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Fibrinogen – a possible extracellular target for inositol phosphates

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Abstract

A potential extracellular target for inositol phosphates and analogues with anticancer properties is identified. Proteins from detergent-solubilised HeLa cell lysates bound to a novel affinity column of *myo*-inositol 1,3,4,5,6-pentakisphosphate (InsP₅) coupled to Affigel-10. One high-affinity ligand was fibrinogen Bβ. Inositol phosphates and analogues were able to elute purified fibrinogen from this matrix. InsP₅ and the inositol phosphate mimic biphenyl 2,3',4,5',6-pentakisphosphate (BiPhP₅) bind fibrinogen *in vitro*, and block the effects of fibrinogen in A549 cell-based assays of proliferation and migration. They are also able to prevent the fibrinogen-mediated activation of phosphatidylinositol 3-kinase. These effects of fibrinogen appear to be mediated through the intercellular adhesion molecule-1 (ICAM-1), as cells not expressing ICAM-1 fail to respond. In contrast, *myo*-inositol hexakisphosphate and the epimeric *scyllo*-inositol 1,2,3,4,5-pentakisphosphate were without effect. These findings are consistent with earlier reports that higher inositol phosphates have anticancer properties. This new mechanism of action and target for these extracellular inositol phosphates to have their effects allows a re-evaluation of earlier data.

Keywords

inositol phosphate; fibrinogen; proliferation; migration; extracellular

Introduction

Inositol-containing compounds are known to play critical roles as intracellular second messengers. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is the precursor to two

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Conflicts of interest

There is no conflict of interest.

key inositol-containing messengers; *myo*-Inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) is a water-soluble messenger that acts on its receptors in the endoplasmic reticulum causing calcium mobilisation from intracellular stores [Streb *et al.*, 1983], whereas the product of phosphoinositide 3-kinase (PI 3-K), PtdInsP_3 remains in the membrane and acts to recruit many proteins involved in proliferation, cancer, inflammation and diabetes [Vanhaesebroeck *et al.*, 2001].

Whilst the intracellular role of particular inositol phosphates has been established, much controversy surrounds their extracellular effects. *In vitro* assays have shown that the addition of high concentrations (5mM) of *myo*-inositol hexakisphosphate (InsP_6) (Figure 1) inhibits proliferation of cancer cell lines [El-Sherbiny *et al.*, 2001]. These findings have been supported in animal models in which InsP_6 provides a protective effect against many cancers [Vucenik *et al.*, 1997]. More recently it has been shown that these effects can be mimicked by other inositol phosphates, particularly *myo*-inositol 1,3,4,5,6-pentakisphosphate (InsP_5) (Figure 1), which appears more effective in decreasing growth rate, metastasis formation and preventing tumour development than InsP_6 [Maffucci *et al.*, 2005; Piccolo *et al.*, 2004] requiring doses of 50 μM *in vitro* and 50mg/kg *in vivo*. These extracellular effects are non-physiological as InsP_5 and InsP_6 levels are normally very low [Letcher *et al.*, 2008] unless a high InsP_6 diet is maintained [Grases *et al.*, 2001]. Intracellular targets for inositol phosphates have been identified, including proteins downstream of PI 3-K [Downes *et al.*, 2005; Komander *et al.*, 2004], and inhibiting these proteins would, indeed, cause a decrease in proliferation. Whether extracellular inositol phosphates can access these proteins has not been determined. Alternative mechanisms as to how these molecules work include binding extracellular proteins or cell-surface receptors. We have, therefore, searched for novel proteins that bind inositol phosphates in order to explain these effects using a novel affinity matrix. Our studies show that inositol phosphates bind fibrinogen and act to antagonise fibrinogen's ability to activate PI 3-K, and cause proliferation and migration.

Whilst many intracellular targets of InsP_5 have been identified, the extracellular targets for InsP_5 remain elusive. The ability of inositol phosphates to bind and chelate fibrinogen could explain their antiproliferative and antimetastatic effects. A similar approach, targeting vascular endothelial growth factor (VEGF) with soluble decoy receptors (aflibercept - or VEGF Trap, Regeneron/Sanofi-Aventis) [Holash *et al.*, 2002] is in phase III clinical trials for the treatment of lung and prostate cancer. The VEGF antibody bevacizumab (Avastin, Genentech/Roche) has already been given FDA approval.

