Inhibition of the Phosphatidylinositol 3-Kinase/Akt Pathway by Inositol Pentakisphosphate Results in Antiangiogenic and Antitumor Effects

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Abstract

The purpose of this study was to investigate the antiangiogenic and in vivo properties of the recently identified phosphatidylinositol 3-kinase (PI3K)/Akt inhibitor Inositol(1,3,4,5,6) pentakisphosphate [Ins(1,3,4,5,6)P5]. Because activation of the PI3K/Akt pathway is a crucial step in the events leading to angiogenesis, the effect of Ins(1,3,4,5,6)P5 on basic fibroblast growth factor (FGF-2)-induced Akt phosphorylation, cell survival, motility, and tubulogenesis in vitro was tested in human umbilical vein endothelial cells (HUVEC). The effect of Ins(1,3,4,5,6)P5 on FGF-2-induced angiogenesis in vivo was evaluated using s.c. implanted Matrigel in mice. In addition, the effect of Ins(1,3,4,5,6)P5 on growth of ovarian carcinoma SKOV-3 xenograft was tested. Here, we show that FGF-2 induces Akt phosphorylation in HUVEC resulting in antiapoptotic effect in serum-deprived cells and increase in cellular motility. Ins(1,3,4,5,6)P5 blocks FGF-2-mediated Akt phosphorylation and inhibits both survival and migration in HUVEC. Moreover, Ins(1,3,4,5,6)P5 inhibits the FGF-2-mediated capillary tube formation of HUVEC plated on Matrigel and the FGF-2-induced angiogenic reaction in BALB/c mice. Finally, Ins(1,3,4,5,6)P5 blocks the s.c. growth of SKOV-3 xenografted in nude mice to the same extent as cisplatin and it completely inhibits Akt phosphorylation in vivo. These data definitively identify the Akt inhibitor Ins(1,3,4,5,6)P5 as a specific antiangiogenic and antitumor factor. Inappropriate activation of the PI3K/Akt pathway has been linked to the development of several diseases, including cancer, making this pathway an attractive target for therapeutic strategies. In this respect, Ins(1,3,4,5,6)P5, a water-soluble, natural compound with specific proapoptotic and antiangiogenic properties, might result in successful anticancer therapeutic strategies. (Cancer Res 2005; 65(18): 8339-49)

Introduction

Angiogenesis, the formation of a mature vasculature from a primitive vascular network, is required for different physiologic events such as development, growth, tissue remodeling, and wound healing (1). On the other hand, angiogenesis is involved in pathologies such as arteriosclerosis and tumor growth. Angiogenesis consists of a multistep process involving capillary endothelial cell migration and proliferation and formation of three-dimensional structures capable of carrying blood (1). Endothelial cell migration and proliferation is induced by growth factors such as vascular endothelial growth factor (VEGF or VEGF-A) and fibroblast growth factor (FGF; ref. 2). Basic FGF (FGF-2) was the first proangiogenic molecule to be identified. FGF-2 and VEGF stimulate survival, proliferation, migration, and differentiation of endothelial cells, although the efficiencies of transduction of these responses are dependent on the cell types (2). Binding of VEGF and FGF-2 to their respective receptors leads to receptor phosphorylation and subsequent activation of signaling proteins such as phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (2). A recent report showed that stimulation of PI3K by FGF receptor is mediated by coordinated recruitment of multiple docking proteins (3).

PI3K catalyzes the phosphorylation of inositol phospholipids at the D3 position to generate 3′-phosphorylated phosphoinositides (4). The 3′-phosphorylated phosphoinositides act by recruiting specific signaling proteins to the plasma membrane in a mechanism mediated by their interaction with some structural protein domains, among which the pleckstrin homology domain is the most studied (5). One of the best-characterized PI3K downstream targets is the serine/threonine protein kinase B, also known as Akt (6), and all studies concerning the role of PI3K in angiogenesis focus their attention on the PI3K/Akt pathway (7). Three members of the Akt family (Akt1, Akt2, and Akt3) have been identified and they are, in general, broadly expressed. Akt activation requires its translocation to the plasma membrane via interaction of its pleckstrin homology domain with 3′-phosphorylated phosphoinositides (8–11) and subsequent phosphorylation at residues Thr308 and Ser473. Phosphorylation at residue Thr308 is catalyzed by the enzyme phosphoinositide-dependent kinase-1 (12, 13), which itself is recruited to the plasma membrane via the interaction of its pleckstrin homology domain with 3′-phosphorylated phosphoinositides (14). The kinase responsible for phosphorylation at residue Ser473 is yet to be definitively identified. Once activated, Akt can phosphorylate several signaling proteins, which, in turn, lead to the choice of cellular proliferation or apoptosis (15–17). Therefore, Akt activation is
considered both necessary and sufficient for cell survival. In particular, Akt promotes survival through inactivation of caspase-9 (18), activation of the transcription factor nuclear factor-κB (19, 20), and phosphorylation of different components of the apoptotic machinery, such as BAD and forkhead transcription factor (FKHR1-1; ref. 21). Overexpression of Akt may therefore contribute to tumor development and progression. The crucial role of the PI3K/Akt pathway in cancer is further supported by the fact that the tumor suppressor PTEN, whose gene is deleted or mutated in a wide variety of human cancers, possesses a 3’-phosphoinositide-phosphatase activity inactivating the PI3K/Akt pathway (22).

