation (~80–90 %) at a concentration of 1 μM (Figure 1A and Table 1). Importantly, as shown in our previous studies, Akt1i and Akt2i displayed only a modest inhibitory effect on Akt phosphorylation at a concentration of 10 μM [12]. Consistent with this, Akt1i and Akt2i had very little effect on insulin-stimulated Akt substrate phosphorylation at this concentration [12]. In contrast, MK-2206 significantly inhibited the insulin-dependent phosphorylation of AS160 (Akt substrate of 160 kDa), TSC2 (tuberous sclerosis complex 2), PRAS40 (proline-rich Akt substrate of 40 kDa), FoxO1 (forkhead box O1) and GSK3 (glycogen synthase kinase 3) α/β at 1 μM (~53–98 %; Table 1) and, at 10 μM, complete inhibition was observed (Figure 1A). To determine the biological efficacy of MK-2206, we next examined its ability to inhibit insulin-stimulated glucose transport and GLUT4 translocation. Strikingly, slight inhibitory effects were already observed at a concentration of 0.1 μM (Figure 2), and at 1 μM, insulin-stimulated glucose transport and GLUT4 translocation were inhibited by ~64 and ~54 % respectively (Figure 2 and Table 1). Intriguingly, even at a dose of 10 μM MK-2206, we still observed a small effect of insulin to stimulate GLUT4 translocation and glucose transport (Figure 2). As to whether this reflects residual Akt activity or the activity of a non-Akt-dependent pathway remains to be determined.

Given that some Akt inhibitors have been described to inhibit glucose uptake independently of their effect on Akt activity [12], we sought to determine whether MK-2206 exerts similar off-target effects in 3T3-L1 adipocytes. We used two approaches, as described previously [12], to determine whether MK-2206 can prevent glucose uptake in 3T3-L1 adipocytes independently of its effect on Akt activity. The first approach makes use of osmotic shock to prevent GLUT4 endocytosis, resulting in the accumulation of GLUT4 at the PM [12,15–17] before exposure of the cells to the inhibitors. In the second approach, adipocytes were exposed to insulin at 37 °C to increase GLUT4 accumulation at the PM. Cells were then cooled to 4 °C, MK-2206 was added for 5 min, and glucose transport was measured at the reduced temperature. The rationale here is that, if the drugs have an inhibitory effect when added at this low temperature, this must denote a specific effect of the compound directly on the transporter or the membrane. At low doses (0.1–1 μM), MK-2206 did not have any off-target effect on glucose uptake (results not shown). At higher doses (10 μM), MK-2206 had a slight (~15 %) inhibitory

