BJ www.biochemj.org

Biochem. J. (2012) 442, 161–169 (Printed in Great Britain) doi:10.1042/BJ20111913

This is a data-enriched, interactive PDF that provides the gateway to a world of information when opened in Utopia Documents Download FREE software now

Effects of acutely inhibiting PI3K isoforms and mTOR on regulation of glucose metabolism *in vivo*

Greg C. SMITH*†, Wee Kiat ONG‡, Gordon W. REWCASTLE†§, Jackie D. KENDALL†§, Weiping HAN‡ and Peter R. SHEPHERD*†[1]

*Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand, †Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand, ‡Laboratory of Metabolic Medicine, Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), Singapore, and §Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand.

In *in vitro* studies class-I PI3Ks (phosphoinositide 3-kinases), class-II PI3Ks and mTOR (mammalian target of rapamycin) have all been described as having roles in the regulation of glucose metabolism. The relative role each plays in the normal signalling processes regulating glucose metabolism *in vivo* is less clear. Knockout and knockin mouse models have provided some evidence that the class-I PI3K isoforms p110α, p110β, and to a lesser extent p110γ, are necessary for processes regulating glucose metabolism and appetite. However, in these models the PI3K activity is chronically reduced. Therefore we analysed the effects of acutely inhibiting PI3K isoforms alone, or PI3K and mTOR, on glucose metabolism and food intake. In the present study impairments in glucose tolerance, insulin tolerance and increased hepatic glucose output were observed in mice treated with the pan-PI3K/mTOR inhibitors PI-103 and NVP-BEZ235. The finding that ZSTK474 has similar effects indicates that these effects are due to inhibition of PI3K rather than mTOR. The p110α-selective inhibitors PIK75 and A66 also induced these phenotypes, but inhibitors of p110β, p110δ or p110γ induced only minor effects. These drugs caused no significant effects on BMR (basal metabolic rate), O_2 consumption or water intake, but BEZ235, PI-103 and PIK75 did cause a small reduction in food consumption. Surprisingly, pan-PI3K inhibitors or p110α inhibitors caused reductions in animal movement, although the cause of this is not clear. Taken together these studies provide pharmacological evidence to support a pre-eminent role for the p110α isoform of PI3K in pathways acutely regulating glucose metabolism.

Key words: glucose metabolism, insulin, mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), phosphoinositide 3-kinase catalytic α polypeptide (PIK3CA).

INTRODUCTION

PI3Ks (phosphoinositide 3-kinases) are a family of eight enzymes that are capable of phosphorylating the D3 position of the inositol head group of phosphoinositides. Although all of these enzymes share a high degree of sequence similarity in the kinase domain, there are significant differences in other domains, and so the PI3Ks have been divided into three classes based on structural similarities [1]. The catalytic domain of the PI3K family also shares a high degree of homology with a family of five serine kinases that are referred to as the PIKKs (phosphoinositide kinase-related kinases) [2]. This family includes mTOR (mammalian target of rapamycin) and ATM (ataxia telangiectasia mutated) [2].

There is a significant body of evidence to indicate that various forms of PI3K play roles in the regulation of glucose metabolism. Class-II PI3Ks are activated by insulin and have also been implicated in mediating insulin-induced increases in glucose uptake [3,4]. The class-III PI3K is not regulated directly by insulin levels, but is regulated by changes in cellular glucose levels [5]. Of the PIKKs, mTOR [6,7] and ATM [8] have been implicated in regulating pathways involved in glucose metabolism. The class-IB PI3Ks may play a role in regulating insulin secretion *in vitro* [9] and *in vivo* [10]. However, the role of class-IA PI3Ks in mediating the effects of insulin on glucose metabolism has been investigated most extensively [11]. A number of approaches have been used to define the role of specific isoforms of class-IA PI3K in the regulation of glucose metabolism. Overexpression of p110α or p110β is sufficient to induce GLUT-4 (glucose transporter type 4) translocation and glucose uptake *in vitro* [12–16]. However, high-level expression of PI3Ks does not prove that a particular PI3K isoform is involved, as forced overexpression of p110 causes not only large increases in PtdIns(3,4,5)P_3, but also in the other D3 inositides, so it is possible that the effects seen are due to the increase in PtdIns3P, PtdIns(3,4)P_2 and PtdIns(3,5)P_2 [17]. Global gene KOs (knockouts) of p110α and a KI (knockin) that creates a kinase dead allele of p110α are embryonically lethal, and data on insulin action have only been obtained from studies of heterozygous mice [18] or tissue-specific PI3K KO models [19,20]. These studies have provided evidence for impairments in glucose metabolism when levels of p110α are chronically reduced. KI mice have also been created in which the kinase activity of p110β is ablated and mice homozygous for this mutation have minor defects in glucose metabolism, implying a role for the catalytic activity of p110β in pathways regulating glucose metabolism [20,21]. However, long-term gene knockdown can cause developmental problems in key glucoregulatory tissues that could contribute to the defects in glucose metabolism, and the results of studies with seemingly similar PI3K KO models do not always produce similar effects on glucose metabolism [19].

Pharmacological inhibitors offer a more direct means of studying the role of the catalytic functions of the PI3K enzymes [22]. A wide range of small molecule inhibitors targeting class-I PI3K isoforms and mTOR have been developed [2,23,24]. A number of these are selective for particular class-I PI3K isoforms and/or mTOR [25–31]. Some of these inhibitors have been used in a limited range of *in vitro* studies of insulin action [26,32,33], but there is very little data available on the *in vivo* effect of these inhibitors on glucose metabolism [26].

Abbreviations used: ATM, ataxia telangiectasia mutated; BMR, basal metabolic rate; GTT, glucose tolerance test; ITT, insulin tolerance test; KI, knockin; KO, knockout; LC-MS/MS, liquid chromatography tandem MS; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PI3KCA, PI3K catalytic α polypeptide; PIKK; phosphoinositide kinase-related kinase; PTT, pyruvate tolerance test.

[1] To whom correspondence should be addressed (email peter.shepherd@auckland.ac.nz).

© 2012 The Author(s)