Very recently, we have evaluated the effect of different inositol polyphosphates on Akt activation and we have shown that Inositol(1,3,4,5,6)pentakisphosphate [Ins(1,3,4,5,6)P5] is specifically able to inhibit Akt phosphorylation and kinase activity, whereas other inositol polyphosphates tested have no effect (23, 24). Consequently, Ins(1,3,4,5,6)P5 specifically promotes apoptosis in lung, ovarian, and breast cancer cell lines (24). In this report, we describe the antiangiogenic properties and the in vivo antitumor effects of Ins(1,3,4,5,6)P5. We show that Ins(1,3,4,5,6)P5 inhibits FGF-2-induced Akt phosphorylation and consequently blocks FGF-2-mediated survival in human umbilical vein endothelial cells (HUVEC). In addition, Ins(1,3,4,5,6)P5 specifically inhibits the FGF-2-mediated cell migration and capillary tube formation of HUVEC plated on Matrigel. Moreover Ins(1,3,4,5,6)P5 inhibits the FGF-2-induced angiogenic reaction in BALB/c mice, indicating that Ins(1,3,4,5,6)P5 is an antiangiogenic factor both in vivo and in vitro. Finally, we report that Ins(1,3,4,5,6)P5 reduces the s.c. growth of the human ovarian carcinoma, SKOV-3, xenografted in nude mice and blocks the Akt phosphorylation in vivo. These data definitively identify the Akt inhibitor Ins(1,3,4,5,6)P5 as a specific antiangiogenic and antitumor factor.

Materials and Methods

Materials

Ins(1,4,5,6)P4 (25) and Ins(1,3,4,5,6)P5 (24) were synthesized as previously reported. Each compound was purified to homogeneity by ion-exchange chromatography on Q-Sepharose Fast Flow resin 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, Ins(1,3,4,5,6)P5 was synthesized on a larger scale via 2-O-benzyl-5-monoinositol (26), purified as before, and then converted into the hexaosum salt by treatment with Dowex 50WX2-100 ion-exchange resin followed by addition of sodium hydroxide (6 equivalents) and lyophilization. Ins(1,2,3,4,5,6)Pi was obtained from Sigma (St. Louis, MO, phytic acid). Anti-pSer473 Akt, anti-pThr308 Akt, and anti-Akt were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD-31 was from PharMingen (San Diego, CA). FGF-2 was from Peprotech (London, United Kingdom). "Akt inhibitor" (IL-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbone) was from Calbiochem (La Jolla, CA; ref. 27). SHS (3-deoxy-2-O-methyl-5-monoinositol 1-[(R)-2-methoxy-3-octadecylcyclopropyl]hydrogen phosphate) was from Alexis (Nottingham, United Kingdom; ref. 28).

Cell Culture

HUVECs were purchased from TCS CellWorks (Buckingham, United Kingdom) and grown in EBM medium (Bio Whittaker, Walkersville, MD) containing 10% fetal bovine serum (FBS) and supplemented with hEGF, hydrocortisone, bovine brain extract, and gentamicin sulfate-amphotericin-B, as suggested (EGM kit, Bio Whittaker). Cells were used at passages 2-6.

Cell Survival

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. HUVECs were left untreated (control) or pretreated with the indicated concentrations of inositol polyphosphates or Akt inhibitors for 30 minutes in M199. FGF-2 (100 ng/mL) was added for further 48 hours in the absence or presence of the inhibitors. Serum was added in some wells as a positive control. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution in M199 (500 μg/mL final concentration) was added to each well for the last 4 hours. After washing, DMSO was added to the wells for 15 minutes, collected, and absorbance (570-650 nm) was determined by using a spectrophotometer.

Acridine orange/ethidium bromide staining. HUVECs were left untreated or pretreated with 50 μmol/L of inositol polyphosphates for 30 minutes in M199. FGF-2 (100 ng/mL) was added for further 48 hours in the absence or presence of the inhibitors. Serum was added in some wells as a positive control. Apoptosis was assessed by adding an acridine orange (100 μg/mL)/ethidium bromide (100 μg/mL; 1:1 v/v) mixture as described (24).

DNA laddering assay. HUVECs grown in Petri dishes were left untreated (control) or pretreated with 50 μmol/L. Ins(1,3,4,5,6)P5 for 30 minutes in M199. FGF-2 (100 ng/mL) was then added in the absence or presence of the inhibitor. Cells kept in medium supplemented with serum were used as a positive control. After 48 hours, cells were detached and DNA laddering assay was done as described (24).

Migration Assay

Cell migration was done in Transwell chambers (tissue culture treated, 10 mm diameter, 8-μm pores; Nunc, Rochester, NY) coated with 100 μg/mL gelatin (0.1% in acetic acid) or 10 μg/mL fibronectin. HUVECs were serum starved in M199 containing 0.5% FBS overnight; pretreated with 100 μmol/mL wortmamin, 10 μmol/L LY294002, or 50 μmol/L of the indicated inositol polyphosphates for 30 minutes and detached. Cells were then resuspended in M199 containing 1% bovine serum albumin ± wortmannin, LY294002, or the inositol polyphosphates, added (25,000 cells/150 μL) to the top of each migration chamber, and allowed to migrate in the presence of 100 ng/mL FGF-2 ± wortmannin, LY294002, or the inositol polyphosphates in the lower chamber. After 4 hours, cells that had not migrated were removed by using a cotton swab, whereas migrated cells were fixed with 4% paraformaldehyde, stained with 1% crytal violet, and counted.

