Published in final edited form as:

Thromb Haemost. 2014 March 3; 111(3): 508–517. doi:10.1160/TH13-06-0484.

PDK1 selectively phosphorylates Thr(308) on Akt and contributes to human platelet functional responses

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Summary

3-phosphoinositide-dependent protein kinase 1 (PDK1), a member of the protein A,G and C (AGC) family of proteins, is a Ser/Thr protein kinase that can phosphorylate and activate other protein kinases from the AGC family, including Akt at Thr308, all of which play important roles in mediating cellular responses. The functional role of PDK1 or the importance of phosphorylation of Akt on Thr308 for its activity has not been investigated in human platelets. In this study, we tested two pharmacological inhibitors of PDK1, BX795 and BX912, to assess the role of Thr308 phosphorylation on Akt. PAR4-induced phosphorylation of Akt on Thr308 was inhibited by BX795 without affecting phosphorylation of Akt on Ser473. The lack of Thr308 phosphorylation on Akt also led to the inhibition of PAR4-induced phosphorylation of two downstream substrates of Akt, viz. GSK3 β and PRAS40. *In vitro* kinase activity of Akt was completely abolished if Thr308 on Akt was not phosphorylated. BX795 caused inhibition of 2-MeSADP-induced or collagen-induced aggregation, ATP secretion and thromboxane generation. Primary aggregation induced by 2-MeSADP was also inhibited in the presence of BX795. PDK1 inhibition also resulted in reduced clot retraction indicating its role in outside-in signalling. These results demonstrate that PDK1 selectively phosphorylates Thr308 on Akt thereby regulating its activity and plays a positive regulatory role in platelet physiological responses.

Keywords

Kinases; platelet pharmacology; signal transduction

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Conflicts of interest

None declared.

Introduction

The platelet has emerged as an important factor in homeostasis within the vasculature. Through an intricate series of signalling pathways, through many different receptors, the platelet can discern the requirement for various degrees of haemostasis without severe thrombosis (1). Adhesion of platelets to exposed collagen from an injured vessel wall, initially through glycoprotein (GP) Ib-IX-V to von Willebrand factor (VWF) (2–4) and followed by GPVI (5) and integrin $\alpha 2\beta 1$ (6), causes platelet activation leading to secretion (ADP, serotonin) and generation of autocrines (thromboxane A₂) which recruit more platelets to the site of injury (7). Transformation of the fibrinogen receptor, $\alpha \text{IIb}\beta 3$, from a resting to an activated state, is a result of this activation and mediates platelet aggregation (8).

The majority of platelet agonists signal through the phosphoinositide 3-kinase (PI3K)/Akt (Protein kinase B) pathway upon G_i stimulation by secreted ADP (9). Activation of this route leads to an increased concentration of phosphatidylinositol-3,4-bisphosphate (PI(3,4)P₂) and phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) in the membrane. These lipids interact with the pleckstrin homology (PH) domain of Akt and 3-phosphoinositide-dependent kinase-1 (PDK1) (10). This recruitment to the membrane causes a major conformational change in Akt and allows the phosphorylation of Thr308 in the activation loop of Akt by PDK1 (11, 12). Although it is widely accepted that Akt regulation is dependent on PI3-kinase activity, it has been reported that, in platelets, Akt can be activated by a partially PI3-kinase independent mechanism (13). All three isoforms of Akt – Akt1, Akt2 and Akt3 – require phosphorylation for activation.

The importance of Akt in platelet function is evident in Akt1- (14), Akt2- (15) and Akt3- (16) deficient mouse models. All demonstrate impaired platelet responsiveness to various agonists. Akt3-deficient mouse platelets, for example, selectively exhibit impaired aggregation and secretion in response to low concentrations of thrombin receptor agonists and thromboxane A₂ (TXA₂), but not collagen or von Willebrand factor (VWF). In contrast, platelets from Akt1- or Akt2-deficient mice are defective in platelet activation induced by thrombin, TXA₂ and VWF, but only Akt1-deficient platelets show significant defects in response to collagen, indicating differences among the Akt isoforms (16). Akt isoforms also play a role in integrin outside-in signalling and platelet spreading (17).