The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

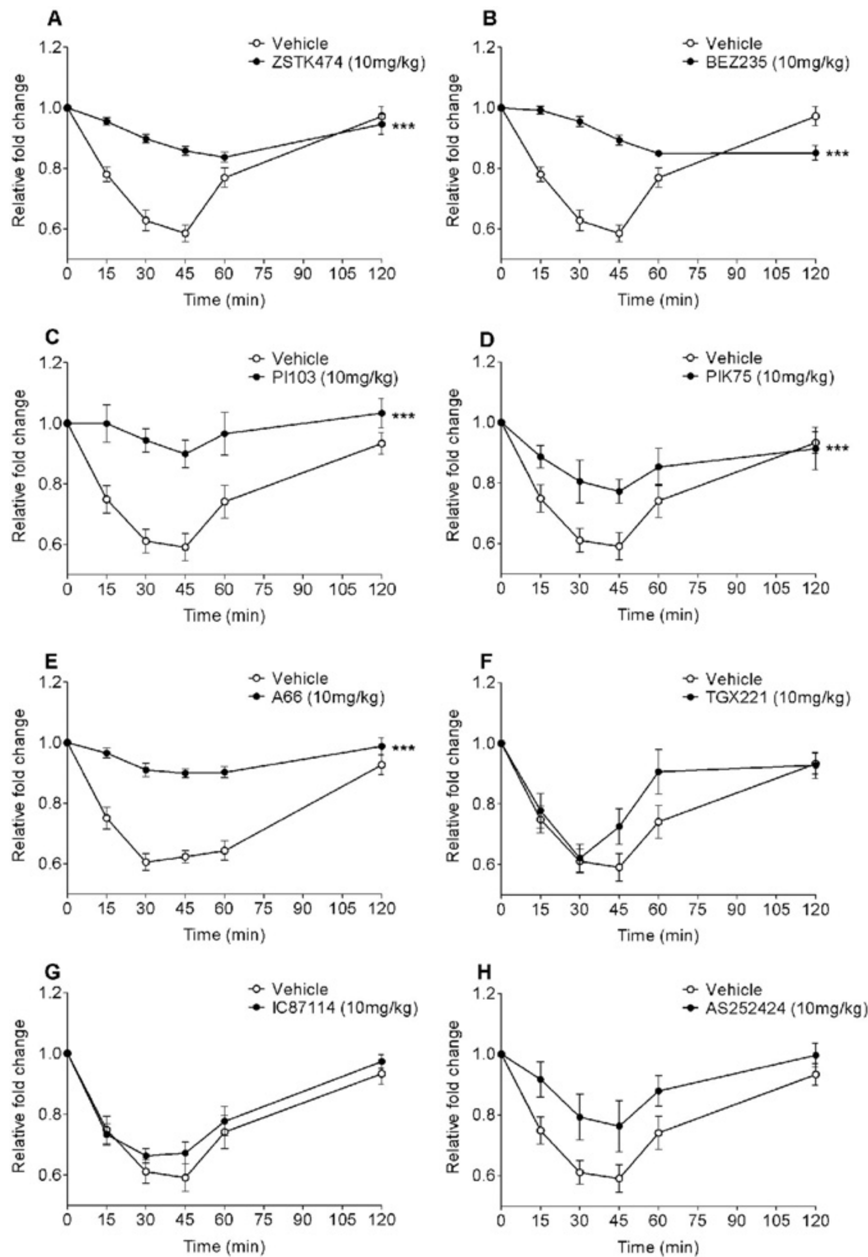

Figure 1 Acute effect of PI3K inhibitors on insulin tolerance

The indicated PI3K inhibitors were administered intraperitoneally (10 mg/kg of body mass) and 1 h later animals were injected with insulin (0.75 units/kg of body mass). Glucose levels in blood were measured in blood samples taken at the indicated time as described in the Experimental section. Results are means ± S.E.M. ($n \geqslant 6$). Statistical significance was determined by repeated measures ANOVA (***$P < 0.001$ compared with the vehicle control animals).

In the present study we have investigated the effects of a range of inhibitors with varying specificity for class-I PI3K isoforms and mTOR on whole-body glucose metabolism in mice. The present study supports a major role for the p110α isoform of PI3K in maintaining glucose homoeostasis *in vivo*. Surprisingly the data also demonstrate that animals treated with a pan-PI3K inhibitor or p110α inhibitors display a marked reduction in movement.

EXPERIMENTAL

Animal model

The GTT (glucose tolerance test), ITT (insulin tolerance test) and PTT (pyruvate tolerance test) studies used male CD1 mice. Metabolic cage studies used male C57Bl/6 mice that were mass and percentage of fat matched into groups using the EchoMRI-100 quantitative magnetic resonance system (Echo Medical Systems). The light/dark cycle was 12 h in all cases and all animals were fed on standard laboratory chow (Harland Teklad). All animal experiments were approved by the Animal Ethics Committees' of Auckland University in New Zealand and the Agency for Science, Technology and Research (A*STAR) Biomedical Science Institutes in Singapore.

Compounds and reagents

The study used ZSTK474 (pan-PI3K inhibitor) [25], PI-103 (pan-PI3K/mTOR inhibitor) [26], BEZ235 (pan-PI3K inhibitor/

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

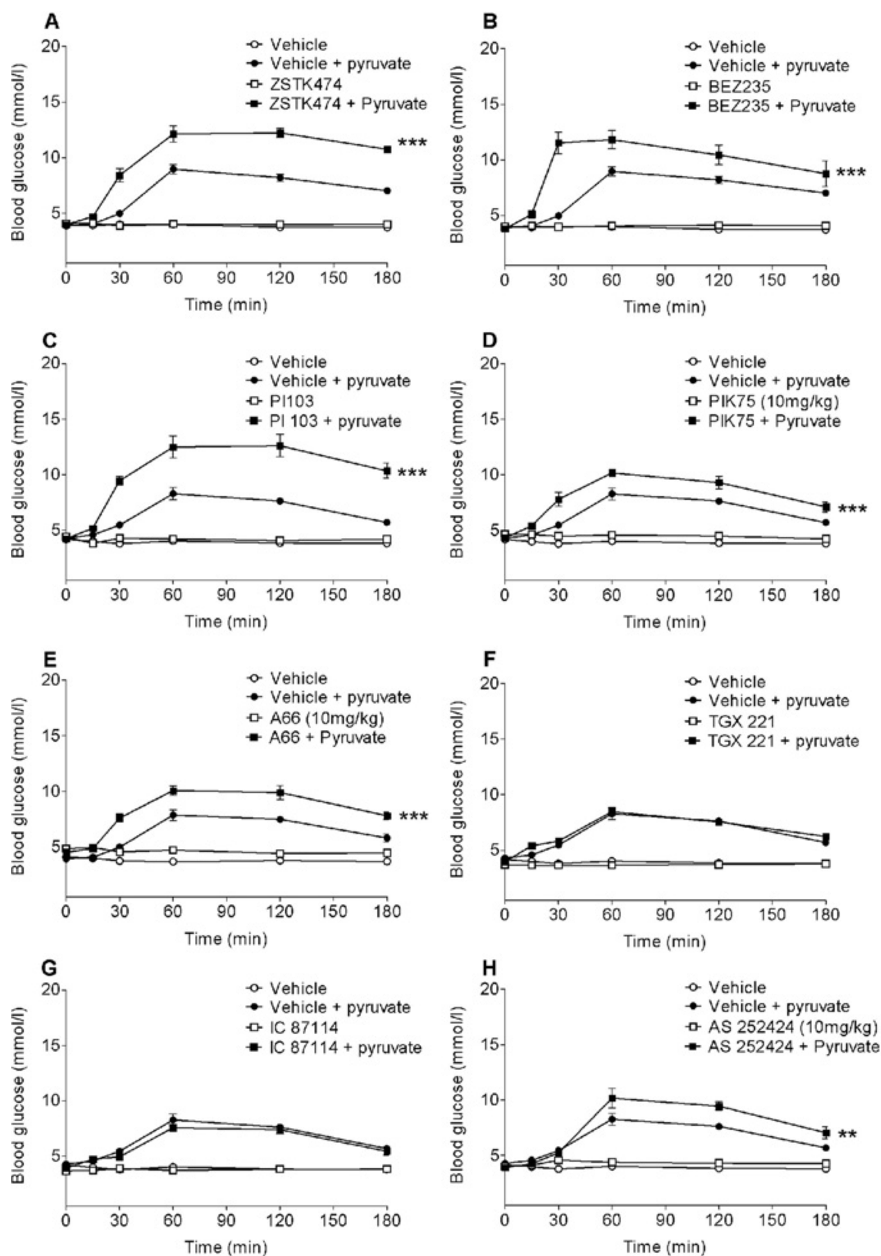

Figure 2 Acute effect of PI3K inhibitors on hepatic glucose output

The indicated PI3K inhibitors were administered intraperitoneally (10 mg/kg of body mass) and 1 h later animals were injected with pyruvate (2 g/kg of body mass). Glucose levels in blood were measured in blood samples taken at the indicated time as described in the Experimental section. Results are means ± S.E.M. ($n \geqslant 6$). Statistical significance was determined by repeated measures ANOVA (***$P < 0.001$ compared with the vehicle control animals).

mTOR) [27], PIK75 (p110α inhibitor) [26], A66 (p110α-selective inhibitor) [28], TGX221 (p110β-selective inhibitor) [29], IC87114 (p110δ-selective inhibitor) [30] and AS252424 (claimed to be a p110γ-selective inhibitor) [31]. These were synthesized in-house as described previously [28,32] or obtained from Symansis. All compounds were greater than 99 % pure by HPLC analysis and NMR data indicated that they were the correct molecules. Unless otherwise stated, other reagents were purchased from Sigma Chemicals.

GTT, ITT and PTT

GTTs, ITTs and PTTs, as well as determinations of insulin levels, were performed as described previously [34], except that male CD1 mice were used instead of rats. For GTTs and PTTs the mice were starved overnight and for the ITT food was withdrawn 2 h prior to the start of the experiments. Drugs were dosed intraperitoneally 1 h after the end of the dark cycle and 1 h prior to the intraperitoneal dosing with glucose or pyruvate (2 g/kg of body mass) or insulin (0.75 unit/kg of body mass).