Materials and Methods

Materials

InsP_5 , *scyllo*-inositol 1,2,3,4,5-pentakisphosphate (*scyllo*- InsP_5) and biphenyl 2,3',4,5',6-pentakisphosphate (BiPhP_5) were prepared as previously reported [Riley *et al.*, 2006; Vandeput *et al.*, 2007]. All compounds were purified to homogeneity by ion-exchange chromatography. The InsP_5 matrix was prepared by conjugation of 2-*O*-(2-aminoethyl)- InsP_5 with Affigel-10 (Bio-Rad UK) using methods previously reported for the corresponding InsP_3 resin [Riley *et al.*, 2004]. See Figure 1 for relevant structures.

Preparation of HeLa cell extracts, binding, elution and identification

Whole HeLa cells were purchased from CilBiotech (Mons, Belgium) and solubilised in a buffer consisting of Tris-HCl, 50mM pH 7.5; NaCl 150mM; Nonidet P40 1%; glycerol 10%; sodium orthovanadate 1mM; ethylenediaminetetraacetic acid 5mM; β -glycerophosphate 5mM; NaF 10mM. Remaining particulate matter was removed by centrifugation, and the supernatant loaded onto the InsP_5 matrix. Following a rigorous wash with buffer containing 1M NaCl, proteins were eluted with a gradient of InsP_6 . The capacity of the resin was approximately 40 μ g protein/packed ml resin. SDS-PAGE of fractions identified fractions containing protein. Proteins with the highest affinity for the matrix (requiring 1mM InsP_6 to elute) were precipitated with HClO_4 (0.6M), washed and sent for analysis at the Fingerprints Proteomics Facility, College of Life Sciences, University of Dundee.

Analysis of tryptic digests identified one major protein bound to the InsP_5 matrix. Two peptides were identified as EGVNDNEEGFFSAR and RARPAKA. Mascot analysis determined these peptides as originating from fibrinogen B β .

Confirmation of binding

Human fibrinogen (F4883, Sigma) was dissolved in 50mM Tris, pH 7.4. 100 μ l of 10mg/ml was loaded onto InsP_5 matrix and washed with 3×5 ml buffer. Protein was eluted with 1 ml aliquots of 1nM – 1mM inositol phosphate or analogue. Finally the column was washed with 2×1 ml 10mM InsP_6 . Protein concentration was determined from each fraction using Coomassie blue reagent.

Cell-based assays

A549 human lung carcinoma cells (European Collection of Animal Cell Cultures) were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 2 mM glutamine, 10% foetal bovine calf serum, and 100 U/ml penicillin and 100 μ g/ml streptomycin. Where appropriate, cells were treated with tumour necrosis factor- α (TNF α) (100ng/ml) 24 hours prior to treatment with fibrinogen, inositol phosphates and analogues were applied at the same time as fibrinogen.

Migration

A549 cells were allowed to grow to confluent monolayers in 12-well tissue culture plates and serum-starved for 24 hours. A wound was created using a p2 pipette tip. Cells were washed 3 times with fresh serum-free DMEM medium and placed in serum free DMEM for 48 hours before fibrinogen was added. Wound widths for each monolayer were measured immediately after the initial wounding and every 24 h afterwards, under microscopic vision with a graded eyepiece for 72 hours, and the distance between the confluent sheets of cells recorded. Where appropriate, cells were treated with TNF α (100ng/ml) 24 hours prior to treatment with fibrinogen. For assays analysing the effects of inositol phosphates and analogues, these were applied at the same time as fibrinogen (100 μ g/ml).

Proliferation

Cells were plated in 96-well tissue culture plates and serum-starved for 24 hours before fibrinogen was added for 48 hours. Proliferation was monitored by the addition of CellTiter 96AQueous cell proliferation reagent (Promega, Southampton, UK) and absorbance (490nm) measured for 0-4 hours. Where appropriate, cells were treated with TNF α (100ng/ml) 24 hours prior to treatment with fibrinogen. For assays analysing the effects of inositol phosphates and analogues, these were applied at the same time as fibrinogen (100 μ g/ml).