Angiogenesis In vitro (Capillary Tube Formation)

Matrigel (Becton Dickinson, Bedford, MA) was added to an eight-chamber slide and allowed to gel for 2 hours at 37°C. Cells were deprived overnight in serum-free medium before detaching. HUVECs (5 × 104) were preincubated with 50 μmol/L of the different inositol polyphosphates or 1 μmol/L of SHS for 20 minutes at 37°C; then, before plating, FGF-2 (100 ng/mL) was added to the cells where necessary. Endothelial cell migration and rearrangement was visualized after 4 to 6 hours and the number of branching points counted. Only points generating at least three tubules were counted. Representative fields were photographed using a Nikon microscope.

Angiogenesis In vivo

BALB/c mice were injected s.c. dorsolaterally with 0.4 mL of Matrigel alone or in combination with 5 μg/mL FGF-2 and/or 50 μmol/L of the different inositol polyphosphates. The injected Matrigel rapidly formed a solid gel that persisted for at least 10 days in mice. The mice were sacrificed after 6 days, the mass of Matrigel was removed along with overlying skin and fixed with 10% formaldehyde for 24 hours before it was embedded in paraffin. The paraffin blocks were then cut into 4-μm-thick sections and processed for immunohistochemistry by using a modification of the avidin-biotin peroxidase complex technique. Briefly, 4-μm tissue sections were deparaffinized, rehydrated, and placed in 3% hydrogen peroxide to inhibit endogenous peroxidase. The tissue sections were trypsinized with 0.1% trypsin and 0.1% CaCl2 for 30 minutes at 37°C to expose the antigenic sites masked by formalin fixation, blocked for 1 hour with 3% normal goat serum, and subsequently incubated with anti-CD-31 for 60 minutes at a dilution of 1:1,000. The sections were then treated with biotinylated secondary antibody for 30 minutes at room temperature followed by avidin biotin complex reagent for 30 minutes and diaminobenzidine for 1 minute. Counterstaining was done with hematoxylin. For a quantitative analysis, capillary density was calculated, in the area immediately below the skin, as mean of the total number of vessels in five independent fields in three sections.
Xenotransplantation of Human Cell Culture in Nude Mice
SKOV-3 cells (3 × 10^6) were injected s.c. (0.2 mL per mouse). Twelve days after tumor implantation, mice were randomized in groups of nine animals each. Ins(1,3,4,5,6)P_5 and Ins(1,2,3,4,5,6)P_6 were dissolved in saline and injected i.p. at the dose of 1 mg per mouse per 0.2 mL. Control mice were treated with 0.2 mL of saline. Cisplatin was given i.v. on days 15 and 22 (q7dx2) at the dose of 4 mg/kg. The drug was dissolved in saline immediately before use. On day 23, two mice per group were killed and the tumor removed for monitoring Akt phosphorylation. The experiment was ended 40 days following tumor implant. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

High-performance Liquid Chromatography Analysis of Inositol Phosphates
[3H]Ins(1,3,4,5,6)P_5 was obtained by phosphorylation of [3H]Ins(1,3,4,5)P_4 (American Radiolabelled Chemicals, Inc., St. Louis, MO). [32P]Ins(1,3,4,5,6)P_5 was obtained by phosphorylation of Ins(1,4,5)P_3 in the presence of γ[32P]ATP. Phosphorylation was obtained by using yeast Inositol Polyphosphate Multi Kinase (29). The radiolabeled inositol phosphates synthesized were purified by high-performance liquid chromatography (HPLC) and desalted as described (30). SKOV-3 cells were incubated with medium containing the radioactive compound for different times before washing with ice-cold PBS. Alternatively, cells were incubated with the radioactive compound for 30 minutes and medium was then removed and replaced with normal medium. After further incubation for the specified times, cells were washed with ice-cold PBS. Inositol phosphates were extracted using 0.6 mL of ice-cold acidic buffer [0.6 mol/L perchloric acid, 0.1 mg/mL Ins(1,3,4,5,6)P_5, 2 mmol/L L EDTA]. The acidic extracts were neutralized with the neutralization efficiency of Ins(1,3,4,5,6)P_5 in inhibiting the PI3K/Akt pathway in endothelial cells. FGF-2 induced Akt phosphorylation in HUVEC with a rapid peak of activation after 10 minutes of stimulation (Fig. 1A). No Akt phosphorylation was detected at 15 minutes of FGF-2 stimulation (Fig. 1B), whereas a second peak of activation was observed at longer times of stimulation (Fig. 1B). When we tested the effect of different inositol polyphosphates, we observed that Ins(1,3,4,5,6)P_5, but not other inositol polyphosphates (Supplementary Fig. 1) inhibited FGF-2-induced Akt phosphorylation (Fig. 1A). Once the efficiency of Ins(1,3,4,5,6)P_5 in inhibiting the PI3K/Akt pathway in endothelial cells was assessed, we tested the effect of this compound in several processes associated with angiogenesis.