Until recently, minimal attention has been given to one of Akt's upstream regulators, PDK1. This may partially be due to the fact that PDK1-deficiency is embryonic lethal (18). In 2009, Chalhoub et al. (19) generated a strategic conditional PDK1 knockout model in the brain using the *Cre-loxP* system. PDK1 inactivation induced strikingly different effects on the regulation of phosphorylated Akt in glia versus neurons, and the authors concluded that there were cell type-specific differences in feedback regulation of the PI3K pathway. Also, while pursuing small molecule inhibitors of PDK1, Najakov et al. proposed a model in which the strength of the upstream signal determined whether a PDK1 inhibitor can block Akt phosphorylation (20), PDK1 inhibition appeared to have different consequences depending on the cell type and agonist employed.

In 2013, Chen et al. (21) generated megakaryocyte/platelet-specific PDK1 knockout mice to investigate the role of PDK1 in platelet activation and thrombus formation. The data indicated that platelet PDK1 activates Akt and inhibits GSK3 β , thereby enhancing thrombin-induced platelet aggregation, clot retraction, platelet spreading on immobilised fibrinogen and thrombin formation.

The effects of inhibition of PDK1 on cancer cell growth *in vitro* and *in vivo* appear to be evident, and this validates PDK1 as a compelling drug target for clinically effective small-molecule anticancer agents (22–24). Therefore, the effects of these inhibitors in other cell systems must be addressed, especially considering the important role PDK1 plays in most signalling cascades.

In this study we chose two small molecule inhibitors of PDK1, BX795 and BX912. These compounds were first described in 2005 (25) and were shown to have greater than a 20-fold selectivity for PDK1 relative to 10 other kinases tested. We assessed their effects on agonist-induced phosphorylation of Akt at Thr308. We have shown that PDK1 is essential for Akt activity and its inhibition diminished agonist-induced platelet aggregation, dense granule secretion, thromboxane formation and clot retraction. Thus PDK1 contributes to human platelet functional responses.

Materials and methods

Reagents

BX795 and BX912 were purchased from B-Bridge International, Inc. (Cupertino, CA, USA). Bisindolylmaleimide 1 (GF 109203X) was from Calbiochem (San Diego, CA, USA). 2-MeSADP, acetylsalicylic acid (ASA), and apyrase (Type V) were from Sigma (St. Louis, MO, USA). AYPGKF was purchased from GenScript Corp. (Piscataway, NJ, USA). Convulxin was purified according to the method of Polgar et al. (54). Collagen, Chronolume (for detection of secreted ATP) and ATP standard were from Chrono-log Corp. (Havertown, PA, USA). Nitrocellulose membrane used was Whatman Protran[®] (Dassel, Germany). All of the primary antibodies used were from Cell Signalling Technology (Beverly, MA, USA). Odyssey blocking buffer was from LI-COR Bioscience (Lincoln, NE, USA). Secondary antibodies DyLight[™] 800-conjugated goat anti-rabbit IgG and DyLight[™] 680-conjugated goat anti-mouse IgG were from Thermo Scientific (Waltham, MA, USA).

Human platelet isolation, aggregation and ATP secretion

Washed human platelets were prepared as previously described (26). The platelet count was adjusted to 2×10^8 /ml. Inhibitors were incubated for 5 minutes (min) at 37°C prior to agonist addition, and aggregation and ATP secretion were measured as previously described (27).

Western blot analysis

Platelets were stimulated with agonists in the presence of vehicle or inhibitor for the indicated time under stirring conditions at 37°C. Samples were prepared for SDS-PAGE and Western blotting as previously described (27).