Analysis of inositol lipid levels

Cells were labelled with 50 μ Ci/ml myo-[3 H] inositol (ARC, Cardiff, UK) in inositol-free DMEM (MP Biomedicals, Cambridge, UK) for 96 h, prior to lysis in 0.6 M HClO $_4$. Inositol lipids were extracted from the HClO $_4$ pellet, prepared, and analysed by high performance liquid chromatography as previously described [Batty and Downes, 1994]. Peaks were ascribed by co-elution of standards in parallel runs. Inositol lipid levels were measured after 5 minutes treatment with fibrinogen. By this time, the short-lived peak of PtdInsP $_3$ had waned, but the sustained plateau of PtdIns(3,4)P $_2$ had become established. Where appropriate, cells were treated with TNF α (100ng/ml) 24 hours prior to treatment with fibrinogen; inositol phosphates and analogues were applied at the same time as fibrinogen. PtdIns(3,4)P $_2$ levels were normalised by dividing by the peak area for PtdIns(3,4)P $_2$ by PtdIns for each individual run.

Clot formation

Thrombin-mediated fibrin formation and clot formation was performed in 96-well plates. Fibrinogen (2 mg/ml) was incubated in the presence of thrombin (0.25-1 μ g/ml) in a buffer comprising Tris, 50mM (pH 7.2); NaCl, 150mM; MgSO $_4$, 1mM; PEG (6000-8000) 0.1%. The turbidity was recorded for a period of 30 minutes as absorbance at 450nm. Data were analysed using SoftMaxPro software (Molecular Devices, Wokingham, UK). Inositol phosphates or analogues were added prior to addition of thrombin.

Results

Identification of protein bound to the InsP $_5$ matrix

HeLa cell extracts were loaded onto the InsP $_5$ matrix and washed with high salt buffer. Protein samples were eluted off the matrix and analysed by HPLC MS/MS. One major peak was identified as containing peptides from fibrinogen B β strand. Subsequently, intact human fibrinogen was tested, and found to bind the matrix. InsP $_5$ and InsP $_6$ readily eluted fibrinogen from the matrix at micromolar concentrations. Scyllo-InsP $_5$ (1mM) was unable to elute the protein from the matrix (data not shown). BiPhP $_5$ was the most effective agent tested, eluting fibrinogen from the matrix at low nanomolar concentrations (Figure 2). These results show that inositol phosphates and analogues specifically bind fibrinogen.

Inositide production

One cell surface receptor for fibrinogen is ICAM-1. Activation of ICAM-1 by fibrinogen raises the concentration of PtdIns(3,4)P $_2$, an intracellular second messenger important in

proliferation and migration, in a dose-dependent manner (Figure 3) with an EC_{50} of 10 μ g/ml. Fibrinogen only caused an increase of $PtdIns(3,4)P_2$ when cells had been pretreated with the pro-inflammatory cytokine $TNF\alpha$. This is in keeping with the observation that expression of ICAM-1 is upregulated in A549 cells following treatment with the cytokine [Rauch *et al.*, 2007]. $InsP_5$ (100 μ M) and $InsP_6$ (100 μ M) were able to partially reverse this increase in $PtdIns(3,4)P_2$ levels, whereas *scyllo*- $InsP_5$ (100 μ M) was without effect. $BiPhP_5$ (100 μ M) was the most effective inhibitor of $PtdIns(3,4)P_2$ production tested (Figure 4). Inositol phosphates or analogues at a concentration of 100 μ M had no effect in the absence of fibrinogen (data not shown).

Proliferation

We have used an MTS-based assay to study fibrinogen-mediated proliferation of A549 cells. Fibrinogen was able to cause a dose-dependent increase in proliferation in cells pretreated with $TNF\alpha$ with an EC_{50} of 30 μ g/ml. A549 cells not treated with $TNF\alpha$ were unaffected by fibrinogen. $InsP_5$ and $InsP_6$ were able to inhibit fibrinogen-mediated proliferation, whereas *scyllo*- $InsP_5$ was without effect. $BiPhP_5$ was a potent inhibitor (Figure 5), with an EC_{50} of 1.2 μ M, compared to an EC_{50} of 20 μ M for $InsP_5$ (Figure 5). Inositol phosphates or analogues at a concentration of 100 μ M had no effect in the absence of fibrinogen (data not shown).

