A novel inhibitor of the PI3K/Akt pathway based on the structure of inositol 1,3,4,5,6-pentakisphosphate

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BACKGROUND: Owing to its role in cancer, the phosphoinositide 3-kinase (PI3K)/Akt pathway is an attractive target for therapeutic intervention. We previously reported that the inhibition of Akt by inositol 1,3,4,5,6-pentakisphosphate (InsP5) results in anti-tumour properties. To further develop this compound we modified its structure to obtain more potent inhibitors of the PI3K/Akt pathway.

METHODS: Cell proliferation/survival was determined by cell counting, sulphorhodamine or acridine orange/ethidium bromide assay; Akt activation was determined by western blot analysis. In vivo effect of compounds was tested on PC3 xenografts, whereas in vitro activity on kinases was determined by SelectScreen Kinase Profiling Service.

RESULTS: The derivative 2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP5) is active towards cancer types resistant to InsP5 in vitro and in vivo. 2-O-Bn-InsP5 possesses higher pro-apoptotic activity than InsP5 in sensitive cells and enhances the effect of anti-cancer compounds. 2-O-Bn-InsP5 specifically inhibits 3-phosphoinositide-dependent protein kinase 1 (PDK1) in vitro (IC50 in the low nanomolar range) and the PDK1-dependent phosphorylation of Akt in cell lines and excised tumours. It is interesting to note that 2-O-Bn-InsP5 also inhibits the mammalian target of rapamycin (mTOR) in vitro.

CONCLUSIONS: InsP5 and 2-O-Bn-InsP5 may represent lead compounds to develop novel inhibitors of the PI3K/Akt pathway (including potential dual PDK1/mTOR inhibitors) and novel potent anti-cancer drugs.

Keywords: phosphoinositide 3-kinase; inositol polyphosphates; protein kinase B-Akt; 3-phosphoinositide-dependent protein kinase 1; apoptosis

Phosphoinositide 3-kinase (PI3K) isoforms catalyse the phosphorylation of the 3-hydroxyl group within the inositol ring of phosphoinositides generating lipid products, which in turn mediate the activation of several proteins (Maffucci and Falasca, 2001; Vanhaesebroeck et al., 2001). The best characterised PI3K effector is the Serine/Threonine kinase protein kinase B (PKB)/Akt, which regulates a plethora of intracellular processes, including cell survival, growth, proliferation, migration and regulation of cell size (Vivanco and Sawyers, 2002; Manning and Cantley, 2007). Upon PI3K activation, interaction between Akt pleckstrin homology (PH) domain and the PI3K product phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) recruits Akt to the plasma membrane, where it is activated through phosphorylation at its residues Thr308 and Ser473. Phosphorylation of Thr308 is mediated by 3-phosphoinositide-dependent protein kinase 1 (PDK1), which itself possesses a PH domain able to bind PtdIns(3,4,5)P3 (Komander et al., 2004). Mutations in PDK1 PH domain that abolish PtdIns(3,4,5)P3 binding strongly inhibit Akt activation in homozygous knock-in embryonic stem cell and knock-in mice (McManus et al., 2004; Bayascas et al., 2008). On the other hand, binding of Akt PH domain to PtdIns(3,4,5)P3 is critical to induce a conformational change that allows PDK1-dependent phosphorylation (Calleja et al., 2007). Among other targets, Akt activates the multi-protein complex mTORC1 containing the enzyme mammalian target of rapamycin (mTOR), which regulates several intracellular functions including cell growth, cell cycle progression and autophagy (Wullschleger et al., 2006). It is interesting to note that a second mTOR-containing complex (mTORC2) is involved in the phosphorylation of Akt at its residue Ser473 as well as its activation (Sarbassov et al., 2005). The mechanism of mTORC2-dependent Akt phosphorylation at Ser473 is still not completely understood, but it does not seem to involve phosphoinositides, as in the case of PDK1, since mTOR does not appear to possess phosphoinositide-binding domains.

Deregulation of PI3K-dependent signalling pathways is linked to the development of cancer (Maehama and Dixon, 1999; Shayesteh et al., 1999; Vivanco and Sawyers, 2002; Bader et al., 2003; Shaw and Cantley, 2006; Vogt et al., 2007) and to increased resistance to treatment with chemotherapeutic agents (Clark et al., 2002; Liang et al., 2003; She et al., 2003). Accumulation of PtdIns(3,4,5)P3 either
due to gain of function of PI3K activity (Vivanco and Sawyers, 2002; Vogt et al, 2007) or loss of the enzyme phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which specifically dephosphorylates PtdIns(3,4,5)P3 (Maehama and Dixon, 1999), has been detected in almost 50% of all tumour types (Carracedo and Pandolfi, 2008). Reducing the levels of PDK1 in PTEN−/− mice strongly protects them from developing a wide range of tumours (Bayascas et al, 2005). Furthermore, it has recently been reported that PDK1 is overexpressed in several human breast cancers and that increased copy number of the gene encoding for PDK1 is associated with upstream pathway lesions and patient survival (Maurer et al, 2009), highlighting the importance of PDK1 in cancer development. Elevated Akt activity has been found in several forms of cancer (Sun et al, 2001; Bacus et al, 2002; Altomare et al, 2004) and evidence suggest that mTORC1 is one of the key effectors in PI3K/Akt-mediated tumourigenesis (Guertin and Sabatini, 2007). The crucial role of mTORC2 in tumorigenesis driven by Pten loss has also been reported (Guertin et al, 2009). The PI3K/Akt pathway is, therefore, at present being considered to be an attractive target for therapeutic intervention, and several compounds targeting the different components of the pathway have been developed or are in development (Vivanco and Sawyers, 2002; Luo et al, 2003; Hennessy et al, 2005; Guertin and Sabatini, 2007; Liu et al, 2009), with some of them currently in clinical trials for cancer treatment (Liu et al, 2009). Toxicity, low therapeutic index, insolubility and aqueous instability have prevented the use of many PI3K and in vivo selectivity profiles (Peifer and Alessi, 2008). Similarly several Akt inhibitors have been designed including compounds targeting its ATP-binding domain or allosteric inhibitors and pseudosubstrates (Luo et al, 2003; Crowell et al, 2007; Lindsley et al, 2008). However, some of these agents show toxic side effects because of non-specific effects or blockade of all Akt isoforms, they often possess poor physicochemical properties and inadequate selectivity profiles (Peifer and Alessi, 2008). Despite severe efforts, there is still a need to develop novel, more potent inhibitors of the PI3K/Akt pathway to overcome problems of lack of specificity and chemoresistance.