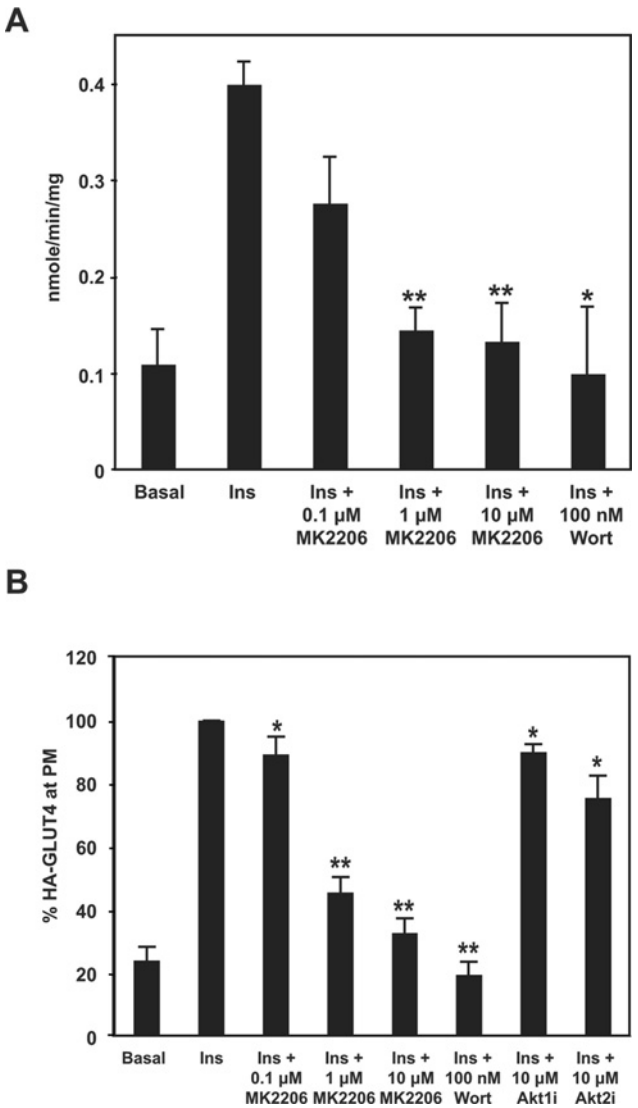

Figure 2 Effect of MK-2206 on insulin-stimulated glucose uptake and GLUT4 translocation to the PM in 3T3-L1 adipocytes

Differentiated 3T3-L1 adipocytes were serum-starved in KRP (Krebs–Ringer–phosphate) buffer for 2 h. Cells treated with the indicated inhibitors were examined for (**A**) glucose uptake and (**B**) GLUT4 translocation assay at 37 °C as described previously [12]. Results are means ± S.D. (n = 3–4). *$P < 0.05$; **$P < 0.01$, insulin-treated compared with insulin (Ins) plus inhibitors. HA, haemagglutinin; Wort, wortmannin.

effect on glucose transport using both approaches, but this was much less than observed using the earlier-generation inhibitors (~35–50 %) [12] (Figures 3A and 3B). To examine whether MK-2206 affects the glucose-transport activity of GLUT1, we made use of human erythrocytes, which express high levels of this transporter [18]. Human erythrocytes treated with cytochalasin B, a potent inhibitor of glucose transport, displayed a 78 % reduction in glucose transport, compared with the vehicle-treated control. Akt1i and Akt2i reduced glucose uptake by ~20–30 % respectively, comparable with that reported in our previous study [12] (Figure 3C). Neither wortmannin nor MK-2206 had any significant effect on glucose transport in human erythrocytes (Figure 3C). These results indicate that the off-target effect of MK-2206, in preventing glucose uptake independent of its effect on Akt, is minor compared with other available Akt inhibitors.