Wound healing

Similar results were obtained in an *in vitro* wound-healing assay that mimics directional cell migration *in vivo* [Pinco *et al.*, 2002]. Fibrinogen was able to effect wound healing in a dose-dependent manner, with EC_{50} of 30 μ g/ml. A549 cells not treated with $TNF\alpha$ were unaffected by fibrinogen. $InsP_5$ and $InsP_6$ were able to inhibit fibrinogen-mediated migration, whereas *scyllo*- $InsP_5$ was without effect. $BiPhP_5$ was the most effective (Figure 5) with an EC_{50} of 6 μ M, compared to an EC_{50} of 100 μ M for $InsP_5$ (Figure 5). Inositol phosphates or analogues at a concentration of 100 μ M had no effect in the absence of fibrinogen (data not shown).

Clot formation

We have also tested the ability of inositol phosphates and analogues to alter clot formation in an *in vitro* assay. Thrombin caused fibrinogen cleavage in a time- and dose-dependent manner, leading to clot formation. The effects on clot formation were unaffected by all inositol phosphates and analogues tested (100 μ M). At higher doses many inositol phosphates were able to reduce the rate of clot formation. As this was mimicked by EDTA at equal doses, these effects were discounted as merely ion chelator properties (data not shown). Inositol phosphates or analogues at a concentration of 100 μ M had no effect in the absence of fibrinogen (data not shown).

Discussion

Inositol phosphate analogues show some potential as anticancer drugs, once the targets have been identified and structure-activity relationships can be derived. The antiproliferative properties of higher inositol phosphates have been observed for many years [Fox and Eberl,

2002; Maffucci *et al.*, 2005; Piccolo *et al.*, 2004]. We now propose a novel mechanism of action for these inositol phosphates: their binding to fibrinogen.

Fibrinogen has previously been shown to drive proliferation [Gardiner and D'Souza, 1997], migration [Rauch *et al.*, 2007] and PI-3kinase activation [Maxwell *et al.*, 2004] of cells in 0.2 – 100 μ M (0.07 – 33 mg/ml). Resting levels of fibrinogen (3 mg/ml) are raised in certain cancer patients [Preston *et al.*, 1998; Roche *et al.*, 2003] making fibrinogen a risk factor in some 60% of deaths in the Western world. During blood coagulation and wound healing, fibrinogen is cleaved by thrombin and polymerises to form a fibrin matrix, providing a stroma that encourages migration and proliferation of connective tissue cells in wound healing and clot formation [Gray *et al.*, 1993]. Fibrinogen can assemble into an extracellular matrix, in which it binds growth factors like basic fibroblast growth factor (bFGF) and VEGF with high affinity [Sahni and Francis, 2000]. The angiogenic and proliferative functions of fibrinogen have also been hijacked by many tumour cell types. Tumours can only grow to approximately 1-2mm³ before they require their own blood supply [Rybarczyk and Simpson-Haidaris, 2000]. By secreting fibrinogen, these cells drive cellular migration and angiogenesis. Secreted fibrinogen can form part of the extracellular matrix, enhancing the effects of certain growth factors, or acting as a proliferative agent alone [Gray *et al.*, 1993; Rybarczyk *et al.*, 2003]. In this respect, regulating the interaction of fibrinogen with other proteins can be a means by which we can influence many basic and complex diseases. Fibrinogen's mitogenic action can also be explained by direct binding to cell-surface receptor proteins. Fibrinogen has been shown to raise PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 levels in cells expressing $\alpha_{IIb}\beta_3$ receptors [Maxwell *et al.*, 2004]. Binding of fibrinogen to ICAM-1 implies that it has a role in the inflammatory process, which involves cell proliferation and migration [Rauch *et al.*, 2007]. ICAM-1 is up-regulated by pro-inflammatory cytokines such as TNF α , interleukin-1 and interferon γ [Hubbard and Rothlein, 2000]. ICAM-1 has been found to be up-regulated on cell membranes in many cancers, such as gastric, pancreatic and breast cancer tissues (e.g. [O'Hanlon *et al.*, 2002]). Interestingly, inhibition of poly (ADP-ribose) polymerase downregulates NF- κ B and, hence ICAM-1, and appears to have anti-metastatic effects [Cuzzocrea *et al.*, 2002].

Fibrinogen is also important in tumour development, enhancing tumour cell migration, as well as having a direct mitogenic action itself. It can also facilitate metastasis by enhancing the sustained adherence and survival of individual tumour cell emboli in the vasculature of target organs [Roche *et al.*, 2003].