Metabolic cage studies

Oxymax/CLAMS (Columbus Instruments) was used to quantify oxygen consumption ($\dot{V}O_2$), CO_2 production ($\dot{V}CO_2$), BMR (basal metabolic rate), food intake, water intake and animal movement as described previously [35]. BMR was expressed as a function of lean body mass as recommended in a previous study [36]. All

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

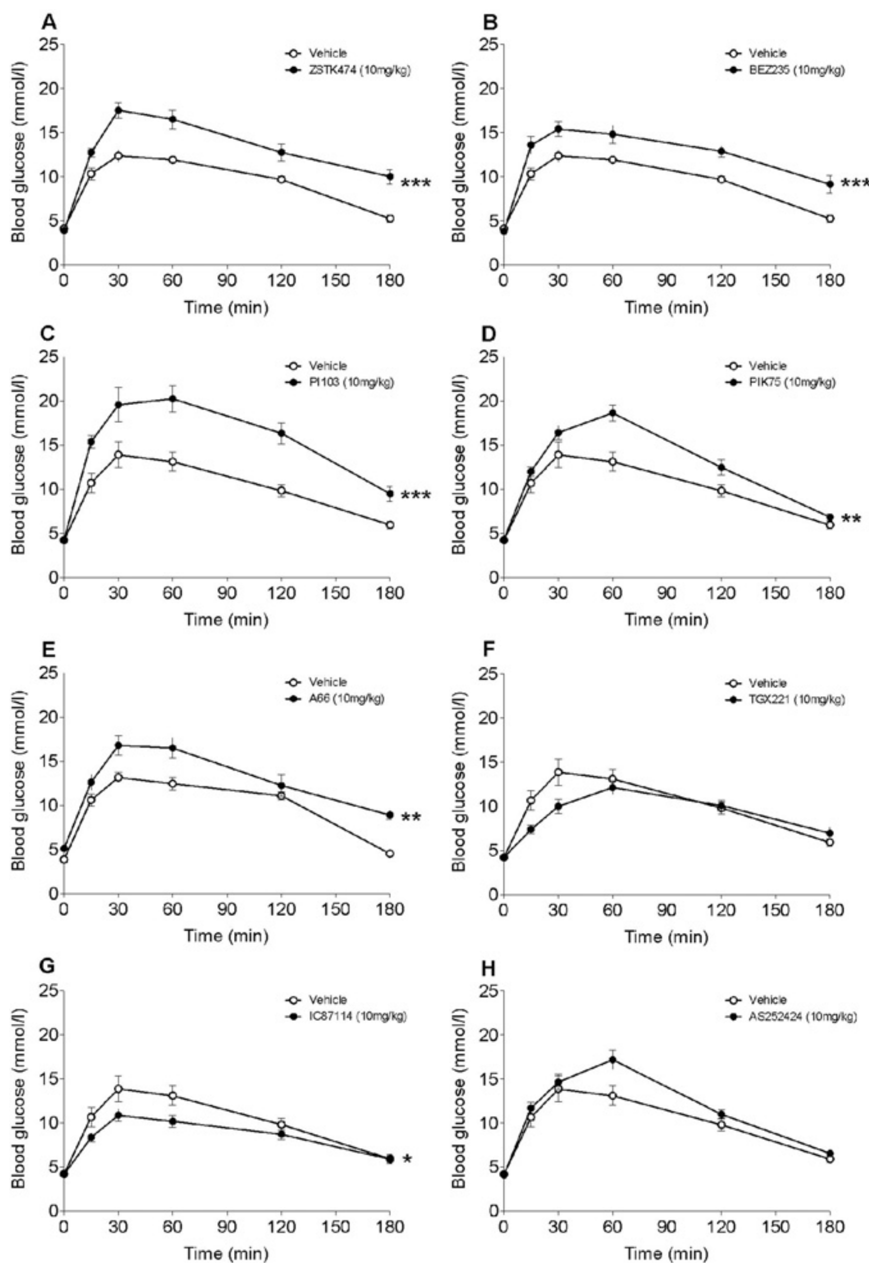

Figure 3 Acute effect of PI3K inhibitors on glucose tolerance

The indicated PI3K inhibitors were administered intraperitoneally (10 mg/kg of body mass) and 1 h later animals were injected with glucose (2 g/kg of body mass). Glucose levels in blood were measured in blood samples taken at the indicated time as described in the Experimental section. Results are means ± S.E.M. ($n \geqslant 6$). Statistical significance was determined by repeated measures ANOVA (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with the vehicle control animals).

data were normalized to total lean mass using the EchoMRI-100 quantitative magnetic resonance system as described previously [35]. Animals were acclimatized for 24 h in cages and the data were collected over the following 24 h.

Analysis of drug levels

Pharmacological kinetics studies were undertaken in fed CD1 male mice (30 g body mass). Animals were administered with the stated PI3K inhibitors via oral gavage or intraperitoneal injection, and terminal blood samples were collected in EDTA blood collection tubes at 15 min, and 1, 2, 4, 6 and 24 h post-drug exposure. All drugs were dissolved in DMSO. Blood was centrifuged (2000 ***g*** for 10 min and 4 °C) and plasma isolated for drug quantification. Drug quantification was undertaken using LC-MS/MS (liquid chromatography tandem MS). Briefly, 300 μl of 100 % methanol was added to 100 μl of plasma. The samples were gently mixed and centrifuged (2000 ***g*** for 10 min and 4 °C). The supernatant was removed and 50 μl was added into vials for LC-MS/MS. The ion-source type was ESI (electrospray ionization) with the following conditions: spray voltage (5500 V), sheath gas pressure (50 units), ion sweep gas pressure (0.0 unit), auxillary gas pressure (2 units), capillary temperature [370 °C and the capillary offset at 35 V. HPLC kinetex columns were used (100 mm × 3 mm, 2.6u C18(2)-HST; Phenomenex]. The run method was isocratic 10 % (0.1 % formic acid in water) and 90 % methanol.