Akt activity assay

Akt activity was measured using the “Akt kinase activity assay kit (nonradioactive)” from Cell Signalling (Cat# 9840) with modifications. Briefly, platelets (2×10^9 /ml; 200 μ l) were pre-incubated with vehicle (DMSO) or varying concentrations of BX795 for 5 min at 37°C. Samples were then activated with 200 μ M AYPGKF for 2 min at 37°C under stirring conditions. Reactions were stopped by addition of lysis buffer included in the kit. Total Akt was immunoprecipitated with Akt (pan) (40D4) mouse monoclonal antibody (Sephacrose bead conjugate) from Cell Signalling for 2 hours at 4°C. The immune complexes were washed three times in 1x lysis buffer and once in kinase assay buffer from the kit. An *in vitro* kinase assay was performed using a GSK-3 fusion protein as substrate in the presence of 200 μ M ATP for 30 min at 30°C. The reaction was stopped by addition of sample buffer and boiled for 5 min. Proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose and probed for phospho-GSK3 β (Ser21/9).

Measurement of TXA₂ generation

Measurement of thromboxane B₂ generation (the stable analogue of TXA₂) was performed as previously described (28).

Clot retraction

Human platelets were isolated as described above and clot retraction measured as previously described (29) with minor modifications. Briefly, 500 μ l platelets (4×10^8 /ml) were added to a glass cuvette and mixed with 1 mM CaCl₂ and 0.1 mg/ml fibrinogen. A total of 0.2 units/ml of thrombin was added to initiate clot retraction. Platelets were allowed to retract at room temperature and photographed at the indicated time points.

Statistics

All densitometric analyses were performed using the Odyssey Image Studio 2 software. For the Akt *in vitro* kinase assay, analysis was performed using Fujifilm Science Lab 2003, Image Gauge, Version 4.22 software (Edison, NJ, USA). KaliedaGraph 2003, Version 3.62 by Synergy software and Prism 2007, Version 5 by Graphpad software were used for statistical analysis and graphic representation.

Results

PDK1 inhibitors block Akt phosphorylation at Thr308

Phosphorylation of Akt at Thr308 has been shown to be a PI3-kinase-dependent event (11, 12). Although the ability of PDK1 to phosphorylate Akt at Thr308 has been known for many years (30), its influence on human platelet functional responses has not been studied. Thus we evaluated two commercially available PDK1 inhibitors – BX795 and BX912. Both contain a common aminopyrimidine backbone and bind to the ATP binding pocket site of PDK1 (25). Both PDK1 inhibitors efficiently inhibited the phosphorylation of Akt at Thr308 when platelets were stimulated with the PAR4 agonist, AYPGKF (Figure 1A, B). BX912 also inhibited the phosphorylation of Akt at Ser473 in a concentration-dependent manner compared with BX795 (Figure 1A, C). In fact, no effect on the Ser473 phosphorylation of

Akt was detected except at 10 μ M BX795, the highest concentration tested. We also measured PAR4-induced phosphorylation of GSK3 β at Ser9 (Figure 1A, D) and PRAS40 at Thr246 (Figure 1A, E), and both inhibitors blocked these phosphorylation sites to the same degree. Interestingly, although BX912 inhibited agonist-induced Akt phosphorylation at Ser473, its effect on platelet functional responses mimicked that of BX795. Therefore, we chose to present only BX795 data in the following work.

To ensure that the PDK1 inhibitor did not affect the kinetics of Akt phosphorylation or dephosphorylation we measured PAR4-induced phosphorylation of Akt at Thr308 (Figure 2A, B) and at Ser473 (Figure 2A, C), GSK3 β at Ser9 (Figure 2A, D), and PRAS40 at Thr246 (Figure 2A, E) for various times in the absence and presence of 1 μ M BX795. At no time was Akt phosphorylated at Thr308 while Akt phosphorylation at Ser473 was unaffected.

PDK1-mediated phosphorylation of Akt on 308 is necessary for its activity

Recent studies have indicated that mTORC2-mediated phosphorylation of Akt at Thr473 is not required for Akt 1 activity (31). However, the role of Akt(Thr308) phosphorylation has not been elucidated. Hence, we evaluated the role of phosphorylation of Akt at Thr308 on its kinase activity using an *in vitro* kinase assay. Human platelets were activated by AYPGKF in the absence and presence of 1 μ M BX795, the cells lysed and total Akt immunoprecipitated. The ability of this immunoprecipitate to phosphorylate a GSK3 β fusion peptide was measured. BX795 inhibited the phosphorylation of GSK3 β in a concentration-dependent manner (Figure 3 A, B). These data demonstrate that phosphorylation of Thr308 on Akt is essential for its kinase activity.