Some of us have previously shown that the biphenyl polyphosphate BiPh P_5 is a potent ligand for the PH domain of Akt and Ins P_3 5-phosphatase as well as for the Ins(1,4,5) P_3 receptor [Mills *et al.*, 2008; Vandeput *et al.*, 2007]. We believe that the effects seen here are not mediated by interaction of BiPh P_5 with intracellular proteins and that the effects of BiPh P_5 can be explained by its interaction with fibrinogen. By limiting the ability of fibrinogen to raise 3-phosphorylated inositol lipid levels, BiPh P_5 , along with Ins P_5 and Ins P_6 would reduce Akt phosphorylation without the need to enter the cytoplasm. We have found good correlation between the ability of inositol phosphates to elute fibrinogen from an Ins P_5 matrix with reduction of PtdIns(3,4) P_2 formation, and inhibition of fibrinogen-mediated proliferation and migration. These studies show that BiPh P_5 has high affinity for

fibrinogen and is a potent inhibitor of fibrinogen-mediated $\text{PtdIns}(3,4)\text{P}_2$ formation, proliferation and cell migration. Previous reports looking at downstream events (e.g. Akt phosphorylation, [Maffucci *et al.*, 2005; Piccolo *et al.*, 2004]) have not measured levels of the intracellular second messengers. The reduction of 3-phosphorylated inositol lipids we have shown can explain the phenomena described in these two and related papers. This includes cell-type specificity, as not all cells express fibrinogen-binding receptors.

In addition to its role in blood clotting, fibrinogen is an important factor in cellular interactions, inflammation, cellular migration and proliferation [Standeven *et al.*, 2005]. The targets for fibrinogen can be varied, but in A549 cells it is clear that ICAM-1 is a receptor whose expression is induced following treatment with interleukin-1, $\text{TNF-}\alpha$ or interferon- γ [Rothlein *et al.*, 1988]. Binding by fibrinogen leads to activation of PI 3-K, raising levels of 3-phosphorylated inositol lipids and phosphorylation of protein targets including Akt and p38 [Rauch *et al.*, 2007]. The physiological roles of fibrinogen have been subverted by certain cancers, making fibrinogen a target for anticancer drugs [Lu *et al.*, 2007].

Fibrinogen is a heparin-binding protein, heparin impeding fibrinogen binding to endothelial cells [Odrlić *et al.*, 1996]. It is noted that other heparin-binding proteins bind higher inositol phosphates. For example, acidic and basic fibroblast growth factors (aFGF and bFGF, also referred to as FGF-1 and FGF-2, respectively) have been long known to bind InsP_6 , which blocks the proliferative effects of bFGF, but not EGF [Morrison *et al.*, 1994]. This chelation of growth factor by inositol phosphates could, again, explain the effects of inositol phosphates on bFGF-mediated angiogenic and proliferative effects later observed by Maffucci *et al.* (2005) [Maffucci *et al.*, 2005]. Many other anticancer drugs have been shown to inhibit fibrinogen binding to leukaemia cells, including cisplatin [Lu *et al.*, 2004], an agent found to enhance the effects of InsP_5 on apoptosis [Piccolo *et al.*, 2004]. Like cisplatin and many frequently used anticancer drugs [Lu *et al.*, 2004], InsP_5 , InsP_6 and BiPhP_5 were found not to inhibit fibrin clot formation unless heroic concentrations were used (data not shown).

The bioavailability of inositol phosphates from diet has recently been questioned [Letcher *et al.*, 2008]. A *Giardia lamblia* InsP_6 kinase-based mass assay was used to detect levels in a variety of biological samples. No InsP_6 was detected in human plasma or urine, suggesting previous reports of micromolar concentrations may have been erroneous. Further analysis to determine whether InsP_6 in tablet form or solution (see [Fox and Eberl, 2002]) or intraperitoneal injection [Maffucci *et al.*, 2005] raises plasma levels needs to be undertaken.

Conclusion

It is clear that inositol phosphates can act as anticancer drugs *in vivo*. Our findings that certain inositol phosphates and analogues are able to prevent formation of $\text{PtdIns}(3,4)\text{P}_2$ in cells treated with fibrinogen identify a new target for study. These inositol phosphates and analogues bind fibrinogen and reverse its actions in proliferation and migration assays. Our results have opened a route to the rational design of novel anti-proliferative inositol phosphate analogues utilising simple *in vitro* assays. Further characterisation of the inositol phosphate binding site within fibrinogen could allow computer-aided design of these

compounds, the development of which has been hampered previously by the need to complete costly animal experiments.