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

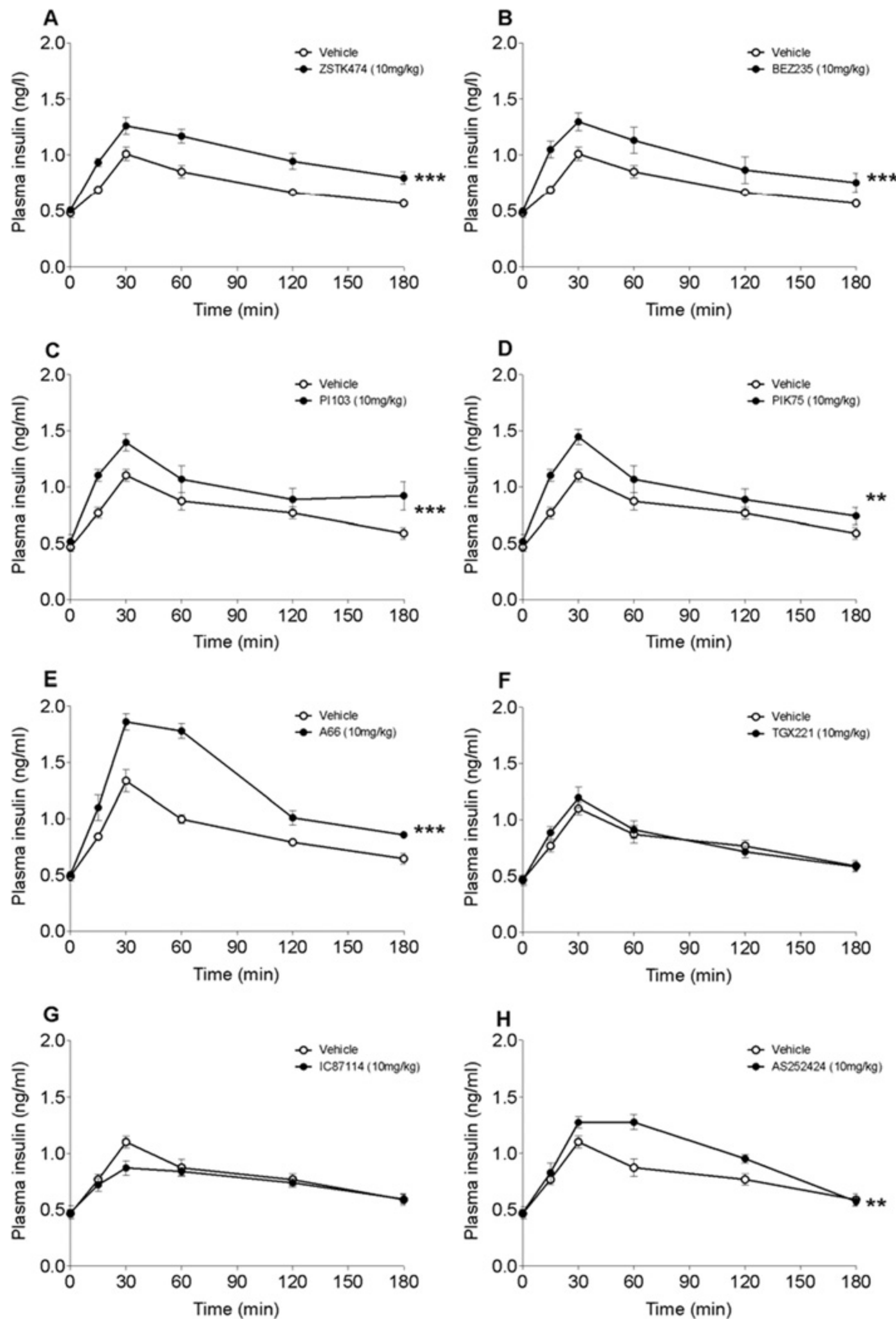

Figure 4 Acute effects of PI3K inhibitors on insulin levels during the GTT

The indicated PI3K inhibitors were administered intraperitoneally (10 mg/kg of body mass) and a GTT was performed as described in Figure 3. Insulin levels were measured in blood samples taken at the indicated time as described in the Experimental section. Results are means ± S.E.M. ($n \geq 6$). Statistical significance was determined by repeated measures ANOVA (** $P < 0.01$ and *** $P < 0.001$ compared with the vehicle control animals).

The flow rate was 0.2 ml/min. Retention times were 2.64 min (PI-103), 2.76 min (TGX221) and 2.35 min (IC87114). Unknown concentrations were determined from the standard curve and internal standard.

RESULTS

Drug pharmacokinetics

We have reported previously pharamacokinetic data for BEZ235 and A66 [28]. In the present paper we report pharmacokinetic data for PI-103, TGX221 and IC87114 following oral or intraperitoneal injection (Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420161add.htm). These studies established that an intraperitoneal dose of 10 mg/kg of body mass gave suitable blood concentrations of drug for short-term metabolic studies.

Effect of inhibitors on whole-body glucose metabolism

The results of the present study show that the pan-PI3K/mTOR inhibitors PI-103 and BEZ235, and the pan-PI3K inhibitor

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

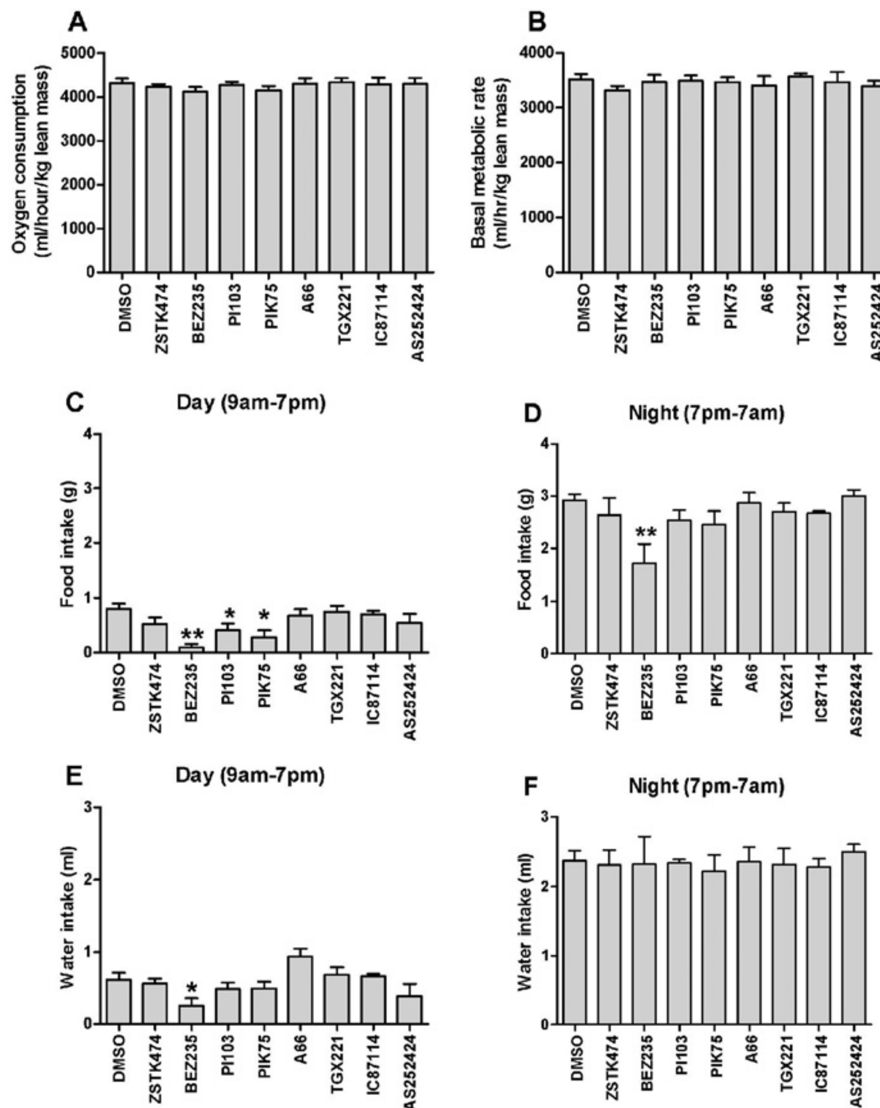

Figure 5 Effect of PI3K inhibitors on oxygen consumption, BMR, food consumption and water intake

Animals were injected with the indicated PI3K inhibitors intraperitoneally (10 mg/kg of body mass) and were observed for 24 h following injection in a CLAMS metabolic cage as described in the Experimental section. The results are shown for oxygen consumption (**A**), BMR (**B**), food intake during the day (**C**), food intake during the night (**D**), water intake during the day (**E**) and water intake during the night (**F**). Results are means ± S.E.M. ($n \geqslant 6$). Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparison test (**P* < 0.05 and ***P* < 0.01 compared with the vehicle control animals).

ZSTK474 severely impaired whole-body glucose metabolism in mice (Figures 1–4). The finding that the drugs induced severe impairments in insulin tolerance (Figure 1) suggests they are causing insulin resistance at the level of one or all of the major insulin target tissues, i.e. muscle, liver or fat. The finding that they all increased production of glucose from pyruvate in a PTT (Figure 2) indicates that gluconeogenesis is increased and provides evidence that insulin action in the liver is impaired. Further evidence that the drugs induce insulin resistance comes from the GTT results which show that all three of these pan-PI3K inhibitors induced significant impairments in the ability of the mice to dispose of a glucose load (Figure 3). Of the isoform-selective class-IA PI3K inhibitors, PIK75 and A66 induced significant impairments in the ITT and GTT, and an increase in glucose production during a PTT (Figures 1–3), with TGX221 and IC87114 having only minor effects. AS252424 caused a significant increase in hepatic glucose production (Figure 2H) and a trend towards an impairment in insulin tolerance (Figure 1H). AS252424 was originally described as a p110γ-selective inhibitor, but the findings above lead us to re-evaluate this and we find that it inhibits p110γ with an IC_{50} value of 17 nM (compared with 30 nM reported in [31]) and p110α with an IC_{50} value of 80 nM (compared with 935 nM reported in [31]). Therefore *in vivo* this inhibitor is likely to be cross-reacting with p110α.