BX795 inhibits agonist-induced platelet functional responses

Since BX795 inhibited Akt kinase activity, we examined its effect on platelet physiological responses. In platelets not pre-treated with aspirin, BX795 inhibited 2-MeSADP-induced aggregation and ATP secretion in a concentration-dependent manner (Figure 4A). To ensure that this effect on aggregation was not due to just inhibition of autocrine formation of thromboxane A₂, we evaluated the role of PDK1 in primary aggregation. In aspirin- treated platelets, where thromboxane generation is blocked, BX795 inhibited 2-MeSADP-induced primary aggregation as well (Figure 4B), indicating that PDK1 inhibition affects the initial stages of aggregation. We also examined the role of PDK1 in PAR4- (Figure 4C) and collagen-induced (Figure 4D) aggregation and ATP secretion. Similar results were obtained, demonstrating that the effects we see with BX795 are independent of the agonist used. This inhibition was also reflected in thromboxane formation induced by 2-MeSADP or collagen in the presence of BX795 (Figure 4E, F).

BX795 does not inhibit PAR4-induced PKC activity

Considering that PDK1 is a master kinase that can phosphorylate and activate a variety of AGC kinases, including several PKC isoforms, we explored the possibility that this may be the reason for agonist-induced inhibition of aggregation and secretion by BX795. We therefore utilised a phospho-(Ser) PKC substrate antibody from Cell Signalling (cat#2261) which is specific to cPKC substrates containing phospho-serine. In addition, we also used a

phospho-threonine antibody since PDK1 is a serine/threonine kinase. This was compared to platelets pre-treated with GF109203X, a pan PKC inhibitor (Figure 5). There was no apparent effect of BX795 on PAR4-induced phosphorylation of PKC substrates or phospho-threonine except at 10 μ M. BX795, the highest concentration tested, indicating that the inhibition of aggregation and secretion we see at 1 μ M BX795 does not appear to be due to a reduction of PKC activity.

PDK1 is involved in clot retraction

Integrin α IIb β 3 outside-in signalling also regulates platelet functional responses including clot retraction which is inhibited by the PI3K inhibitor LY294002 (32). Since PDK1 is dependent on 3-phosphoinositides we tested the effect of BX795 on outside-in signalling. Clot retraction was measured in the presence of increasing concentrations of BX795 (Figure 6) and was dramatically delayed or and totally inhibited at 300 nM in the time frame measured. These data suggest that outside-in signalling is strongly influenced by PDK1.

Discussion

The PI3-kinase/Akt signalling pathway is involved in response to numerous stimuli and plays a key role in regulation of many functions including cell growth, proliferation, apoptosis and angiogenesis (18). Abnormal activation of this pathway is very common in cancer and an increasing number of inhibitors have targeted downstream effectors of PI3-kinase including Akt and PDK1 (33).

PDK1, a 63 kDa protein, was first purified from tissue extracts as an enzyme that could phosphorylate Thr308 in the activation loop of Akt in the presence of PtdIns(3,4,5)P₃ (12,34). PDK1 can phosphorylate the activation site of at least 23 AGC kinases including all isoforms of Akt, S6K, serum- and glucocorticoid-regulated kinase (SGK), p90 ribosomal protein S6 kinases (RSK), and several isoforms of PKC (e.g. PKD δ , PKC ζ and PKC β II).