Figure 1. Ins(1,3,4,5,6)P_5 inhibits FGF-2-induced Akt phosphorylation and survival of endothelial cells. We have recently described the proangiogenic properties of the natural compound Ins(1,3,4,5,6)P_5 (24) and defined its mechanism of action through inhibition of the PI3K/Akt pathway. To determine whether Ins(1,3,4,5,6)P_5 might have antiangiogenic properties, we first tested whether Ins(1,3,4,5,6)P_5 was able to inhibit Akt activation in endothelial cells. FGF-2 induced Akt phosphorylation in HUVEC with a rapid peak of activation after 10 minutes of stimulation (Fig. 1A). No Akt phosphorylation was detected at 15 minutes of FGF-2 stimulation (Fig. 1B), whereas a second peak of activation was observed at longer times of stimulation (Fig. 1B). When we tested the effect of different inositol polyphosphates, we observed that Ins(1,3,4,5,6)P_5, but not other inositol polyphosphates (Supplementary Fig. 1) inhibited FGF-2-induced Akt phosphorylation (Fig. 1A). Once the efficiency of Ins(1,3,4,5,6)P_5 in inhibiting the PI3K/Akt pathway in endothelial cells was assessed, we tested the effect of this compound in several processes associated with angiogenesis.
Angiogenesis is a process involving cellular survival, migration, proliferation, and differentiation. Therefore, we tested the effect of Ins(1,3,4,5,6)P_5 on FGF-2-induced cell survival in HUVEC. As shown in Fig. 1C, FGF-2 was able to induce survival in serum-starved HUVEC. Pretreatment of HUVEC with Ins(1,3,4,5,6)P_5 inhibited FGF-2-mediated survival in a dose-dependent manner (Fig. 1C). Treatment with Ins(1,3,4,5,6)P_5 in the absence of FGF-2 did not affect survival, indicating that Ins(1,3,4,5,6)P_5 had no toxic effects (Fig. 1C). The observation that two commercially available Akt inhibitors ("Akt inhibitor" from Calbiochem and SH5 from Alexis) had a similar inhibitory effect confirmed that the PI3K/Akt pathway is involved in FGF-2-induced cell survival (Fig. 1C). The effect of Ins(1,3,4,5,6)P_5 was very specific because treatment with 50 μmol/L of other inositol polyphosphates, such as Ins(1,2,3,4,5,6)P_6 and Ins(1,4,5,6)P_6, was inactive (Fig. 1D). Taken together, these data indicate that Ins(1,3,4,5,6)P_5 inhibits survival of endothelial cells by blocking Akt activation.

To better characterize the mechanism of action of Ins(1,3,4,5,6)P_5, we checked whether it may have a proapoptotic effect in endothelial cells, as we have reported for different cancer cell lines (24). We therefore did apoptosis assays in HUVEC in the absence or presence of Ins(1,3,4,5,6)P_5 and FGF-2. Acridine orange/ethidium bromide assays confirmed that FGF-2 protected HUVEC from apoptosis induced by serum deprivation and its effect was comparable with that of serum (Fig. 2A and B). Ins(1,3,4,5,6)P_5 prevented the FGF-2-mediated inhibition of apoptosis in serum-deprived HUVEC, whereas no effect was observed in cells treated with Ins(1,2,3,4,5,6)P_6 (Fig. 2A and B). The proapoptotic effect of Ins(1,3,4,5,6)P_5 was confirmed by analyzing cell morphology (Fig. 2C) and by DNA laddering assays (Fig. 2D). Taken together, these data indicate that Ins(1,3,4,5,6)P_5 has proapoptotic effects in HUVEC and can overcome the FGF-2-mediated cell survival.