One possible explanation for defects in glucose metabolism could be an inhibitory effect on insulin release as such effects have been reported previously *in vitro* [37]. However, insulin levels did not decrease in the drug-treated animals during the GTT (Figure 4). In fact insulin levels rose in the case of the pan-PI3K inhibitors and PIK75 and A66, in line with the impaired glucose tolerance as would be expected in an insulin-resistant state. Therefore, although a small effect on insulin release can not be ruled out, the drugs certainly don't completely block insulin release.

We were also interested to investigate whether acute administration of these PI3K inhibitors might affect energy expenditure and so we performed metabolic cage studies.

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

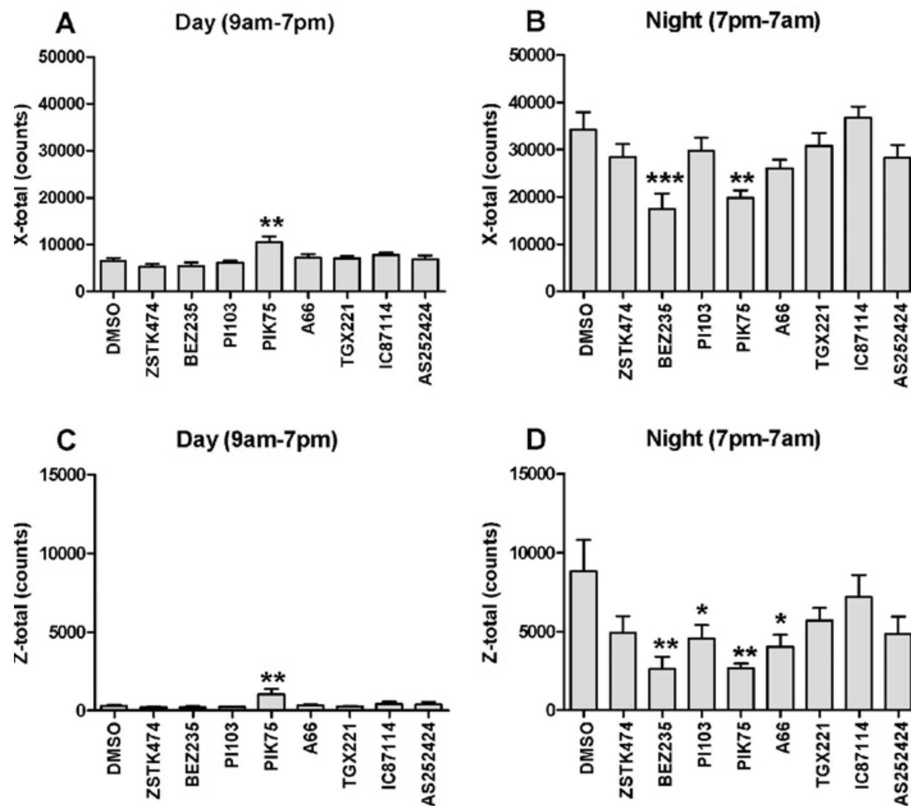

Figure 6 Effects of PI3K inhibitors on movement

The indicated PI3K inhibitors were administered intraperitoneally (10 mg/kg of body mass) and measurements of animal movement were made over a 24 h period in metabolic cages as described in the Experimental section. The results are shown for the total X-counts during the day (**A**), total X-counts during the night (**B**), total Z-counts during the day (**C**) and total Z-counts during the night (**D**). Results are means ± S.E.M. ($n \geqslant 6$). Statistical significance was determined by one-way ANOVA and Dunnett's multiple comparison test ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the vehicle control animals).

These studies did not find any changes in BMR or oxygen consumption (Figure 5). Neither were there major changes in water consumption. However, BEZ235 induced significant reductions in food intake in both the light and dark cycle, whereas PI-103 and PIK75 caused significant decreases in food intake during the light cycle (Figure 5).

During the metabolic cage studies, data were also obtained on animal movement. Surprisingly this showed that a number of the inhibitors induced a reduction in movement and that this was an acute effect of the drugs (Figure 6). The reduction in movement was mainly due to a reduction in Z-movement (i.e. up–down movement) (Figure 6). It is notable that the pan-PI3K inhibitors PI-103 and BEZ235, and both of the p110α-selective inhibitors, were the inhibitors that caused the largest effects.

DISCUSSION

The present study shows that the pan-PI3K/mTOR inhibitors PI-103 and BEZ235 have dramatic effects on whole-body glucose metabolism. This extends the findings of Knight et al. [26] who demonstrated that PI-103 induced impairments in insulin tolerance. The present study also shows that PIK75 caused a serious impairment of glucose metabolism in mice. This also extends the findings of Knight et al. [26] who only looked at insulin tolerance. They concluded that this was evidence for an important role for p110α in regulating glucose metabolism *in vivo*. However, PIK75 is a suboptimal inhibitor to use for such studies as it has a number of off-target effects, including inhibition of p110γ and a number of protein kinases. However, the effects of PI-103 and BEZ235 are most likely not to be due to inhibition of mTOR as ZSTK474, which inhibits class-I PI3K isoforms, but not mTOR, has very similar effects. Furthermore, it is unlikely to be due to inhibition of class-II PI3Ks as PI-103 and PIK75 do not inhibit these isoforms [26]. Using a number of different inhibitors with different profiles against protein kinases also guards against the possibility that the effect of the drugs might be due to off-target effects. Furthermore, we find PI-103, BEZ235 and ZSTK474 (Supplementary Table S1 at http://www.BiochemJ.org/bj/442/bj4420161add.htm) and A66 [28] have very low levels of off-target activity.

The present study is the first to examine the effect of a selective p110α inhibitor (A66) on glucose metabolism *in vivo*. We find that A66 impairs all measures of *in vivo* insulin action, almost to the same level as the pan-PI3K inhibitors. This provides strong pharmacological evidence that p110α is the most important isoform in the pathways acutely regulating glucose metabolism, and that functional redundancy between PI3K isoforms is unlikely to be a major feature of major pathways regulating glucose metabolism *in vivo* [32]. The effects of A66 on glucose metabolism are a phenocopy of mice heterozygous for global expression of a kinase-dead form of p110α [18]. However, even though A66 is inhibiting p110α globally, the results of the present study are also remarkably similar to those seen in mice in which the *Pik3ca* gene had been deleted either acutely or chronically only in liver [19]. Taken together with our PTT results this suggests that a major site of action of the p110α in regulating the effects of insulin on glucose metabolism is in liver.

An area where our studies do not correlate with genetic studies is with regard to p110β inhibition. Two previous studies have

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

analysed the role of p110β in glucose metabolism using genetic models. One of these was a KI model, which created a kinase-dead form of p110β [21], whereas the other ablated p110β specifically in liver [20]. Both of these models showed impairments of glucose tolerance and insulin tolerance, as well increased hepatic glucose output, which is a similar phenotype to that seen in our studies with pan-PI3K inhibitors. However, we only see minor changes in glucose metabolism in animals treated with TGX221 and these do not achieve statistical significance. This is supported by the studies of Knight et al. [26] who found that the p110β inhibitor TGX115 did not affect insulin tolerance in mice. One explanation could be that the defects in glucose metabolism seen in the genetic studies [20] may be caused by long-term effects of the loss of p110β function, which are not seen with acute inhibition of the catalytic activity of the enzyme. Another explanation would be that our results support a non-catalytic role for the p110β in pathways controlling metabolism in the liver, as has previously been suggested [20].

The finding of the present study that some of the drugs induce a small reduction in food intake differs from previous studies in genetic mouse models [18,38] and our own studies in which isoform selective PI3K inhibitors were directly injected into the brain [39]. Those studies have indicated that a reduction in p110α and p110β activity in the brain actually leads to increased food intake rather than a decrease. It is not clear why the drugs in the present study did not induce a similar effect, but it may be related to the fact that they were administered peripherally and so they may not be crossing the blood–brain barrier to a sufficient extent to achieve such effects. Also, the reduced food intake does not necessarily mean a reduced appetite as the reduction in movement may be preventing the animals from eating.

The reduction in movement seen in mice treated with pan-PI3K inhibitors or the p110α-selective inhibitors is interesting. A similar reduction in movement was observed in mice in which the *p110α* gene had been deleted in the liver [19]. One interpretation of this would be that p110α plays some previously unsuspected role in regulating movement, but it is also possible that it is a side effect of the off-target actions of the drugs. Further studies will be required to resolve this issue.