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Abbreviations used

BiPhP₅	biphenyl 2,3',4,5',6-pentakisphosphate
bFGF	basic fibroblast growth factor
DMEM	Dulbecco's modified Eagle's medium
ICAM-1	intercellular adhesion molecule-1
InsP₃	<i>myo</i> -inositol 1,4,5-trisphosphate
InsP₅	<i>myo</i> -inositol 1,3,4,5,6-pentakisphosphate
InsP₆	<i>myo</i> -inositol hexakisphosphate
PtdIns(4,5)P₂	phosphatidylinositol 4,5-bisphosphate
PI 3-K	phosphoinositide 3-kinase
scyllo-InsP₅	<i>scyllo</i> -inositol 1,2,3,4,5-pentakisphosphate
TNFα	tumour necrosis factor α
VEGF	vascular endothelial growth factor

References

- Batty IH, Downes CP. The inhibition of phosphoinositide synthesis and muscarinic-receptor-mediated phospholipase C activity by Li⁺ as secondary, selective, consequences of inositol depletion in 1321N1 cells. *Biochem. J.* 1994; 297:529–537. [PubMed: 8110190]
- Cuzzocrea S, McDonald MC, Mazzon E, Dugo L, Serraino I, Threadgill M, Caputi AP, Thiernemann C. Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly (ADP-ribose) polymerase, in a rodent model of lung injury. *Biochem. Pharmacol.* 2002; 63:293–304. [PubMed: 11841805]
- Downes CP, Gray A, Fairservice A, Safrany ST, Batty IH, Fleming I. The regulation of membrane to cytosol partitioning of signalling proteins by phosphoinositides and their soluble headgroups. *Biochem Soc Trans.* 2005; 33:1303–1307. [PubMed: 16246104]
- El-Sherbiny YM, Cox MC, Ismail ZA, Shamsuddin AM, Vucenik I. G0/G1 arrest and S phase inhibition of human cancer cell lines by inositol hexaphosphate (IP₆). *Anticancer Res.* 2001; 21:2393–2403. [PubMed: 11724298]
- Fox CH, Eberl M. Phytic acid (IP₆), novel broad spectrum anti-neoplastic agent: a systematic review. *Complement. Ther. Med.* 2002; 10:229–234. [PubMed: 12594974]
- Gardiner EE, D'Souza SE. A mitogenic action for fibrinogen mediated through intercellular adhesion molecule-1. *J. Biol. Chem.* 1997; 272:15474–15480. [PubMed: 9182580]
- Grases F, Simonet BM, Vucenik I, Prieto RM, Costa-Bauza A, March JG, Shamsuddin AM. Absorption and excretion of orally administered inositol hexaphosphate (IP₆ or phytate) in humans. *Biofactors.* 2001; 15:53–61. [PubMed: 11673644]