**Ins(1,3,4,5,6)P_5 inhibits FGF-2-induced cell migration.** We next tested the effect of Ins(1,3,4,5,6)P_5 on cell migration in HUVEC. FGF-2-induced cell migration in HUVEC was assessed by using gelatin-coated (Fig. 3A and B) or fibronectin-coated (see below) Transwell chambers. Pretreatment with Ins(1,3,4,5,6)P_5 specifically reduced the FGF-2-mediated cell migration on gelatin similarly to
the PI3K inhibitors wortmannin and LY294002 (Fig. 3A). No inhibition was observed when we used Ins(1,2,3,4,5,6)P₆ and Ins(1,4,5,6)P₃ (Fig. 3A). Ins(1,3,4,5,6)P₃ on its own had no effect on cell migration (Fig. 3B). Inhibition of Akt activation was observed by monitoring the phosphorylation state of Akt in parallel experiments (Fig. 3C). The observation that pretreatment with wortmannin and LY294002 (Fig. 3A) had a similar inhibitory effect on FGF-2-dependent migration suggested that this process is mediated by activation of a PI3K-dependent pathway. It is important to notice that in these particular experiments, a slight inhibition of Akt phosphorylation was observed in cells treated with Ins(1,2,3,4,5,6)P₆ (Fig. 3C) and this is likely due to a partial conversion of Ins(1,2,3,4,5,6)P₆ to Ins(1,3,4,5,6)P₅ at such long time of treatment as recently proposed (see Discussion). Nevertheless, the minimal inhibition of Akt activation induced by Ins(1,2,3,4,5,6)P₆ (whereas Ins(1,4,5,6)P₃ and Ins(1,4,5,6)P₅, used at the same concentration had no effect (Fig. 4). The involvement of Akt in this process was confirmed by a similar inhibition obtained using the Akt inhibitor SH5 (Fig. 4). Representative fields are shown in Fig. 4A, whereas a quantitative analysis of the same experiment is shown in Fig. 4B. Neither Ins(1,3,4,5,6)P₅ nor SH5 on their own had some effect on tubulogenesis (Supplementary Fig. 2) confirming that they were specifically inhibiting FGF-2-mediated effect. To test whether Ins(1,3,4,5,6)P₅ inhibits not only morphogenesis (in vitro) but also angiogenesis (in vivo), we injected BALB/c mice with FGF-2 along with Matrigel with or without several different inositol polyphosphates. After 5 days, the gels were removed, embedded, sectioned, and stained with anti-CD-31 antibody for the presence of blood vessels. As shown in Fig. 5A, Matrigel supplemented with FGF-2 induced an angiogenic reaction associated with the chemotactic response. Interestingly, in the presence of 50 μmol/L Ins(1,3,4,5,6)P₅, we observed a clear inhibition of the FGF-2-induced angiogenic response, whereas under the same conditions, no effect was observed in the presence of Ins(1,2,3,4,5,6)P₆, Ins(1,4,5,6)P₅, Ins(1,3,4,5,6)P₃, or Ins(3,4,5,6)P₃ (Supplementary Fig. 3). No blood vessel formation was detected in the presence of the different inositol polyphosphates but in the absence of FGF-2 (Supplementary Fig. 3). Capillary density was calculated, in the area immediately below the skin, as mean of the total number of vessels in five independent fields in three sections. Taken together, these data indicate that Ins(1,3,4,5,6)P₅ specifically has antiangiogenic properties in both in vitro and in vivo assays.

**Ins(1,3,4,5,6)P₅ inhibits growth of human ovarian carcinoma SKOV-3 xenograft.** The proapoptotic and antiangiogenic effects of Ins(1,3,4,5,6)P₅ strongly suggested that this compound might have antitumor properties. We therefore tested the antineoplastic activity of Ins(1,3,4,5,6)P₅ on SKOV-3 human ovarian carcinoma implanted s.c. in nude mice. Twelve days after inoculation of cells, one group of mice was treated with Ins(1,3,4,5,6)P₅, a second one with Ins(1,2,3,4,5,6)P₆, a third one with cisplatin, a drug commonly used in treatment of ovarian carcinoma, and a control group treated with vehicle alone. Measurements of tumors volume are graphed in Fig. 6A. Very interestingly, we observed that Ins(1,3,4,5,6)P₅ clearly reduced tumor growth (Fig. 6A) to the same extent than cisplatin (Fig. 6A). On the contrary, Ins(1,2,3,4,5,6)P₆ only minimally slowed tumor growth during the first days of treatment but had no further effect during the

Figure 3. Ins(1,3,4,5,6)P₅ inhibits the FGF-2-induced migration of HUVEC. A. Transwell assays on gelatin-coated chambers were done on cells pretreated with 100 nmol/L wortmannin, 10 μmol/L LY294002, or 50 μmol/L of the indicated inositol polyphosphates in the presence of 100 ng/mL FGF-2. Columns, mean of at least five independent experiments and are expressed as percentage of each control; bars, ± SE. *, P < 0.05; **, P < 0.01. B, Transwell assays on gelatin-coated chambers were done on cells pretreated with 50 μmol/L of Ins(1,3,4,5,6)P₃ in the presence of 100 ng/mL FGF-2. Columns, mean of at least three independent experiments and are expressed as the number of migrated cells per field; bars, ± SE. In such experimental conditions, 300 ± 25 cells per field migrated upon FGF-2 stimulation: this represented a 2-fold increase of migration over basal (143 ± 16 cells per field). *, P < 0.01. C, Western blotting analysis of lysates from HUVEC pretreated with 50 μmol/L of the indicated inositol polyphosphates. After detachment, cells were plated on gelatin-coated wells and allowed to adhere for 4 hours in the presence of 100 ng/mL FGF-2 alone or in combination with the indicated inositol polyphosphates. Total, phosphorylation of Akt at residue Ser473 was assessed by using a specific antibody. Filter was then stripped and reprobed with an anti-Akt antibody. Arrow points the band corresponding to total Akt. Bottom, densitometry analysis was carried out and the levels of pSer473 were normalized to the corresponding total amount of Akt.
following days (Fig. 6A). No sign of toxicity, as judged by parallel monitoring body weight, was observed in Ins(1,3,4,5,6)P5-treated mice. A reduction in tumor weight was clearly appreciable in Ins(1,3,4,5,6)P5-treated mice at the end of the experiment (Fig. 6B). Importantly, a complete inhibition of Akt phosphorylation at both residue Ser473 (Fig. 6C) and Thr308 (Fig. 6D) was observed in Ins(1,3,4,5,6)P5-treated mice after 12 days of treatment. Strikingly, the Ins(1,3,4,5,6)P5-induced inhibition was even higher than the cisplatin-induced reduction (Fig. 6C and D). A slight inhibition of Akt phosphorylation was observed in Ins(1,2,3,4,5,6)P6-treated mice (Fig. 6C and D), although such effect was clearly not sufficient to completely block tumor growth (Fig. 6A-B). This is the first demonstration that Ins(1,3,4,5,6)P5 can inhibit Akt activation and block the tumor growth in vivo.