In summary, the results of the present study provide strong pharmacological evidence to support the contention that p110α activity is necessary for the pathways regulating glucose metabolism *in vivo*. The results also show that acute dosing with pan-PI3K and p110α inhibitors have effects on food intake and animal movement, indicating that the these effects should be monitored in human clinical trials using PI3K inhibitors.

AUTHOR CONTIBUTION

All authors contributed to the experimental design. Gordon Rewcastle and Jackie Kendall synthesized the chemicals used. Greg Smith performed the experiments. Peter Shepherd wrote the paper.

ACKNOWLEDGEMENTS

We thank Woo-Jeong Lee (Department of Molecular Medicine and Pathology, University of Auckland, Medical School, Auckland, New Zealand) for PI3K assays.

FUNDING

This work was supported by the Maurice Wilkins Centre for Molecular Biodiscovery (to P.R.S.), the Health Research Council of New Zealand [grant numbers 06/062A and 09/388 (to P.R.S. and G.W.R.)], A*STAR Biomedical Research Council (to W.H.) and a joint research grant of A*STAR-HRC of New Zealand [grant number A*STAR-NZ HRC JGC 10-023 (to P.R.S. and W.H.)]. G.S. is funded by a Foundation for Research, Science and Technology post-doctoral research fellowship. P.R.S. is a founder of Symansis.

REFERENCES

1 Shepherd, P. R., Withers, D. J. and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem. J. **333**, 471–490
2 Marone, R., Cmiljanovic, V., Giese, B. and Wymann, M. P. (2008) Targeting phosphoinositide 3-kinase: moving towards therapy. Biochim. Biophys. Acta **1784**, 159–185
3 Falasca, M., Hughes, W. E., Dominguez, V., Sala, G., Fostira, F., Fang, M. Q., Cazzolli, R., Shepherd, P. R., James, D. E. and Maffucci, T. (2007) The role of phosphoinositide 3-kinase C2α in insulin signaling. J. Biol. Chem. **282**, 28226–28236
4 Brown, R. A., Domin, J., Arcaro, A., Waterfield, M. D. and Shepherd, P. R. (1999) Insulin activates the α-isoform of class II phosphoinositide 3-kinase. J. Biol. Chem. **274**, 14529–14532
5 Byfield, M. P., Murray, J. T. and Backer, J. M. (2005) hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. J. Biol. Chem. **280**, 33076–33082
6 Shepherd, P. R., Navé, B. T. and Siddle, K. (1995) Insulin stimulation of glycogen synthesis and glycogen synthase activity is blocked by wortmannin and rapamycin in 3T3-L1 adipocytes: evidence for the involvement of phosphoinositide 3-kinase and p-70 ribosomal protein-S6 kinase. Biochem. J. **305**, 25–28
7 Duvel, K., Yecies, J. L., Menon, S., Raman, P., Lipovsky, A. I., Souza, A. L., Triantafellow, E., Ma, Q., Gorski, R., Cleaver, S. et al. (2010) Activation of a metabolic gene regulatory network downstream of mTOR complex 1. Mol. Cell **39**, 171–183
8 Cosentino, C., Grieco, D. and Costanzo, V. (2011) ATM activates the pentose phosphate pathway promoting anti-oxidant defence and DNA repair. EMBO J. **30**, 546–555
9 Aoyagi, K., Ohara-Imaizumi, M., Nishiwaki, C., Nakamichi, Y. and Nagamatsu, S. (2010) Insulin/phosphoinositide 3-kinase pathway accelerates the glucose-induced first-phase insulin secretion through TrpV2 recruitment in pancreatic β-cells. Biochem. J. **432**, 375–386
10 MacDonald, P. E., Joseph, J. W., Yau, D., Diao, J., Asghar, Z., Dai, F., Oudit, G. Y., Patel, M. M., Backx, P. H. and Wheeler, M. B. (2004) Impaired glucose-stimulated insulin secretion, enhanced intraperitoneal insulin tolerance, and increased β-cell mass in mice lacking the p110γ isoform of phosphoinositide 3-kinase. Endocrinology **145**, 4078–4083
11 Shepherd, P. R. (2005) Mechanisms regulating phosphoinositide 3-kinase signalling in insulin sensitive tissues. Acta Physiol. Scand. **183**, 3–12
12 Ueki, K., Algenstaedt, P., Mauvais-Jarvis, F. and Kahn, C. R. (2000) Positive and negative regulation of phosphoinositide 3-kinase-dependent signaling pathways by three different gene products of the p85α regulatory subunit. Mol. Cell. Biol. **20**, 8035–8046
13 Asano, T., Kanda, A., Katagiri, H., Nawano, M., Ogihara, T., Inukai, K., Anai, M., Fukushima, Y., Yazaki, Y., Kikuchi, M. et al. (2000) p110β is up-regulated during differentiation of 3T3-L1 cells and contributes to the highly insulin-responsive glucose transport activity. J. Biol. Chem. **275**, 17671–17676
14 Tanti, J., Gremeaux, T., Grillo, S., Calleja, V., Klippel, A., Williams, L. T., Obberghen, E. V. and Brustel, Y. L. (1996) Overexpression of a constitutively active form of phosphatidylinositol 3-kinase is sufficient to promote GLUT4 translocation in adipocytes. J. Biol. Chem. **271**, 25227–25232
15 Frevert, E. U., Bjorbaek, C., Venable, C. L., Keller, S. R. and Kahn, B. B. (1998) Targeting of constitutively active phosphoinositide 3-kinase to GLUT4-containing vesicles in 3T3-L1 adipocytes. J. Biol. Chem. **273**, 25480–25487
16 Martin, S. S., Haruta, T., Morris, A. J., Klippel, A., Williams, L. T. and Olefsky, J. M. (1996) Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3-L1 adipocytes. J. Biol. Chem. **271**, 17605–17608
17 Klippel, A., Reinhard, C., Kavanaugh, W. M., Apell, G., Escobedo, M. and Williams, L. T. (1996) Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal transducing kinase pathways. Mol. Cell. Biol. **16**, 4117–4127
18 Foukas, L. C., Claret, M., Pearce, W., Okkenhaug, K., Meek, S., Peskitt, E., Sancho, S., Smith, A. J. H., Withers, D. J. and Vanhaesebroeck, B. (2006) Critical role for the p110α phosphoinositide-3-OH kinase in growth and metabolic regulation. Nature **441**, 366–370
19 Sopasakis, V. R., Liu, P., Suzuki, R., Kondo, T., Winnay, J., Tran, T. T., Asano, T., Smyth, G., Sajan, M. P., Farese, R. V. et al. (2010) Specific roles of the p110α isoform of phosphatidylinsositol 3-kinase in hepatic insulin signaling and metabolic regulation. Cell Metab. **11**, 220–230
20 Jia, S., Liu, Z., Zhang, S., Liu, P., Zhang, L., Lee, S. H., Zhang, J., Signoretti, S., Loda, M., Roberts, T. M. and Zhao, J. J. (2008) Essential role of PI3K-p110b in cell growth, metabolism and tumorogenesis. Nature **454**, 776–779
21 Ciraolo, E., Iezzi, M., Marone, R., Marengo, S., Curcio, C., Costa, C., Azzolino, O., Gonella, C., Rubinetto, C., Wu, H. et al. (2008) Phosphoinositide 3-kinase p110β activity: key role in metabolism and mammary gland cancer but not development. Sci. Signaling **1**, ra3
22 Weiss, W. A., Taylor, S. S. and Shokat, K. M. (2007) Recognizing and exploiting differences between RNAi and small-molecule inhibitors. Nat. Chem. Biol. **3**, 739–744
23 Workman, P., Clarke, P. A., Raynaud, F. I. and van Montfort, R. L. (2010) Drugging the PI3 kinome: from chemical tools to drugs in the clinic. Cancer Res. **70**, 2146–2157
24 Liu, P., Cheng, H., Roberts, T. M. and Zhao, J. J. (2009) Targeting the phosphoinositide 3-kinase pathway in cancer. Nat. Rev. Drug Discovery **8**, 627–644
25 Kong, D. and Yamori, T. (2007) ZSTK474 is an ATP-competitive inhibitor of class I phosphatidylinositol 3 kinase isoforms. Cancer Sci. **98**, 1638–1642