PDK1 has been described as a “master” regulator of AGC kinase signal transduction (35) and the “hub” of intracellular signaling (36). We chose two pharmacological inhibitors of PDK1, BX795 and BX912, which were first described by Feldman et al. (25). BX795 showed an IC₅₀ value of 0.006 μ M and BX912 had an IC₅₀ value of 0.012 μ M in regard to PDK1 inhibition. They also analysed the effects of BX795 and BX320 on Akt(Ser473) phosphorylation in extracts prepared from compound-treated PC-3 cells. Both these compounds reduced the levels of Akt(Ser473) phosphorylation although the potency appeared to be lower than that for inhibition of Akt(Thr308) levels. PDK1 activity appeared to be critical for phosphorylation of both Thr308 and Ser473 on Akt, at least in PC-3 cells. However, they could not exclude the possibility that the less potent inhibition of Akt(Ser473) phosphorylation displayed by BX compounds resulted from their non-selective action on other kinases. How BX compounds may influence Akt S473 phosphorylation through inhibition of PDK1 is not known. PDK1 may modulate other proteins that affect mTORC2, the protein responsible for phosphorylating Akt on S473. However, under conditions where agonist-induced Akt(Thr308) phosphorylation was negligible, platelet aggregation, ATP secretion, thromboxane formation and clot retraction were dramatically reduced. Akt activity was also abolished. Agonist-induced phosphorylation of GSK3 β and

PRAS40, two downstream substrates of Akt, was also reduced, demonstrating the need for this phosphorylation site in affecting downstream substrates. Clot retraction in the presence BX795/BX912 was also repressed indicating that PDK1 may play a role in regulating integrin α IIb β 3 outside-in signalling.

Inhibition of activation of downstream substrates of Akt may have been the reason for the inhibitory effects of BX795. We have studied two of them, GSK3 β and PRAS40. The kinase activity of GSK3 β is thought to be constitutive and negatively regulated by phosphorylation at Ser9 (37, 38). Phosphorylation of Ser9 by Akt has been linked to decreased GSK3 β activity that may then release inhibition of GSK3 β 's substrates (39–41). We demonstrate inhibition of agonist-induced phosphorylation of GSK3 β in the presence of BX795 and inhibition of platelet functional responses indicating that the lack of GSK3 β phosphorylation and thus inhibition of its downstream substrates may be responsible for our results. However, ablation of GSK3 β in platelets leads to an increase in platelet functional responses as demonstrated by Li et al. (42) and Moore et al. (43). This may be because of the total absence of GSK3 β 's inhibitory effect. PRAS40, a novel Akt target, was first described in platelets in 2011 (31) and its function in platelets is yet to be resolved. In other cells PRAS40 is known for its ability to regulate the mammalian target of rapamycin complex 1 (mTORC1). However Moore et al. (31), using PP242 and Torin1, two mTOR inhibitors, found no significant effect on platelet aggregation. These authors measured PRAS40 phosphorylation at Thr246 and found that it was predominantly an Akt2 substrate. We have used an (pan) Akt antibody in our studies that could not differentiate Akt isoforms.

The role PDK1 plays in the activation of PKCs is less certain. It has been suggested that, upon synthesis, classical PKCs (cPKCs) interact with PDK1 and become phosphorylated at their activation loop (44). This stabilises cPKCs and permits the phosphorylation at their hydrophobic motifs thus acquiring full active conformation. If this stabilisation does not occur, cPKCs can aggregate and/or degrade. This has been verified in embryonic stem cells deficient in PDK1 where the levels of PKCs are drastically reduced (45). These data demonstrate that phosphorylation by PDK1 is necessary for correct folding of PKCs. To confirm that inhibition of PDK1 did not affect agonist-induced stimulation of PKCs we looked for any differences in the phosphorylation of PKC substrates by using a phospho-(Ser) PKC substrate antibody. This was compared to platelets pre-treated with GF109203X, a pan PKC inhibitor. There was no appreciable effect on phosphorylated substrates of PKC in the presence on BX795. However, we cannot eliminate the possibility that inhibition of aggregation and ATP secretion was due to inhibition of a specific PKC isoform.