Ins(1,3,4,5,6)P5 internalization and dephosphorylation in SKOV-3 cells. Data thus far did not rule out the possibility that Ins(1,3,4,5,6)P5 might be converted into different metabolites that were ultimately responsible for the observed antitumor effect. Therefore, we decided to check the intracellular stability of exogenously added Ins(1,3,4,5,6)P5 over time and to check whether Ins(1,3,4,5,6)P5 was converted intracellularly to different phosphorylated metabolites. SKOV-3 were then incubated with [32P]Ins(1,3,4,5,6)P5 for different times and inositol phosphates were extracted and analyzed by HPLC to detect the different radioactive inositol compounds. First of all, we observed that Ins(1,3,4,5,6)P5 was indeed able to enter SKOV-3 in a time-dependent manner (Fig. 7A) and this represents a clear demonstration that, although highly negatively charged, inositol polyphosphates can cross the plasma membrane and be internalized by cells as we reported for the first time (23) and was later confirmed by other groups (31). Furthermore, we observed that the turnover of Ins(1,3,4,5,6)P5 was quite slow, because only a 5.0% of the total Ins(1,3,4,5,6)P5 was converted into different metabolites after 30 minutes of incubation and only a 6.2% was converted after 1 hour of incubation (Fig. 7B). The slow turnover of Ins(1,3,4,5,6)P5 was confirmed by pulse-chase experiments done by incubating SKOV-3 with [3H]Ins(1,3,4,5,6)P5 for 30 minutes followed by incubation in the absence of the radioactive compound for different times. Parallel control experiments were done by using [3H]Ins(1,3,4,5)P4. Inositol phosphates were extracted at different times of incubation and analyzed by HPLC. As shown in Fig. 7C, the incorporated [3H]Ins(1,3,4,5)P4 was very stable and a 84.6% of total [3H]Ins(1,3,4,5,6)P5 was still detectable intracellularly even after 5 hours of incubation (Fig. 7C). On the contrary, [3H]Ins(1,3,4,5)P4 was rapidly and almost completely metabolized with only a 7% of total still detectable after 5 hours of incubation (Fig. 7C).

These data clearly indicate that Ins(1,3,4,5,6)P5 is rapidly and efficiently internalized by cells and is only minimally converted into different metabolites strongly suggesting that the observed antitumor effects were due to its activity and were not mediated by conversion to different phosphorylated forms.
Discussion

Regulation of cell proliferation and cell survival in cancer involves a complex interplay among steroid hormones, growth factors, and their receptors. Understanding the signaling pathways involved in these processes may help in finding predictive factors for tumor aggressiveness and therapy resistance. Among the different pathways activated by growth factor receptors, signals transmitted by PI3K and Akt have proven important for cell survival in many cell types, and genetic and biochemical evidence suggest that inappropriate activation of the PI3K/Akt pathway is linked to the development of cancer (32–35). Altered expression or mutation of several components of this pathway has been implicated in the development and progression of human cancer (36). In particular, it has been reported that PI3K mutations identified in human cancer are oncogenic (37). Indeed, amplification of the gene coding for the p110 catalytic subunit of PI3K has been observed in different tumor types (34, 38), and activating somatic mutations in its regulatory subunit have been found in primary ovarian and colon tumors (39). In addition, amplification of Akt2 can occur in about 40% of breast cancer specimens as well as ovarian and pancreatic cancers (40–42), and Akt phosphorylation is frequently detected in ovarian cancer (43). The most compelling evidence for the involvement of the PI3K/Akt pathway in human cancer comes from studies of the PTEN tumor suppressor gene that encodes a dual specificity protein phosphatase, which also possesses a phosphoinositide 3-phosphatase activity (22). In several human cancers, the PTEN gene has been found to be deleted or mutated, indicating that PTEN loss occurs in a wide spectrum of human cancers and that this is correlated, among other effects, with a constitutive activation of the PI3K/Akt pathway. Interestingly, an elevated Akt activity is associated with increased cellular resistance to treatment with chemotherapeutic agents (44), and recent data suggest that Akt may be a potential target for enhancing the response to radiotherapy in patients with breast cancer (45). Indeed, inhibition of this pathway has been shown to facilitate apoptosis and to sensitize cells to cytotoxic drugs in experimental studies (24, 46).

The link between activation of the PI3K/Akt pathway and cancer makes this pathway an attractive target for therapeutic intervention strategies (47). Indeed, it has been reported that the PI3K inhibitors wortmannin and LY294002 possess antitumor activity in vitro and in vivo, although their general toxicity and lack of selectivity, together with the instability of wortmannin and the insolubility of LY294002, mean that neither has very promising pharmaceutical potential (48–50). In addition, other compounds have emerged over the past few years as potential inhibitors of the PI3K/Akt pathway such as alkylphospholipids or phosphoinositide ether lipid analogues (49, 50). Limitations to the use of these compounds as chemotherapeutic agents include difficulties with solubility, chemical stability, and toxicity (49, 50). Given that administration of standard chemotherapy agents is usually associated with at least mild toxicity, the possible use of nontoxic, natural compounds to target the PI3K/Akt pathway and prevent cancer is attractive.