- Gray AJ, Bishop JE, Reeves JT, Laurent GJ. A alpha and B beta chains of fibrinogen stimulate proliferation of human fibroblasts. *J. Cell Sci.* 1993; 104:409–413. [PubMed: 8505369]
- Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc. Natl. Acad. Sci. U S A.* 2002; 99:11393–11398. [PubMed: 12177445]
- Hubbard AK, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radic. Biol. Med.* 2000; 28:1379–1386. [PubMed: 10924857]
- Komander D, Fairservice A, Deak M, Kular GS, Prescott AR, Peter Downes C, Safrany ST, Alessi DR, van Aalten DM. Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. *EMBO J.* 2004; 23:3918–3928. [PubMed: 15457207]
- Letcher AJ, Schell MJ, Irvine RF. Do mammals make all their own inositol hexakisphosphate? *Biochem J.* 2008; 416:263–270. [PubMed: 18684107]
- Lu DY, Chen XL, Ding J. Treatment of solid tumors and metastases by fibrinogen-targeted anticancer drug therapy. *Med. Hypotheses.* 2007; 68:188–193. [PubMed: 16956730]
- Lu DY, Chi J, Lin LP, Huang M, Xu B, Ding J. Effect of anti-cancer drugs on the binding of ¹²⁵I-Fibrinogen to two leukaemia cell lines in vitro. *J. Int. Med. Res.* 2004; 32:488–491. [PubMed: 15458280]
- Maffucci T, Piccolo E, Cumashi A, Iezzi M, Riley AM, Saiardi A, Godage HY, Rossi C, Broggin M, Iacobelli S, Potter BV, Innocenti P, Falasca M. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway by inositol pentakisphosphate results in antiangiogenic and antitumor effects. *Cancer Res.* 2005; 65:8339–8349. [PubMed: 16166311]
- Maxwell MJ, Yuan YP, Anderson KE, Hibbs ML, Salem HH, Jackson SP. SHIP1 and Lyn kinase negatively regulate integrin alpha(IIB)beta(3) signaling in platelets. *J. Biol. Chem.* 2004; 279:32196–32204. [PubMed: 15166241]
- Mills SJ, Vandeput F, Trusselle MN, Safrany ST, Erneux C, Potter BVL. Benzene polyphosphates as tools for cell signalling: Inhibition of inositol 1,4,5-trisphosphate 5-phosphatase and interaction with the PH domain of protein kinase B alpha. *Chembiochem.* 2008; 9:1757–1766. [PubMed: 18574825]
- Morrison RS, Shi EG, Kan M, Yamaguchi F, McKeenan W, Rudnickanawrot M, Palczewski K. Inositolhexakisphosphate (InsP₆) - an Antagonist of Fibroblast Growth-Factor Receptor-Binding and Activity. *In Vitro Cell. Dev. Biol. Anim.* 1994; 30A:783–789. [PubMed: 7881632]
- O'Hanlon DM, Fitzsimons H, Lynch J, Tormey S, Malone C, Given HF. Soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in breast carcinoma. *Eur. J. Cancer.* 2002; 38:2252–2257. [PubMed: 12441261]
- Odrliin TM, Francis CW, Sporn LA, Bunce LA, Marder VJ, Simpson-Haidaris PJ. Heparin-binding domain of fibrin mediates its binding to endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 1996; 16:1544–1551. [PubMed: 8977460]
- Piccolo E, Vignati S, Maffucci T, Innominato PF, Riley AM, Potter BV, Pandolfi PP, Broggin M, Iacobelli S, Innocenti P, Falasca M. Inositol pentakisphosphate promotes apoptosis through the PI 3-K/Akt pathway. *Oncogene.* 2004; 23:1754–1765. [PubMed: 14755253]
- Pinco KA, He W, Yang JT. alpha 4,beta 1 integrin regulates Lamellipodia protrusion via a focal complex/focal adhesion-independent mechanism. *Mol. Biol. Cell.* 2002; 13:3203–3217. [PubMed: 12221126]
- Preston T, Slater C, McMillan DC, Falconer JS, Shenkin A, Fearon KC. Fibrinogen synthesis is elevated in fasting cancer patients with an acute phase response. *J. Nutr.* 1998; 128:1355–1360. [PubMed: 9687556]
- Rauch BH, Muschenborn B, Braun M, Weber AA, Schror K. ICAM-1 and p38 MAPK mediate fibrinogen-induced migration of human vascular smooth muscle cells. *Eur. J. Pharmacol.* 2007; 577:54–57. [PubMed: 17904546]
- Riley AM, Dozol H, Spiess B, Potter BVL. 2-O-(2-aminoethyl)-myo-inositol 1,4,5-trisphosphate as a novel ligand for conjugation: physicochemical properties and synthesis of a new Ins(1,4,5)P-3 affinity matrix. *Biochem. Biophys. Res. Commun.* 2004; 318:444–452. [PubMed: 15120621]