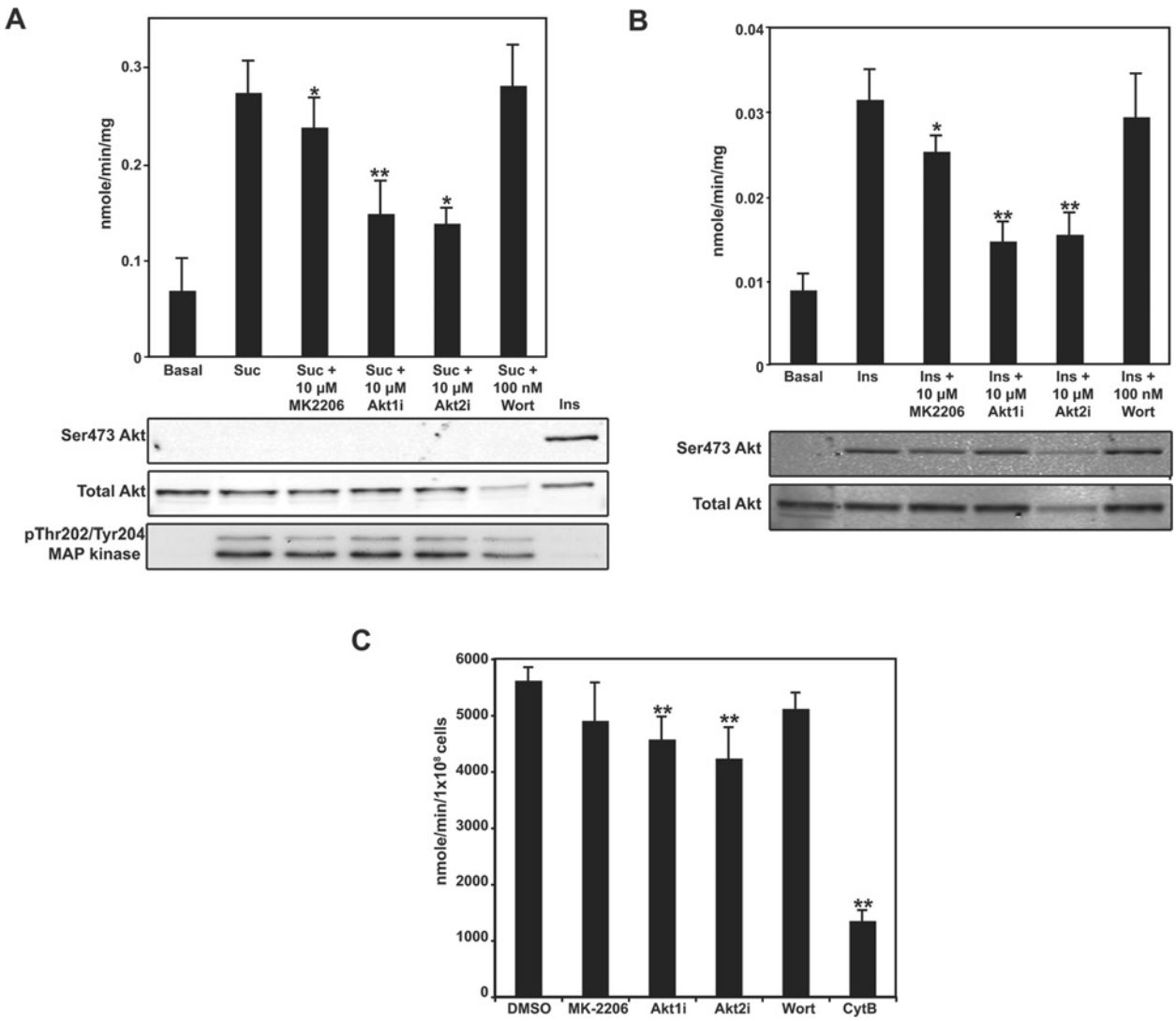

Figure 3 MK-2206 inhibits glucose uptake independent of its effect on Akt signalling in 3T3-L1 adipocytes but not in human erythrocytes

(**A**) 3T3-L1 adipocytes were treated with 0.45 M sucrose (Suc) for 30 min before 5 min of treatment with either 0.1 % DMSO or 10 μM MK-2206, Atk1i, Akt2i or 100 nM wortmannin (Wort). Glucose uptake was performed accordingly. Total cell lysates were immunoblotted with antibodies against pSer^{473} Akt, total Akt or pThr^{202}/Tyr^{204} MAPK (mitogen-activated protein kinase). (**B**) 3T3-L1 adipocytes were treated with 100 nM insulin (Ins) for 20 min at 37 °C and immediately chilled on ice for 2 min before exposure to either 0.1 % DMSO or 10 μM MK-2206, Atk1i or Akt2i 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 antibodies against pSer^{473} Akt and total Akt. Results are means $\pm$ S.D. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$, insulin/sucrose-treated compared with insulin/sucrose plus inhibitors. (**C**) Purified human erythrocytes were treated with DMSO (0.1 %), MK-2206 (10 μM), Akt1i (10 μM), Akt2i (10 μM), wortmannin (100 nM, Wort) or cytochalasin B (25 μM, CytB) for 5 min. Glucose-uptake assays were performed on ice as described previously [12]. Results are means $\pm$ S.D. ($n = 3$–4). $^{**}P < 0.01$ compared with DMSO treatment.