A few years ago we were the first to propose an alternative mechanism to block Akt activation based on the inhibition of its PH domain-mediated translocation to the plasma membrane (Berrie et al, 2000). The critical role of the PH domain in Akt-driven tumourigenesis has recently been highlighted by the detection of a somatic mutation in Akt1 PH domain resulting in Akt1 activation in breast, colorectal and ovarian cancers (Carpten et al, 2005). It is interesting to note that this mutant is able to induce leukaemia in mice (Carpten et al, 2007). Our strategy was based on the hypothesis that specific exogenous inositol polyphosphates can compete with PtdIns(3,4,5)P3 by binding to Akt PH domain, and thus prevent recruitment to the plasma membrane and activation of Akt (Berrie and Falasca, 2000). Indeed we reported that inositol 1,3,4,5,6-pentakisphosphate (InsP5) specifically blocks Akt activation and possesses pro-apoptotic (Razzini et al, 2000; Piccolo et al, 2004), anti-angiogenic and anti-tumour activity in vivo (Maffucci et al, 2005). In addition, some of us recently demonstrated the targeting of the Akt PH domain with an unusual inositol polyphosphate mimic (Mills et al, 2007). Other phosphatidylinositol-based Akt inhibitors also act by inhibiting Akt targeting to the plasma membrane, including ether lipid analogues and PH domain-targeting inhibitors (Kozikowski et al, 2003; Gills et al, 2006; Crowell et al, 2007) such as perifosine, the most developed Akt inhibitor currently available (Kondapaka et al, 2003).

In order to explore early structure-activity relationships for InsP5 and possibly obtain more potent and specific inhibitors of the PI3K/Akt pathway, we synthesised novel compounds based on the InsP5 structure. Here we show that the derivative 2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP5) exhibits more efficient and potent activity than InsP5 not only in inhibiting Akt phosphorylation but also in inducing apoptosis in different human cancer cell lines. It is interesting to note that 2-O-Bn-InsP5 promotes apoptosis in cell lines normally resistant to treatment with InsP5 and markedly inhibits the in vivo growth of InsP5-resistant xenografts. Kinase profiling analysis reveals that 2-O-Bn-InsP5 strongly inhibits PKD1 activity in vitro with an IC50 in the low nanomolar range. This is mirrored by the inhibition of Akt phosphorylation at its residue Thr308 in 2-O-Bn-InsP5-treated cells and in tumours from 2-O-Bn-InsP5-treated mice. Furthermore, the effect of 2-O-Bn-InsP5 is highly specific, as this compound only inhibits PKD1 and to a lesser extent mTOR in a panel of almost 60 kinases. These data represent the first attempt to exploit InsP5 as a potential lead compound for the development of potent small molecule inhibitors of the PI3K/Akt pathway.

MATERIALS AND METHODS

Materials

Inositol 1,3,4,5,6-pentakisphosphate was synthesised as previously reported (Godage et al, 2006). 2-O-Bn-InsP5 was synthesised in a similar manner from 2-O-benzyl-myo-inositol. Each compound was purified to homogeneity by ion-exchange chromatography on Q-Sepharose Fast Flow resin (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and used as the triethylammonium salt, which was fully characterized by 31P and 1H spectroscopy and accurately quantified by total phosphate assay. For the in vivo experiments, InsP5 and 2-O-Bn-InsP5 were each converted into the hexaosodium salt by treatment with Dowex 50WX2-100 ion-exchange resin (Sigma-Aldrich, Gillingham, Dorset, UK), followed by addition of sodium hydroxide (6 equivalents) and lyophilisation. Sulphorhodamine (SRB), curcumin, paclitaxel and 4-hydroxy-tamoxifen were purchased from Sigma-Aldrich; anti-Phospho Ser473 Akt, anti-Phospho Thr308 Akt and anti-Akt from (Cell Signalling Technologies, Danvers, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines

SKOV-3 and PC3 were cultured in RPMI 1640; all other cell lines were cultured in DMEM. Media were supplemented with 10% FBS, penicillin/streptomycin and glutamine.