Recently, we have successfully used an alternative strategy to specifically block the PI3K/Akt pathway based on the mechanism of membrane targeting mediated by the interaction of pleckstrin homology domains with the lipid products of PI3K (23, 24). In particular, we have used inositol polyphosphates, the water-soluble head groups of phosphoinositides, to specifically block the recruitment of Akt to the plasma membrane and therefore inhibit its activation. This strategy was based on the hypothesis that, by binding to the Akt pleckstrin homology domain, specific exogenous inositol polyphosphates can compete with phosphatidylinositol[3,4,5]trisphosphate [PI(3,4,5)P3] and therefore prevent PI(3,4,5)P3-dependent recruitment to the plasma membrane (51).

The use of inositol polyphosphates as potential anticancer agents has been supported by several studies indicating that Ins(1,3,4,5,6)P5 possesses antitumor activity in vitro and in vivo (52). However, the very high concentrations required for Ins(1,3,4,5,6)P5 to be active (1-5 mmol/L) suggest a lack of selectivity of this compound, although it is noteworthy that, even at these concentrations, inositol polyphosphates do not seem to have toxic effects (52). Although several mechanisms have been proposed including through PI3K inhibition, little is still known about the mechanisms by which Ins(1,3,4,5,6)P5 exerts its anticancer actions. Interestingly, it has been recently reported that Ins(1,3,4,5,6)P5 enters HeLa cells and is dephosphorylated to lower forms, mainly Ins(1,3,4,5,6)P5, that in turn is able to induce

Figure 5. Ins(1,3,4,5,6)P5 has antiangiogenic properties in vivo. A, representative images of tissue sections in the presence of FGF-2 alone or FGF-2 along with 50 μmol/L Ins(1,3,4,5,6)P5. B, quantitative analysis: capillary density was calculated in the area immediately below the skin, as mean of the total number of vessels in five independent fields in three sections. * P < 0.01.
apoptosis (31). Indeed, among the different inositol polyphosphates tested in HeLa cells, Ins(1,3,4,5,6)P$_6$ specifically inhibited Akt phosphorylation and activity, showed proapoptotic properties, and was more active than Ins(1,2,3,4,5,6)P$_6$. These data are in agreement with our previous work (24) and suggest that the anticancer activity of Ins(1,2,3,4,5,6)P$_6$ is actually due to its dephosphorylation to lower forms that are potent in inducing apoptosis. It is noteworthy that in this report, we observed that Ins(1,2,3,4,5,6)P$_6$ does not seem to have an effect on Akt phosphorylation when tested in short-time experiments (Supplementary Fig. 1), whereas it seems to slightly inhibit Akt at longer incubations (Fig. 3C and D). The observation that such an effect is visible only at longer incubation strongly suggests that it is likely due to a slight conversion of such inositol polyphosphate to different forms, likely Ins(1,3,4,5,6)P$_5$. Indeed, a degradation of Ins(1,2,3,4,5,6)P$_6$ to lower phosphorylated forms has been already observed in cancer cells although with a slow kinetic because the bulk of such compound seems to be in the hexakisphosphate form even after 6 hours of incubation (31). Such a slow conversion of Ins(1,2,3,4,5,6)P$_6$ to lower phosphorylated forms may easily explain the only minimal effect of Ins(1,2,3,4,5,6)P$_6$ on Akt inhibition and, more important, the lack of effect on both the FGF-2-induced migration and tumor cell growth; that is, the amount of active compound derived from degradation of Ins(1,2,3,4,5,6)P$_6$ is not sufficient to mediate a physiologic effect. This might also explain the requirement of very high concentrations for Ins(1,2,3,4,5,6)P$_6$ to be active. In this respect, our data corroborate the reported anticancer properties of Ins(1,2,3,4,5,6)P$_6$ that are possibly due to its conversion to Ins(1,3,4,5,6)P$_5$. Indeed, Ins(1,3,4,5,6)P$_5$ seems the most stable among the lower phosphorylated forms of inositol polyphosphates. In fact, the accurate HPLC analysis done in this report shows that the turnover of Ins(1,3,4,5,6)P$_5$ is very slow, because we observed that only 5.0% of the total [32P]Ins(1,3,4,5,6)P$_5$ was converted into different metabolites after 30 minutes of incubation and only 6.2% was converted after 1 hour of incubation (Fig. 7B). The slow turnover of Ins(1,3,4,5,6)P$_5$ was confirmed by pulse-chase experiments showing that the incorporated [3H]Ins(1,3,4,5,6)P$_5$ was very stable and 84.6% of total [3H]Ins(1,3,4,5,6)P$_5$ was still detectable intracellularly after 5 hours of incubation (Fig. 7C). On the contrary, [3H]Ins(1,3,4,5)P$_4$ was almost completely metabolized with only 7% of the total still detectable after 5 hours of incubation (Fig. 7C). This is consistent with our reported data obtained in SCLC where conversion of [3H]Ins(1,3,4,5)P$_4$ was even more rapid (23). Taken together, these data clearly indicate that Ins(1,3,4,5,6)P$_5$ is rapidly and efficiently internalized by cells. The observation that the inhibitory effects of Ins(1,3,4,5,6)P$_5$ were observed already at short times of incubation and that only a small percentage of the compound is further converted into different metabolites even
after long time of incubation strongly suggest that the observed antitumor effects are directly attributable to Ins(1,3,4,5,6)P_5 and are not mediated by conversion to different phosphorylated forms.