DISCUSSION

Inhibiting Akt for prevention of tumour growth and proliferation was proposed to be an approach for cancer therapeutics [5–7]. However, as indicated in the present and other studies, inhibiting Akt activity may have significant effects on biological processes in cell types other than cancer cells [12,13]. Notably, given that Akt is essential for the translocation of GLUT4 to the PM to facilitate glucose uptake [9], as well as for other metabolic processes, inhibiting Akt may cause insulin resistance. Since insulin resistance triggers compensatory hyperinsulinaemia, which may itself create an improved growth milieu for tumour cells, one has to be mindful of this potentially serious complication. Exacerbating this problem even further, we previously described an off-target effect of an early generation of Akt inhibitors to block the transport activity of facilitative glucose transporters [12]. Hence, in combination, the off-target effect on glucose transport and the metabolic side effects of these compounds raises some potential concerns. In the present paper, we report on the efficacy and potency of a newer generation of Akt inhibitors and show that MK-2206 has both improved potency and reduced off-target effects, making this a potentially better treatment option.

It has been reported that the potency of MK-2206 as an Akt inhibitor is improved based on its inhibition of proliferation in several cancer cell lines in the low micromolar range [14]. Given that we had previously reported potent effects of Akt inhibitors to reduce glucose transport independently of Akt [12], it was of interest to determine whether these improved effects of MK-2206 were due to improved effects on Akt or transport. This is relevant since tumour cells exhibit a profound increase in glucose transport to sustain their increased energy needs and GLUT1 plays a major role in this effect [19,20]. In our previous study, we showed that

Akt inhibitors inhibit both GLUT1 and GLUT4 transport activity and so this raised the possibility that the anti-tumour effects of these compounds was in part mediated via their inhibitory effects on transport independently of Akt [12]. Intriguingly, this does not appear to be the case because MK-2206 exhibits improved specificity which, when combined with its improved potency, will probably delimit the impact of its transport-inhibitory properties.

However, the improved potency of MK-2206 to inhibit Akt in both tumour cells and adipocytes indicates that this compound has not overcome the potential off-target effects on metabolism. Indeed, in the present paper, we report markedly improved potency of MK-2206 to inhibit insulin-stimulated glucose transport and GLUT4 translocation, two major metabolic actions of insulin in these cells. Thus it seems reasonable to surmise that the effective dose of MK-2206 required to prevent tumour growth in patients may also lead to insulin resistance. To circumvent this problem, it may be possible to engineer Akt inhibitors that have improved specificity for cancer cells. This may involve strategies that can target drugs specifically to cancer cells or Akt inhibitors that have improved specificity. Intriguingly, certain kinds of human tumours appear to involve hyperactivation of specific Akt isoforms [21]. Increased Akt1 has been linked to gastric and breast cancers [22,23], whereas Akt2 was reported to be involved in ovarian, pancreatic and colorectal cancers [24–26], and Akt3 has been associated with breast cancer, prostrate cancer and melanoma [27,28]. Therefore further development of isoform-specific inhibitors of Akt could potentially serve as a more targeted and specific therapeutic treatment. For example, if amplification/activation of a specific isoform of Akt were identified in a tumour, using an inhibitor that targets that specific isoform may be beneficial. This is especially true for Akt2, since it has a greater involvement in metabolism compared with other Akt isoforms. Inhibitors that specifically target Akt1 and/or Akt3 may potentially eliminate the metabolic side effects of current Akt inhibitors that target both Akt1 and Akt2.

In summary, we conclude that MK-2206 is a more potent inhibitor of Akt compared with previous inhibitors, and its specificity is significantly improved with modest off-target effects on glucose transport independent of Akt. MK-2206 provides an optimal tool for studying the effects of Akt *in vitro*.

AUTHOR CONTRIBUTION

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

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