Cell survival and apoptosis assays

Cells seeded in a 24-well plate were treated with the indicated compounds in serum free DMEM or DMEM containing 0.5% FBS (PC3). After 72 h, the number of surviving cells was assessed by manual cell counting or by using the cell counter CDA-500 (Sysmex, Milton Keynes, UK). Alternatively, after 48 h, the number of apoptotic cells was assessed by acridine orange/ethidium bromide assay as described (Piccolo et al, 2004; Maffucci et al, 2005). SRB test was carried out in SKOV-3 and PC3 seeded in 96-well plate (3800 cells per well or 3800 cells per well, respectively) after 72 h of treatment as described (Cappella et al, 2001).
**In vivo studies**

Male nude athymic CD-1 nu/nu mice (8-weeks old) were obtained from Harlan (San Pietro al Natisone, Italy) and maintained under specific pathogen-free conditions with food and water provided *ad libitum*. The general health status of the animals was monitored daily. Procedure involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

**Toxicity assay**

Male nude CD-1 mice were treated with a single dose of 750 mg kg⁻¹ InsP₅ or 2-O-Bn-InsP₅/mouse administrated intra-peritoneally (i.p.). Each group consisted of 2–3 mice. Body weight, deaths and any other sign of toxicity and changes in behaviour (such as motility, eating and drinking habits) were recorded.

**Anti-tumour activity assay**

Exponentially growing PC3 cells were harvested, washed twice and resuspended in PBS at a concentration of 2.5 × 10⁶ cells ml⁻¹. A suspension of 5 × 10⁶ PC3 cells was injected subcutaneously (s.c.) into the left flank of the recipient mice. When tumours reached a size of ~70 mm³ (approximately 15 days after tumour cell implant), mice were divided into seven groups (n = 7). InsP₅ and 2-O-Bn-InsP₅ were administered by daily i.p. injections at different doses of 12.5–25–50 mg kg⁻¹ day⁻¹ for 14 consecutive days. Control mice were treated with water in an equal volume. The diameters of s.c. growing tumours were measured with a caliper twice a week and the experiment was ended at day 28 after the implantation.

**Data analysis and in vivo tumour parameters**

The volume of s.c. growing tumours was calculated by the formula: Tumour weight (mg) = (length × width²)/2. Differences in s.c. tumour growth between the treatment groups were evaluated with a one-way ANOVA followed by Fisher’s test using the StatView statistical package (SAS Institute, Cary, NC, USA). The percentage of tumour growth was calculated as T/C% = (RTV-treated animals/RTV-control animals) × 100, where RTV was the mean relative tumour volume calculated as RTV = Vₓ/V₀. Vₓ was the tumour volume on the day of measurement and V₀ was the tumour volume at the beginning of the treatment. The percentage of tumour weight inhibition (TWI%) was calculated using the formula: TWI% = 100 – T/C%. The log cell kill (LCK) was calculated using the formula: LCK = T – C/3.32 × Td, where T – C is the tumour growth delay calculated as the difference in median time (in days) required for the tumours in the treatment (T) and control group (C) to reach a predetermined size (i.e., 1000 mg). Td is the tumour volume doubling time in days, determined in the exponential growth phase of the control group from a best-fit straight line. Median doubling time was 3 days in control animals.

**Western blot**

Mice with s.c. growing tumours were treated with a single dose of InsP₅ and 2-O-Bn-InsP₅ (50 mg kg⁻¹) or vehicle. Animals were killed 24 h after treatment and tumour samples were collected and snap frozen. Frozen specimens of tumour tissue were homogenised with a Polytron homogeniser in a lysis buffer (ratio 1:1 w/v) containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% Nonidet NP-40, 250 mM NaCl, 50 mM NaF and proteases and phosphatase inhibitors. After centrifugation at 13 000 r.p.m. for 10 min at 4°C, 80 μg of protein was separated on SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). Membranes were probed with the indicated antibodies.

**Protein kinase profiling**

Effect of the indicated compounds on the activity of various kinases was assessed by SelectScreen Kinase Profiling Service (Invitrogen-Life Technologies, Paisley, UK). Assays were performed using 1 μM of the tested compounds and ATP concentration as indicated in the corresponding tables. In the case of InsP₅, a screen using 10 μM of the compound was also carried out, as indicated in the corresponding table.

**RESULTS**

**Synthesis of novel potential inhibitors of the PI3K/Akt pathway and in vitro screening**

We have recently reported that InsP₅ is a novel inhibitor of the PI3K/Akt pathway, which possesses pro-apoptotic, anti-angiogenic and anti-tumour activity (Razzini et al., 2000; Piccolo et al., 2004; Maffucci et al., 2005). To explore the design of novel inhibitors of the PI3K/Akt pathway, potentially more active than InsP₅, we decided to modify the structures of either Ins(1,3,4,5)P₄ or InsP₅. Different strategies were used to modify the parent molecules and several compounds were synthesised and tested. For Ins(1,3,4,5)P₄-related compounds, modifications were made at C-6 because X-ray structures of the Akt (Thomas et al., 2002) and PDK1 (Komander et al., 2004) PH domains in complex with Ins(1,3,4,5)P₄ indicated that the 6-hydroxy group of Ins(1,3,4,5)P₄ is not directly involved in binding. Furthermore, the X-ray structure of Akt PH domain showed that a tyrosine residue near the 6-OH of bound Ins(1,3,4,5)P₄ might interact with an aromatic group. In the case of InsP₅ analogs, modifications were on either the 2-O-atom or the 5-phosphate, thus maintaining the symmetry of the parent molecule. The derivatives were first tested for their ability to inhibit Akt activation in cell lines characterised by constitutive activation of the PI3K/Akt pathway and with a reported sensitivity to InsP₅, namely ovarian cancer cells SKOV-3 and breast cancer cells SKBR3 (Piccolo et al., 2004; Maffucci et al., 2005). In this original screening we observed that the InsP₅, derivative 2-O-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (Figure 1A, named 2-O-Bn-InsP₅) showed the highest efficiency in inhibiting Akt activation in all cell lines tested (data on the other derivatives will be published elsewhere). More specifically, we found that 2-O-Bn-InsP₅ inhibited Akt phosphorylation at its residue Ser473 more efficiently than InsP₅ in SKOV-3, being already active after 8 h of treatment and at a concentration of 20 μM (Figure 1B). Inhibition of Akt phosphorylation at residue Thr308 was also detected (Figure 1B). More interestingly, we found that 2-O-Bn-InsP₅ was able to block Akt phosphorylation in cell lines resistant to InsP₅, such as prostate cancer cells PC3 (Figure 1C) and pancreatic cancer cells ASPC1 (results not shown). Taken together these data indicate that structural modification at the C-2 of InsP₅ can enhance its inhibitory properties towards Akt activation.