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

26 Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B. et al. (2006) A pharmacological map of the PI3-K family defines a role for p110α in insulin signalling. Cell **125**, 1–15
27 Maira, S. M., Stauffer, F., Brueggen, J., Furet, P., Schnell, C., Fritsch, C., Brachmann, S., Chene, P., De Pover, A., Schoemaker, K. et al. (2008) Identification and characterization of NVP-BEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent *in vivo* antitumor activity. Mol. Cancer Ther. **7**, 1851–1863
28 Jamieson, S., Flanagan, J. U., Kolekar, S., Buchanan, C., Kendall, J. D., Lee, W. J., Rewcastle, G. W., Denny, W. A., Singh, R., Dickson, J. et al. (2011) A drug targeting only p110α can block phosphoinositide 3-kinase signalling and tumour growth in certain cell types. Biochem. J. **438**, 53–62
29 Jackson, S. P., Schoenwaelder, S. M., Goncalves, I., Nesbitt, W. S., Yap, C. L., Wright, C. E., Kenche, V., Anderson, K. E., Dopheide, S. M., Yuan, Y. et al. (2005) PI 3-kinase p110β: a new target for antithrombotic therapy. Nat. Med. **11**, 507–514
30 Sadhu, C., Masinovsky, B., Dick, K., Sowell, C. G. and Staunton, D. E. (2003) Essential role of phosphoinositide 3-kinase δ in neutrophil directional movement. J. Immunol. **170**, 2647–2654
31 Pomel, V., Klicic, J., Covini, D., Church, D. D., Shaw, J. P., Roulin, K., Burgat-Charvillon, F., Valognes, D., Camps, M., Chabert, C. et al. (2006) Furan-2-ylmethylene thiazolidinediones as novel, potent, and selective inhibitors of phosphoinositide 3-kinase γ. J. Med. Chem. **49**, 3857–3871
32 Chaussade, C., Rewcastle, G. W., Kendall, J. D., Denny, W. A., Cho, K., Gronning, L. M., Chong, M. L., Anagnostou, S. H., Jackson, S. P., Daniele, N. and Shepherd, P. R. (2007) Evidence for functional redundancy of class IA PI3K isoforms in insulin signalling. Biochem. J. **404**, 449–458
33 Kim, J. E., Shepherd, P. R. and Chaussade, C. (2009) Investigating the role of class-IA PI 3-kinase isoforms in adipocyte differentiation. Biochem. Biophys. Res. Commun. **379**, 830–834
34 Smith, G. C., Chaussade, C., Vickers, M., Jensen, J. and Shepherd, P. R. (2008) Atypical antipsychotic drugs induce derangements in glucose homeostasis by acutely increasing glucagon secretion and hepatic glucose output in the rat. Diabetologia **51**, 2309–2317
35 Lou, P. H., Yang, G., Huang, L., Cui, Y., Pourbahrami, T., Radda, G. K., Li, C. and Han, W. (2010) Reduced body weight and increased energy expenditure in transgenic mice over-expressing soluble leptin receptor. PLoS ONE **5**, e11669
36 Butler, A. A. and Kozak, L. P. (2010) A recurring problem with the analysis of energy expenditure in genetic models expressing lean and obese phenotypes. Diabetes **59**, 323–329
37 Gao, Z. Y., Konrad, R. J., Collins, H., Matschinsky, F. M., Rothenberg, P. L. and Wolf, B. A. (1996) Wortmannin inhibits insulin secretion in pancreatic islets and β-TC3 cells independent of its inhibition of phosphatidylinositol 3-kinase. Diabetes **45**, 854–862
38 Al-Qassab, H., Smith, M. A., Irvine, E. E., Guillermet-Guibert, J., Claret, M., Choudhury, A. I., Selman, C., Piipari, K., Clements, M., Lingard, S. et al. (2009) Dominant role of the p110β isoform of PI3K over p110α in energy homeostasis regulation by POMC and AgRP neurons. Cell Metab. **10**, 343–354
39 Tups, A., Anderson, G. M., Rizwan, M., Augustine, R. A., Chaussade, C., Shepherd, P. R. and Grattan, D. R. (2010) Both p110α and p110β isoforms of phosphatidylinositol 3-OH-kinase are required for insulin signalling in the hypothalamus. J. Neuroendocrinol. **22**, 534–542
40 Cohen, P. (2010) Guidelines for the effective use of chemical inhibitors of protein function to understand their roles in cell regulation. Biochem. J. **425**, 53–54

Received 26 October 2011/21 November 2011; accepted 5 December 2011
Published as BJ Immediate Publication 5 December 2011, doi:10.1042/BJ20111913

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

Biochem. J. (2012) **442**, 161–169 (Printed in Great Britain) doi:10.1042/BJ20111913

online data

SUPPLEMENTARY ONLINE DATA

Effects of acutely inhibiting PI3K isoforms and mTOR on regulation of glucose metabolism *in vivo*

Greg C. SMITH*†, Wee Kiat ONG‡, Gordon W. REWCASTLE†§, Jackie D. KENDALL†§, Weiping HAN‡ and Peter R. SHEPHERD*†[1]

*Department of Molecular Medicine and Pathology, University of Auckland, Auckland, New Zealand, †Maurice Wilkins Centre for Molecular Biodiscovery, University of Auckland, Auckland, New Zealand, ‡Laboratory of Metabolic Medicine, Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A*STAR), Singapore, and §Auckland Cancer Society Research Centre, University of Auckland, Auckland, New Zealand.

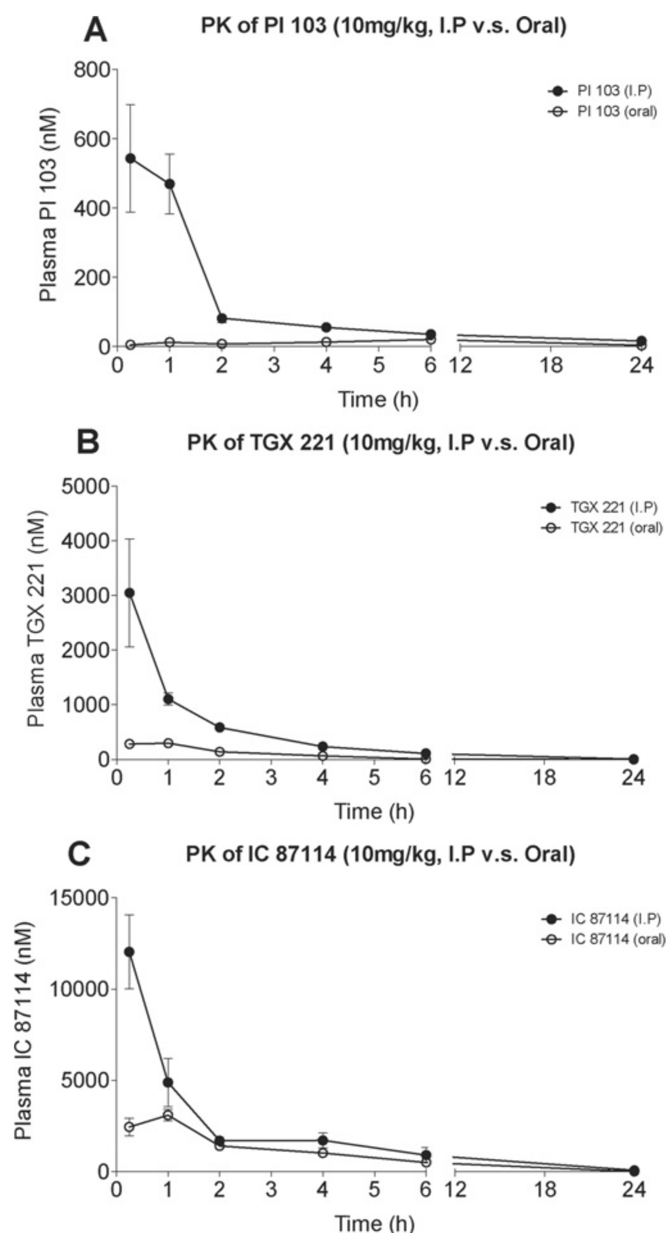

Figure S1 Levels of drug in mouse blood after administration

I.P, intraperitoneal.