Several pieces of evidence indicate that Ins(1,3,4,5,6)P_5 promotes apoptosis through inhibition of the PI3K/Akt pathway. In fact, the specificity of Ins(1,3,4,5,6)P_5 activity is confirmed by our experimental evidence indicating that this compound is active in human cancer cell lines characterized by an elevated PI3K/Akt activity, whereas it is inactive in other cell lines tested (23, 24). Our previous work confirmed a strict correlation between the Ins(1,3,4,5,6)P_5-mediated cell survival and survival in HUVEC. The observation that other commercially available Akt inhibitors induced a similar inhibitory effect suggested that Akt is involved in the FGF-2-mediated cell survival and therefore that the Ins(1,3,4,5,6)P_5-mediated effect on survival occurs through inhibition of the PI3K/Akt pathway. More specifically, we observed that Ins(1,3,4,5,6)P_5 promoted apoptosis, assessed both by acridine orange/ethidium bromide and by DNA laddering assays. In addition, we observed that Ins(1,3,4,5,6)P_5 inhibited FGF-2-induced cell migration and angiogenesis, as assessed both in vitro and in vivo. This is the first study reporting an antiangiogenic activity for Ins(1,3,4,5,6)P_5. Although the antiangiogenic effect of Ins(1,3,4,5,6)P_5 can be partly explained by the observed proapoptotic properties, it is likely that the complete inhibition of angiogenesis is the result of the Ins(1,3,4,5,6)P_5-mediated reduction of FGF-2-induced migration as well. Therefore, the antiangiogenic properties reported represent a clear advance in understanding the properties of Ins(1,3,4,5,6)P_5.

The proapoptotic and antiangiogenic properties of Ins(1,3,4,5,6)P_5 led us to the hypothesis that this compound might have antitumor effects. Indeed, when we examined its potential antineoplastic activity on SKOV-3 human ovarian carcinoma implanted s.c. in nude mice, we found that Ins(1,3,4,5,6)P_5 blocked tumor growth. These data make a significant advance compared with our previous work in particular because they validate the effects of Ins(1,3,4,5,6)P_5 in an in vivo model. Strikingly, not only did we observe that Ins(1,3,4,5,6)P_5 inhibited the growth of the human ovarian carcinoma SKOV-3 xenografts but also that the effect of Ins(1,3,4,5,6)P_5 was comparable to that of cisplatin, the drug commonly used for ovarian cancer treatment. Furthermore, a clear and total inhibition of Akt phosphorylation at both residues Ser^373 and Thr^383 was observed in Ins(1,3,4,5,6)P_5-treated mice. This represents the first demonstration of an in vivo effect of Ins(1,3,4,5,6)P_5 on Akt activation.

Inhibition of the Akt pathway may prove highly effective in future treatment regimens, although likely in combination with canonical cytotoxic drugs. Indeed, our previous work showed that in vitro Ins(1,3,4,5,6)P_5 enhances the proapoptotic effects of cisplatin and etoposide in ovarian and lung cancer cells, respectively (24), supporting a role for Ins(1,3,4,5,6)P_5 as a compound able to sensitize cancer cells to the action of commonly used anticancer drugs. It is important to underline that inositol polyphosphates, including Ins(1,3,4,5,6)P_5, are naturally occurring substances that are present in most legumes, and in wheat bran and nuts (53). Therefore, our study reveals a new pharmacologically active nutrient ("nutraceutical") and underlines the importance of the use of certain foods in the prevention of cancer. Furthermore, the observation that inositol polyphosphates are easily absorbed by oral administration (52) makes Ins(1,3,4,5,6)P_5 even more promising in terms of therapeutic potential.

![Figure 7. Ins(1,3,4,5,6)P_5 internalization and dephosphorylation in SKOV-3 cells.](image-url)
Taken together, these data indicate that specific blockade of the PI3K/Akt pathway by Ins(1,3,4,5,6)P5 results in proapoptotic and antiangiogenic effects, and they show for the first time that Ins(1,3,4,5,6)P5 is able to inhibit tumor growth in vivo. It is noteworthy that the concentration of Ins(1,3,4,5,6)P5 used in our in vivo experiment is very low (50 mg/kg) and that we do not detect any toxic effects, as judged by monitoring body weight. These properties, together with the fact that Ins(1,3,4,5,6)P5 is a water-soluble, natural compound, suggest that Ins(1,3,4,5,6)P5 may overcome problems concerning solubility, chemical stability, and toxicity that are currently limiting the use of other potential inhibitors of the PI3K/Akt pathway. The identification of novel properties of Ins(1,3,4,5,6)P5 together with the in vivo data shown in this article not only are consistent with our previous work (23, 24) but also represent a huge step forward in validating Ins(1,3,4,5,6)P5 as a potent and specific functional inhibitor of the PI3K/Akt pathway that may be useful in the treatment of human cancers whose progression is driven by PI3K activation or PTEN gene alterations. Therefore, we believe that Ins(1,3,4,5,6)P5 may be a very promising agent to develop rapidly and bring to clinical testing.

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