**Analysis of the biological activity of 2-O-Bn-InsP₅**

We next compared the effects of 2-O-Bn-InsP₅ and InsP₅ on proliferation/survival of cancer cells in vitro. Treatment with 2-O-Bn-InsP₅ strongly reduced the number of surviving SKBR3 (Figure 2A) and SKOV-3 (Figure 2B) assessed by cell counting. In particular, 2-O-Bn-InsP₅ was more active than InsP₅ in both the cell lines. Acridine orange/ethidium bromide assay confirmed that the percentage of apoptotic cells was higher in 2-O-Bn-InsP₅-treated compared with InsP₅-treated SKBR3 (Figure 2C) and SKOV-3 (Figure 2D). Based on the data on Akt phosphorylation we then decided to analyse the effect of 2-O-Bn-InsP₅ on the survival of cell lines normally very resistant to InsP₅ treatment. 2-O-Bn-InsP₅ was more potent than InsP₅, at a concentration of 50 μM in pancreatic cancer cells BxPc-3 (Figure 3A), whereas it was more...
active than InsP₅ at almost all concentrations tested in pancreatic cancer cells ASPC1 (Figure 3B). A stronger activity of 2-O-Bn-InsP₅ compared with InsP₅ was also observed in breast cancer cells MDA-MB-468 (Figure 3C) and in PC3 (Figure 3D), consistent with data on Akt phosphorylation. Higher activity of 2-O-Bn-InsP₅ in PC3 cells was also observed in SRB assays (Figure 3E). It is important to note that although InsP₅ had no effect in PC3 at concentrations up to 50 μM, concentrations of 200 – 300 μM were eventually able to mimic the effect of 2-O-Bn-InsP₅ in PC3 cells (Figure 3F), thus suggesting that 2-O-Bn-InsP₅ is acting on the same intracellular pathway as InsP₅. Taken together these data demonstrate that addition of a benzyl group to the axial 2-O atom of InsP₅ potentiates the pro-apoptotic properties of the compound not only in cells sensitive to InsP₅ but also in cells normally very resistant to treatment with the parent inositol compound.

**In vivo anti-tumour activity of 2-O-Bn-InsP₅ on InsP₅-resistant xenografts**

We then decided to test the therapeutic efficacy of 2-O-Bn-InsP₅ in human tumour xenografts characterised by the activation of PI3K/Akt pathway and higher sensitivity to 2-O-Bn-InsP₅ compared with InsP₅. We specifically implanted PC3 cells in nude mice and 15 days after the implantation we treated groups of mice with different concentrations (12.5, 25 and 50 mg kg⁻¹) of InsP₅ or 2-O-Bn-InsP₅ for 14 consecutive days (from day 15 to day 28). Tumour growth was followed for further 12 days after the end of the treatment (upto day 40). Data revealed that 2-O-Bn-InsP₅ at doses of 12.5 and 25 mg kg⁻¹ clearly decreased the growth of tumours compared with untreated mice, although the differences were statistically significant only on the last day of measurement (Figure 4A and C). A strong reduction in tumour growth was obtained in the group treated with 50 mg kg⁻¹ 2-O-Bn-InsP₅, with a statistically significant difference vs controls detectable from day 22 after tumour cells implant onwards (Figure 4A and C). Data on *in vivo* anti-tumour activity parameters relative to 2-O-Bn-InsP₅ are shown in Figure 4G, bottom table. More than 50% inhibition of tumour weight was achieved in the 50 mg kg⁻¹ -treated group, with a tumour growth delay (T – C) of almost 9 days between this group and the untreated (control) group. In agreement with our *in vitro* data, we observed that InsP₅ had no effect on concentrations up to 50 mg kg⁻¹ (Figure 4B). At the end of the experiment, western blot analysis revealed that 24 h-treatment with 2-O-Bn-InsP₅ markedly reduced Akt phosphorylation at its residue Ser473 in all 2-O-Bn-InsP₅-treated mice (Figure 4D). It is noteworthy that no evidence of toxicity was observed in different groups of mice at all the tested doses of either 2-O-Bn-InsP₅ or InsP₅ and the body weight of the treated animals was not different from the
Bn-InsP5 did not inhibit (percentage of inhibition activity towards mTOR (Table 1A, Supplementary Table 1). 2-O-inhibit any of the tested kinases (Supplementary Table 3) including more efficient blockade of Akt phosphorylation in vivo kinases and at a concentration of 10
Table 1). InsP 5 showed a reduced inhibitory effect on PDK1 isoforms tested or any Akt isoforms (Table 1B, Supplementary Table 1). Further-
more, 2-O-Bn-InsP 5, InsP 5 inhibit any of the class I PI3K members of the MAPK family (Supplementary Table 1). Further-
myo
M, InsP5 did not significantly cause any major toxic effect untreated mice throughout the entire experiment (Figure 4E). Moreover, a single treatment with a very high dose of 2-O-Bn-InsP 5 or InsP 5 (750 mg kg
1) did not cause any major toxic effect (Figure 4F). Taken together these data demonstrate that 2-O-Bn-InsP 5 is able to inhibit growth of InsP 5-resistant tumours through a more efficient blockade of Akt phosphorylation in vivo.