- Riley AM, Trusselle M, Kuad P, Borkovec M, Cho J, Choi JH, Qian X, Shears SB, Spiess B, Potter BVL. scyllo-inositol pentakisphosphate as an analogue of myo-inositol 1,3,4,5,6-pentakisphosphate: Chemical synthesis, physicochemistry and biological applications. *Chembiochem*. 2006; 7:1114–1122. [PubMed: 16755629]
- Roche Y, Pasquier D, Rambeaud JJ, Seigneurin D, Duperray A. Fibrinogen mediates bladder cancer cell migration in an ICAM-1-dependent pathway. *Thromb. Haemost.* 2003; 89:1089–1097. [PubMed: 12783123]
- Rothlein R, Czajkowski M, Oneill MM, Marlin SD, Mainolfi E, Merluzzi VJ. Induction of Intercellular-Adhesion Molecule-1 on Primary and Continuous Cell-Lines by Pro-Inflammatory Cytokines - Regulation by Pharmacologic Agents and Neutralizing Antibodies. *J. Immunol.* 1988; 141:1665–1669. [PubMed: 3137261]
- Rybarczyk BJ, Lawrence SO, Simpson-Haidaris PJ. Matrix-fibrinogen enhances wound closure by increasing both cell proliferation and migration. *Blood*. 2003; 102:4035–4043. [PubMed: 12920033]
- Rybarczyk BJ, Simpson-Haidaris PJ. Fibrinogen assembly, secretion, and deposition into extracellular matrix by MCF-7 human breast carcinoma cells. *Cancer Res.* 2000; 60:2033–2039. [PubMed: 10766195]
- Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. *Blood*. 2000; 96:3772–3778. [PubMed: 11090059]
- Standeven KF, Ariens RA, Grant PJ. The molecular physiology and pathology of fibrin structure/function. *Blood Rev.* 2005; 19:275–288. [PubMed: 15963835]
- Streb H, Irvine RF, Berridge MJ, Schulz I. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature*. 1983; 306:67–69. [PubMed: 6605482]
- Vandeput F, Combettes L, Mills SJ, Backers K, Wohlkonig A, Parys JB, De Smedt H, Missiaen L, Dupont G, Potter BVL, Erneux C. Biphenyl 2,3',4,5',6-pentakisphosphate, a novel inositol polyphosphate surrogate, modulates Ca^{2+} responses in rat hepatocytes. *Faseb J.* 2007; 21:1481–1491. [PubMed: 17264160]
- Vanhaesebroeck B, Leever SJ, Ahmadi K, Timms J, Katso R, Driscoll PC, Woscholski R, Parker PJ, Waterfield MD. Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 2001; 70:535–602. [PubMed: 11395417]
- Vucenik I, Yang GY, Shamsuddin AM. Comparison of pure inositol hexaphosphate and high-bran diet in the prevention of DMBA-induced rat mammary carcinogenesis. *Nutr. Cancer*. 1997; 28:7–13. [PubMed: 9200144]

Figure 1. Structures of inositol phosphates and analogues used in this study

Figure 2. Ability of inositol phosphates and analogues to elute fibrinogen from InsP₅ matrix
Fibrinogen was loaded onto the InsP₅ matrix and the column washed extensively with buffer. Aliquots (1ml) of increasing concentrations of inositol phosphate or analogue (BiPhP₅ (filled circles), InsP₅ (open circles) or InsP₆ (filled squares) were used to elute the protein from the resin. Each column was washed with 2 × 1ml 10mM InsP₆ to ensure all fibrinogen was eluted from the column. *Scyllo*-InsP₅ was unable to elute the protein from the resin (data not shown). Samples from each batch of eluent were tested for protein content using Coomassie blue reagent. Data are representative of 2-4 independent experiments.

Figure 3. Ability of fibrinogen to cause increases in PtdIns(3,4)P₂, wound healing and proliferation

Fibrinogen was assessed for its ability to effect cellular functions in TNF α -treated A549 cells. Cells were treated with fibrinogen for: 5 minutes before quenching for PtdIns(3,4)P₂ analysis (filled squares); 24-72 hours for migration assays to determine wound healing (open squares); and 48 hours for MTS assays to determine proliferation (filled circles). Data are presented mean $\pm$ se of 3-4 independent experiments.

Figure 4. Ability of inositol phosphates and analogues to inhibit fibrinogen-mediated increases in PtdIns(3,4)P₂, wound healing and proliferation

The ability of InsP₆, InsP₅, *scyllo*-InsP₅ and BiPhP₅ (100μM of each) to inhibit the effects of fibrinogen (100μg/ml) on PtdIns(3,4)P₂ formation (black bars), wound healing (grey bars) and proliferation (clear bars) in TNFα-treated A549 cells. Data are presented as percentage of untreated control, mean ± se of 3-6 independent experiments. * p<0.05 compared to fibrinogen control (Dunnett's post hoc multiple comparison test).

Figure 5. Dose-response curves for BiPhP₅ and InsP₅ to inhibit fibrinogen-mediated proliferation and migration

The ability of BiPhP₅ (squares) and InsP₅ (circles) to inhibit fibrinogen (100μg/ml) - mediated proliferation (filled points) and migration (clear points) in TNFα-treated A549 cells. Data are presented mean of 3-6 independent experiments. Error bars are omitted for clarity.