[1] To whom correspondence should be addressed (email peter.shepherd@auckland.ac.nz).

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

Table S1 Effect of compounds on enzyme activities

Results are the percentage enzyme activity remaining after treatment with 10 μM of the drug. Enzyme assays were performed by the National Centre for Protein Kinase Profiling, University of Dundee, Dundee, U.K. Results are means of duplicate determinations. AMPK, AMP-activated protein kinase; ASK1, apoptosis signal regulating kinase 1; BRSK, BR serine/threonine kinase; BTK, Bruton agammaglobulinemia tyrosine kinase; CAMK, calcium/calmodulin-dependent protein kinase; CAMKK, CAMK kinase; CHK, checkpoint kinase; CDK, cyclin-dependent kinase; CK, casein kinase; CLK2, CDC-like kinase 2; CSK, c-Src tyrosine kinase; DAPK1, death-associated protein kinase 1; DYRK, dual-specificity tyrosine-(Y)-phosphorylation-regulated kinase; EF2K, eukaryotic elongation factor-2 kinase; EPH, Ephrin type receptor; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor receptor 1; GCK, glucokinase (hexokinase 4); GSK3, glycogen synthase kinase 3; HIPK1, homeodomain interacting protein kinase 1; IGF-1R, insulin-like growth factor 1 receptor; IKK, Inhibitor of nuclear factor κ-B kinase; IR, insulin receptor; IRR, insulin receptor-related receptor; IRAK4, interleukin-1 receptor-associated kinase 4; JAK2, Janus kinase 2; JNK, c-Jun N-terminal kinase; Lck, lymphocyte-specific protein tyrosine kinase; LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; MKK, MKK kinase; MNK, MAPK-interacting serine/threonine kinase; MAPKAP, MAPK-associated protein; MARK, microtubule affinity-regulating kinase; MEKK1, MAPK/ERK kinase kinase 1; MELK, maternal embryonic leucine zipper kinase; MINK1, misshapen-like kinase 1; MLK, mixed lineage kinase; MSK1, mitogen- and stress-activated protein kinase 1; MST, mammalian STE20-like protein kinase; NEK, NIMA (never in mitosis gene a)-related kinase; PAK, p21 protein (Cdc42/Rac)-activated kinase; PKB, protein kinase B; PDK1, pyruvate dehydrogenase kinase 1; PHK, phosphorylase kinase; PKA, protein kinase A; PKC, protein kinase B; PKD, protein kinase D; PLK1, polo-like kinase 1; PRAK, p38-regulated/activated protein kinase; PRK2, protein-kinase C-related kinase 2; RIPK2, receptor-interacting serine-threonine kinase 2; ROCK2, Rho-associated coiled-coil-containing protein kinase 2; RSK, ribosomal S6 kinase; SGK1, serum/glucocorticoid regulated kinase 1; S6K1, S6 kinase 1; SmMLCK, smooth muscle myosin light chain kinase; Src, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue; SRPK1, SRSF protein kinase 1; SYK, spleen tyrosine kinase; TAK1, transforming growth factor-β-activated kinase 1; TAO1, TAO kinase 1; TBK1, TANK [TRAF (tumour-necrosis-factor-receptor-associated factor)-associated nuclear factor κB activator]-binding kinase 1; TRKa, TRK1-transforming tyrosine kinase protein; TTK, TTK protein kinase; YES1, v-yes-1 Yamaguchi sarcoma viral oncogene homologue 1; VEGFR, vascular endothelial growth factor receptor.

Enzyme	PI-103 (10 μM)	AS252424 (10 μM)	BEZ235 (10 μM)	ZSTK474 (10 μM)
MKK1	105	70	116	83
MKK2	115	77	86	95
MKK6	107	99	93	83
ERK1	112	85	106	106
ERK2	106	104	114	105
JNK1	102	99	105	93
JNK2	100	85	89	87
JNK3	101	90	93	88
p38α MAPK	109	86	111	89
p38β MAPK	100	74	96	95
p38γ MAPK	76	65	101	92
p38δ MAPK	84	38	100	107
ERK8	87	10	94	92
RSK1	101	88	97	95
RSK2	96	40	81	83
PDK1	107	97	98	92
PKBα	78	97	126	123
PKBβ	114	40	106	84
SGK1	92	59	98	98
S6K1	90	43	109	98
PKA	100	66	82	76
ROCK2	91	70	84	85
PRK2	71	88	74	82
PKCα	93	102	87	86
PKCζ	98	96	123	87
PKD1	120	55	107	96
MSK1	105	54	102	89
MNK1	97	48	101	96
MNK2	90	62	94	101
MAPKAP-K2	90	80	99	91
MAPKAP-K3	100	63	110	97
PRAK	77	64	93	82
CAMKKb	114	51	61	87
CAMK1	99	46	100	114
SmMLCK	48	37	109	91
PHK	84	61	95	94
DAPK1	30	8	107	75
CHK1	115	87	146	90
CHK2	85	53	80	88
GSK3b	48	10	76	79
CDK2-Cyclin A	94	89	74	77
PLK1	93	90	95	75
Aurora A	92	95	104	99
Aurora B	95	71	101	94
LKB1	62	52	105	103
AMPK	99	39	99	92
MARK1	91	88	97	106
MARK2	107	90	86	87
MARK3	88	67	100	94
MARK4	108	63	81	89
BRSK1	109	56	85	161
BRSK2	99	44	89	88

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.

Table S1 Continued

Enzyme	PI-103 (10 μM)	AS-252424 (10 μM)	BEZ235 (10 μM)	ZSTK474 (10 μM)
MELK	104	91	35	91
NUAK1	98	73	44	90
CK1	100	85	96	76
CK2	102	25	79	54
DYRK1A	59	3	93	78
DYRK2	83	20	79	93
DYRK3	64	18	56	87
NEK2a	86	136	145	106
NEK6	87	94	95	83
IKKb	74	73	92	94
IKKe	85	40	86	84
TBK1	91	70	107	102
PIM1	72	7	92	97
PIM2	90	7	94	92
PIM3	41	7	87	76
SRPK1	116	39	106	92
EF2K	99	91	110	96
HIPK1	63	29	89	100
HIPK2	18	3	101	63
HIPK3	82	23	102	98
CLK2	25	16	53	85
PAK2	106	89	87	100
PAK4	70	65	87	92
PAK5	112	49	90	84
PAK6	108	71	105	93
MST2	115	60	89	92
MST4	114	45	100	88
GCK	91	53	12	104
MINK1	116	24	75	102
MEKK1	97	56	67	92
MLK1	65	53	67	86
MLK3	57	32	62	97
TAO1	84	18	81	93
ASK1	101	110	106	90
TAK1	79	48	67	97
IRAK4	119	114	78	102
RIPK2	72	19	28	89
TTK	66	56	91	88
Src	99	96	121	104
Lck	85	110	54	89
CSK	101	100	100	84
YES1	92	36	69	72
BTK	82	45	20	98
JAK2	93	60	24	79
SYK	69	55	72	85
EPH-A2	113	51	110	106
EPH-A4	103	74	105	87
EPH-B1	131	94	133	96
EPH-B2	105	100	77	106
EPH-B3	127	52	117	113
EPH-B4	86	89	99	105
FGF-R1	118	42	106	102
HER4	114	68	117	103
IGF-1R	54	75	72	87
IR	80	94	99	104
IRR	89	36	80	88
TrkA	93	79	59	83
VEGFR	92	22	80	87

Received 26 October 2011/21 November 2011; accepted 5 December 2011
Published as BJ Immediate Publication 5 December 2011, doi:10.1042/BJ20111913

© 2012 The Author(s)
The author(s) has paid for this article to be freely available under the terms of the Creative Commons Attribution Non-Commercial Licence (http://creativecommons.org/licenses/by-nc/2.5/) which permits unrestricted non-commercial use, distribution and reproduction in any medium, provided the original work is properly cited.