In vitro kinase profiling of InsP 5 and 2-O-Bn-InsP 5
To determine the mechanism responsible for the higher activity of 2-O-Bn-InsP 5, we decided to carry out a protein kinase activity screen for InsP 5 and 2-O-Bn-InsP 5 (SelectScreen Kinase Profiling Service, Invitrogen-Life Technologies). Among almost 60 protein kinases screened, 2-O-Bn-InsP 5 (1 μM) showed a very high inhibitory activity towards PDK1 (79% inhibition) and a lower activity towards mTOR (Table 1A, Supplementary Table 1). 2-O-Bn-InsP 5 did not inhibit (percentage of inhibition < 40%) any of all the other tested kinases, including AGC kinases, such as GSK3, RSK, S6K and members of the PKC family, AMPK and several members of the MAPK family (Supplementary Table 1). Furthermore 2-O-Bn-InsP 5 did not directly inhibit any of the class I PI3K isoforms tested or any Akt isoforms (Table 1A, Supplementary Table 1). InsP 5 showed a reduced inhibitory effect on PDK1 compared with 2-O-Bn-InsP 5 (Table 1B and C, Supplementary Table 2 and 3). As 2-O-Bn-InsP 5, when tested on a panel of over 50 kinases and at a concentration of 10 μM, InsP 5 did not significantly inhibit any of the tested kinases (Supplementary Table 3) including Akt isoforms (Table 1B). In contrast to 2-O-Bn-InsP 5, InsP 5 did not inhibit mTOR, even when tested at a concentration of 10 μM (Supplementary Table 3). Comparing the effect of 1 μM of different natural inositol polyphosphates on PDK1, InsP 5 possessed the highest inhibitory activity towards PDK1 (71% inhibition) with only Ins(1,3,4,5)P 4 also showing some effect (56% inhibition). None of the other polyphosphates had any significant effect (Table 1C). These data indicate that 2-O-Bn-InsP 5 and InsP 5 inhibit PDK1 very specifically, with 2-O-Bn-InsP 5 possessing the highest inhibitory activity towards PDK1. Indeed results from SelectScreen Kinase Profiling Service (Invitrogen-Life Technologies) 10-point titration revealed that the IC 50 of InsP 5 towards PDK1 was 613 nM whereas the corresponding IC 50 of 2-O-Bn-InsP 5 was a striking 26.5 nM (Table 1A and B). These data clearly indicate that 2-O-Bn-InsP 5 is a novel, potent and highly selective PDK1 inhibitor. This is consistent with the detected inhibition of Thr308 phosphorylation in 2-O-Bn-InsP 5-treated SKOV-3 and PC3 cells (Figure 1B and C) and 2-O-Bn-InsP 5-treated mice (Figure 4D). Furthermore, 2-O-Bn-InsP 5, but not InsP 5, is able to inhibit mTOR selectively in vitro with an IC 50 of 1.3 μM (Table 1A).

In vitro effects of 2-O-Bn-InsP 5 in combination with anti-cancer compounds
Parallel RNAi and compound screens have recently revealed that PDK1 is a critical determinant of sensitivity to tamoxifen in breast...
Based on this result we decided to investigate whether inhibition of PDK1 by 2-O-Bn-InsP₅ was able to sensitise MCF7 to the pro-apoptotic effect of tamoxifen. Our data revealed that treatment with 4-OH tamoxifen (the active metabolite of tamoxifen) for 72 h reduced the number of surviving cells, whereas 2-O-Bn-InsP₅ had little effect (Figure 5A). It is interesting to note that the combination of 2-O-Bn-InsP₅ and 4-OH tamoxifen strongly enhanced the effect of 4-OH tamoxifen or 2-O-Bn-InsP₅ alone (Figure 5A). We then tested the effects of 2-O-Bn-InsP₅ in combination with several natural anti-cancer compounds. The concentrations of the different compounds used in these experiments were minimally effective based on preliminary dose-response experiments (results not shown). A combination of 2-O-Bn-InsP₅ and curcumin, a component of turmeric (Curcuma longa), strongly reduced the number of surviving PC3 cells, resulting in a more than additive effect (Figure 5B) and was able to enhance the effect of curcumin in ASPC1 (Figure 5C) and in MDA-MB-468 (Figure 5D). A combination of 2-O-Bn-InsP₅ and paclitaxel clearly reduced the number of surviving MDA-MB-468 (Figure 5D), SKOV-3 (Figure 5E) and PC3 (Figure 5F) cells compared with the corresponding single treatments. An additive effect was detected when combining 2-O-Bn-InsP₅ with rapamycin in SKOV-3 (Figure 5E) and PC3 (Figure 5F) cells. These data clearly indicate that the combination of 2-O-Bn-InsP₅ and curcumin or paclitaxel can sensitize MCF7 and PC3 cells to the pro-apoptotic effects of 4-OH tamoxifen.

Figure 3 2-O-benzyl-myoinositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP₅) possesses pro-apoptotic activity in cell lines resistant to inositol 1,3,4,5,6-pentakisphosphate (InsP₅). (A–D) BxPc-3 (A), ASPC1 (B), MDA-MB-468 (C) and PC3 (D) were treated for 72 h with the indicated concentrations of InsP₅ or 2-O-Bn-InsP₅. The number of surviving cells was assessed by cell counting. Data are mean ± s.e. of n = 3 (A), n = 6 (B), n = 3 (C) and n = 4 (D) independent experiments carried out in duplicate. * = P < 0.01; ** = P < 0.05. (E, F) PC3 were treated with the indicated concentrations of InsP₅ and 2-O-Bn-InsP₅ (E) or increasing concentrations of InsP₅ (F). After 72 h the number of surviving cells was assessed by SRB assay. Data are mean ± s.e. of n = 2 independent experiments.

cancer cells MCF7 (Iorns et al, 2009). Based on this result we decided to investigate whether inhibition of PDK1 by 2-O-Bn-InsP₅ was able to sensitize MCF7 to the pro-apoptotic effect of tamoxifen. Our data revealed that treatment with 4-OH tamoxifen (the active metabolite of tamoxifen) for 72 h reduced the number of surviving cells, whereas 2-O-Bn-InsP₅ had little effect (Figure 5A). It is interesting to note that the combination of 2-O-Bn-InsP₅ and 4-OH tamoxifen strongly enhanced the effect of 4-OH tamoxifen or 2-O-Bn-InsP₅ alone (Figure 5A). We then tested the effects of 2-O-Bn-InsP₅ in combination with several natural anti-cancer compounds. The concentrations of the different compounds used in these experiments were minimally effective based on preliminary dose-response experiments (results not shown). A combination of 2-O-Bn-InsP₅ and curcumin, a component of turmeric (Curcuma longa), strongly reduced the number of surviving PC3 cells, resulting in a more than additive effect (Figure 5B) and was able to enhance the effect of curcumin in ASPC1 (Figure 5C) and in MDA-MB-468 (Figure 5D). A combination of 2-O-Bn-InsP₅ and paclitaxel clearly reduced the number of surviving MDA-MB-468 (Figure 5D), SKOV-3 (Figure 5E) and PC3 (Figure 5F) cells compared with the corresponding single treatments. An additive effect was detected when combining 2-O-Bn-InsP₅ with rapamycin in SKOV-3 (Figure 5E) and PC3 (Figure 5F) cells. These data clearly indicate that the combination of 2-O-Bn-InsP₅ and curcumin or paclitaxel can sensitize MCF7 and PC3 cells to the pro-apoptotic effects of 4-OH tamoxifen.
InsP$_5$ and natural anti-cancer compounds results in additive or more than additive effects, and therefore suggest that 2-O-Bn-InsP$_5$ can potentially be used in combination with natural compounds to increase their anti-cancer activity.

**DISCUSSION**

The *in vivo* anti-tumour activity of InsP$_5$ together with the lack of toxicity observed using this compound (Maffucci *et al.*, 2005),
sensitive to InsP<sub>5</sub>. In this original screen, 2-O-Bn-InsP<sub>5</sub> was not only more efficient than the parent molecule in inhibiting Akt phosphorylation and inducing apoptosis in InsP<sub>5</sub>-sensitive cell lines but it was also able to induce apoptosis in InsP<sub>5</sub>-resistant cell lines including pancreatic cancer cells. The pro-apoptotic activity of 2-O-Bn-InsP<sub>5</sub> detected in pancreatic cancer cells represents an extremely important result taking into account the high resistance of this cancer to chemotherapeutic treatment and the urgent need for novel therapeutics in clinical treatment. Furthermore, 2-O-Bn-InsP<sub>5</sub> was able to inhibit the in vivo growth of InsP<sub>5</sub>-resistant prostate cancer xenografts. It is noteworthy that, although 2-O-Bn-InsP<sub>5</sub> acted in the micromolar range in in vitro studies, it was able to inhibit tumour growth in vivo at 12.5, 25 and 50 mg kg<sup>-1</sup>, doses commonly used to test the in vivo effect of potential anti-tumour compounds. In particular, 2-O-Bn-InsP<sub>5</sub> induced a tumour weight inhibition of 52% in prostate cancer xenografts when dosed at 50 mg kg<sup>-1</sup> once daily for 14 days.

We then decided to investigate in more detail the mechanisms of action of InsP<sub>5</sub> and 2-O-Bn-InsP<sub>5</sub> and to explain the higher activity of 2-O-Bn-InsP<sub>5</sub> compared with the parent molecule. Our previous and current data demonstrated that the in vitro and in vivo properties of InsP<sub>5</sub> and 2-O-Bn-InsP<sub>5</sub> were because of reduced Akt phosphorylation and activation (Piccolo et al., 2004; Maffucci et al., 2005). Results from the kinase profiling assays here show that InsP<sub>5</sub> and 2-O-Bn-InsP<sub>5</sub> do not inhibit Akt kinase activity itself in vitro, whereas they are both able to directly inhibit PDK1 kinase activity (albeit with different potency), thus indicating that the detected Akt inhibition is due to blockade of the activity of its upstream regulatory kinase. This is consistent with the observed enhanced activity of 2-O-Bn-InsP<sub>5</sub> compared with InsP<sub>5</sub>, likely due to its higher inhibitory activity towards PDK1. Furthermore, this difference could explain the strong effect of 2-O-Bn-InsP<sub>5</sub> in InsP<sub>5</sub>-resistant cell lines such as the PTEN mutant cells MDA-MB-468 and PC3, and would be consistent with the proposed key role of PDK1 in tumourigenesis driven by PTEN loss (Bayascas et al., 2005). It should be noted that the resistance to InsP<sub>5</sub> treatment in these cells is consistent with the delayed onset of inhibition in cells with mutant PTEN observed using the ether lipid analogues (Castillo et al., 2004). The observation that higher concentrations of InsP<sub>5</sub> are eventually able to mimic the pro-apoptotic effect of 2-O-Bn-InsP<sub>5</sub> in these cells further supports the conclusion that the different activity is because of different potency of the two compounds towards PDK1. In this respect, it is interesting to notice that the addition of the benzyl group to InsP<sub>5</sub> confers such a higher inhibitory activity towards PDK1 on the derivative 2-O-Bn-InsP<sub>5</sub>. It would be interesting to investigate whether the resulting small increase in the hydrophobicity of the molecule enhances its activity possibly by improving its binding to PDK1. Moreover, it is noteworthy that such a modification confers 2-O-Bn-InsP<sub>5</sub> a selective inhibitory activity towards mTOR in vitro, providing crucial information to develop novel specific dual PI3K/mTOR inhibitors. Indeed one intriguing possibility is that 2-O-Bn-InsP<sub>5</sub> is more active than InsP<sub>5</sub> because of its unique capability to inhibit simultaneously and very specifically PDK1 and mTOR. In particular the dual activity of 2-O-Bn-InsP<sub>5</sub> can explain its effect

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**Table 1** Results from SelectScreen kinase profiling service (Invitrogen–Life Technologies)

| Compound | [ATP] tested (μM) | Kinase tested | IC<sub>50</sub> (nM) |
|----------|-------------------|---------------|---------------------|
| (A)      |                   |               |                     |
| 2-O-Bn-InsP<sub>5</sub> | 100              | PDK1          | 265                 |
| 2-O-Bn-InsP<sub>5</sub> | 75               | Akt1 (PKBz)   | >10000              |
| 2-O-Bn-InsP<sub>5</sub> | 200              | Akt2 (PKBβ)   | 77000               |
| 2-O-Bn-InsP<sub>5</sub> | 10               | FRAP (mTOR)   | 1300                |
| (B)      |                   |               |                     |
| InsP<sub>5</sub> | 100              | PDK1          | 613                 |
| InsP<sub>5</sub> | 75               | Akt1 (PKBz)   | >10000              |
| InsP<sub>5</sub> | 200              | Akt2 (PKBβ)   | 38300               |
| (C)      |                   |               | % Inhibition-mean   |
| Ins(1,4,5)P<sub>3</sub> | 100             | PDK1          | 17                  |
| Ins(1,3,4,5)P<sub>4</sub> | 100             | PDK1          | 56                  |
| Ins(1,4,5,6)P<sub>4</sub> | 100             | PDK1          | 33                  |
| Ins(3,4,5,6)P<sub>4</sub> | 100             | PDK1          | 35                  |
| InsP<sub>5</sub> | 100              | PDK1          | 71                  |
| Ins(1,2,3,4,5,6)P<sub>6</sub> | 100         | PDK1          | 18                  |

Abbreviations: InsP<sub>5</sub> = inositol 1,3,4,5,6-pentakisphosphate; 2-O-Bn-InsP<sub>5</sub> = 2-O-benzyl-myro-inositol 1,3,4,5,6-pentakisphosphate. 10-point titration for 2-O-Bn-InsP<sub>5</sub>: 10-point titration for InsP<sub>5</sub>. Single point for the indicated inositol polyphosphates. A concentration of 1 μM of the compounds was used in the assays.

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**Figure 4** 2-O-benzyl-myro-inositol 1,3,4,5,6-pentakisphosphate (2-O-Bn-InsP<sub>5</sub>) possesses anti-tumour activity on inositol 1,3,4,5,6-pentakisphosphate (InsP<sub>5</sub>)-resistant xenografts and it is not associated with toxicity in vivo. Male athymic CD-1 nu/nu mice were inoculated subcutaneously (s.c.) with PC3 and treated with the indicated concentrations of either InsP<sub>5</sub> or 2-O-Bn-InsP<sub>5</sub> from day 15 after cell implantation. The inositol compounds (12.5–50 mg kg<sup>-1</sup> day<sup>-1</sup>) and vehicle (water) were given daily by intraperitoneal (i.p.) injections for 14 consecutive days (days 15–28). Tumour size was assessed twice weekly. (A, B) Tumour growth in 2-O-Bn-InsP<sub>5</sub>-treated mice (A) and InsP<sub>5</sub>-treated mice (B) compared with control group measured for the duration of the experiment. Results are expressed as mean ± S.E.M. (C) Top: Table showing all P-values for the indicated doses of 2-O-Bn-InsP<sub>5</sub> and InsP<sub>5</sub>. Bottom: In vivo anti-tumour activity parameters. The percentage of tumour weight inhibition (TWI%), the tumour growth delay (T–C) and the log cell kill (LCK) were calculated as described in the MATERIALS AND METHODS section. The highest inhibition of tumour volume is reported. (D) Mice with s.c. growing tumours were treated with a single dose (50 mg kg<sup>-1</sup>) of InsP<sub>5</sub>, 2-O-Bn-InsP<sub>5</sub> or water (control). Tumours were excised 24 h after treatment. Phosphorylation of Akt at its residues Ser473 and Thr308 was assessed by using specific antibodies. Membranes were then stripped and re-probed with an anti-Akt antibody. (E) Body weights of mice treated with 2-O-Bn-InsP<sub>5</sub> or InsP<sub>5</sub> for 14 consecutive days. (F) Body weights of mice treated with a single dose of 750 mg kg<sup>-1</sup> InsP<sub>5</sub> or 2-O-Bn-InsP<sub>5</sub>.
Translational Therapeutics

Curcumin + phosphorylation, indicating that 2-
the inhibitory activity towards Akt, by preventing its Ser473
et al prostate cancer (Guertin et al, 2010). Furthermore, a potential
key role of mTORC2 in the development of loss of Pten-driven
prostate cancer cells PC3, consistent with the recently reported
in prostate cancer cells PC3, consistent with the recently reported
role of mTORC2 in the development of loss of Pten-driven
prostate cancer (Guertin et al, 2009). Furthermore, a potential
2-O-Bn-InsP₅-mediated inhibition of mTORC2 would also increase
the inhibitory activity towards Akt, by preventing its Ser473
phosphorylation, indicating that 2-O-Bn-InsP₅ represents a useful
compound to be tested in cancer types specifically dependent
on mTOR activation. Studies have recently revealed the existence
of a negative-feedback loop by which mTORC1 inhibition leads
to upregulation of Akt (Manning, 2004; O’Reilly et al, 2006)
and/or ERK/MAPK pathway (Carracedo et al, 2008), activating
proliferative and anti-apoptotic signals in certain cancer types.
The possibility of blocking PDK1 and mTOR simultaneously in these tumours is likely to be very effective therefore, because of its dual inhibitory activity towards both enzymes, it would be interesting to investigate the effect of 2-O-Bn-InsP$_5$ in these cellular contexts.

Our future strategies to design novel compounds will take into consideration the possibility that, besides its direct inhibitory activity towards PDK1 and possibly mTOR, binding of 2-O-Bn-InsP$_5$ to non-catalytic domains may affect the activity of kinases or their mechanism of activation in vivo. Indeed in our previous work we proposed that InsP$_5$ can inhibit Akt activation by binding to Akt PH domain and preventing Akt recruitment to the plasma membrane (Berrie and Falasca, 2000). It was also proposed that inositol phosphates can bind PDH1 PH domain and retain this kinase in the cytosol, preventing Akt phosphorylation at Thr308 (Komander et al, 2004). These data suggest that the detected inhibitory effect of both 2-O-Bn-InsP$_3$ and InsP$_5$ on Akt activation in vivo can result from a combination of a direct effect on PDK1 kinase activity and effect on Akt/PDK1 recruitment to the plasma membrane. Similarly, the possibility that in vivo the inositol polyphosphates can bind and increase the activity of phosphatases which regulate Akt, such as PH domain leucine-rich repeat protein phosphatases 1 and 2 (Gao et al, 2005; Brognard et al, 2007) will be taken into consideration in our future strategies. In this respect it would be interesting to develop binding experiments of cellular lysates to immobilized 2-O-Bn-InsP$_3$ and InsP$_5$ to determine whether the compounds only bind PDK1, as indicated by the kinase profiling assays, or the in vivo mechanisms of action is more complex. These experiments would also give more information of whether the compounds may indirectly act on other kinases without directly affecting their catalytic activity.

It must be noted that, like InsP$_5$, 2-O-Bn-InsP$_5$ is a water soluble compound and it is well tolerated in vivo even at concentrations 15 times higher the active dose. In addition, combination of 2-O-Bn-InsP$_5$ with other anti-cancer compounds including natural compounds results in additive or more than additive effects, indicating that such a compound (or derivatives) may prove particularly useful in combinatorial therapies. In particular 2-O-Bn-InsP$_5$ increases the effect of tamoxifen in breast cancer cells MCF7, consistent with the reported role of PDK1 inhibition in tamoxifen sensitisation (Iorns et al, 2009). It is worth mentioning that our in vitro assays revealed that InsP$_5$ itself is able to inhibit PDK1 (although less than 2-O-Bn-InsP$_5$). This raises the interesting possibility that the endogenous intracellular InsP$_5$ may act as an endogenous PDK1 inhibitor and regulator of the PI3K/Akt pathway. This hypothesis is currently being investigated in our laboratory.

In conclusion, here we have described the in vitro and in vivo properties of 2-O-Bn-InsP$_5$, a derivative of InsP$_5$, which possesses similar solubility and lack of toxicity in vivo but enhanced pro-apoptotic and anti-tumour activity compared with the parent molecule. In particular 2-O-Bn-InsP$_5$ possesses specific inhibitory activity towards PDK1. Data also indicate that 2-O-Bn-InsP$_5$ can inhibit mTOR, at least in vitro. It is interesting to note that InsP$_5$ does not possess such an inhibitory activity towards mTOR, thus suggesting that comparison of the two molecules can give useful information towards developing specific dual PDK1/mTOR inhibitors. Taken together these data indicate that InsP$_5$ and 2-O-Bn-InsP$_5$ may represent promising models for further development of novel anti-cancer drugs.

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