Moore, et al. (41) recently published that thrombin-mediated GSK3 β / α phosphorylation is regulated by PKC α at early time points and by Akt at later time points. We assessed the phosphorylation state of GSK3 β in thrombin/ADP-activated platelets pretreated with BX795 or GF109203X (data not shown). Our data revealed a reduction in GSK3 β (Ser9) phosphorylation in the presence of BX795 at early time points compared to platelets pretreated with GF109203X where this phosphorylation was absent. Although bisindolyl maleimide-based inhibitors are reported to be PKC specific, literature has shown this not to be the case. Komander et al. (46) published that BIM compounds can also inhibit PDK1 directly. Although these were not cell-based assays, care must still be taken in drawing

conclusions based on inhibitors, Whether the reduction in GSK3 β (Ser9) phosphorylation is due to a direct effect of BX795 on PDK1, a direct effect of GF109203X on PDK1 or an indirect effect on PKC is difficult to discern.

Although initially developed as a PDK1 inhibitor, recent studies have found that BX795 can also inhibit TANK-binding kinase-1 (TBK1) and closely related I κ B kinase ϵ (IKK ϵ) (47). Besides being a major protein involved in responsiveness to inflammatory cytokines (48–50), it has been demonstrated that TBK1 directly activates Akt by phosphorylation of the hydrophobic motif (Thr308) and the activation loop (Ser473) independently of PDK1 and mTORC2 (51, 52). This contribution of TBK1 to Akt activation appears to be non-redundant to the PDK1/mTORC2 pathway (51). Our laboratory has found TBK1 in human platelets (unpublished observation). If, in fact, BX795 was inhibiting TBK1 activity, and consequently Akt phosphorylation, we should observe inhibition of both Akt(Ser473) and Akt(Thr308) phosphorylation sites. As shown in Figure 1, we see total inhibition of the Akt(Thr308) phosphorylation site while Akt(Ser473) remains undisturbed. We cannot rule out, however, the possibility that TBK1 may play a role in Akt activation.

Recently Chen et al. (21) published a work using mice with a platelet-specific PDK1 deletion. Aggregation of PDK-1-deficient platelets was abnormal in response to low levels of α -thrombin, U46619 and ADP. Phosphorylation of Akt at Thr308 and GSK3 β at Ser9 was totally abolished in PDK 1-deficient platelets in response to the agonists listed above. Akt phosphorylation at Ser473 was diminished but not ablated in these platelets. The authors concluded, however, that Akt phosphorylated at Ser473 had no contribution to thrombin-induced platelet activation. We were concerned about the differences in pAkt(Ser473) phosphorylation in the PDK1^{-/-} mice compared to BX795 where there was no inhibition. We therefore imitated the ablation of PDK1 by pre-treating murine platelets with varying concentrations of BX795 prior to activation by thrombin (data not shown). BX795 inhibited agonist-induced Akt(Thr308) phosphorylation in a concentration-dependent manner similar to that seen in human platelets (Figure 1C). However, the agonist-induced phosphorylation of Akt(Ser473) was also inhibited in a concentration-dependent manner, contrary to what we see in human platelets. We believe that there may be a species difference. This effect is not uncommon. When first describing these PDK1 small molecule inhibitors, Feldman et al. (23) noted “PDK1 can play a key role in Ser473-Akt phosphorylation, depending on the cell type and/or the signalling pathways activated. The phosphorylation of Ser473-Akt in PC-3 cells, which is promoted through loss of PTEN requires PDK1 activity, whereas phosphorylation of Ser473-Akt in PANC1 cells, which is stimulated by IGF-1 appears to depend on other factors.” Iwanami et al. (53) also observed that “Western blotting analyses of cerebella and hippocampi in various models revealed up-regulation of Akt(Ser473) phosphorylation in PDK1-deleted glia, but not PDK1-deleted neurons.” It was concluded “deletion of PDK1 differentially activates PI3 kinase signalling in glia relative to neurons, raising the concept that the signalling feedback pathways are wired differently in different CNS cell types.” It is possible that murine platelets and human platelets may have different feedback pathways. Nonetheless, we are in agreement with Chen et al. (51) that Akt phosphorylated at Ser473 does not contribute to platelet function. Chen et al. (51) also concluded that PDK1 regulates activation through integrin α IIB β 3-mediated outside-in signalling as PDK1 deficiency severely interfered with platelets spreading on immobilised

fibrinogen and delayed clot retraction. All this data was collected using murine platelets. Our data was collected using human platelets with PDK1 inhibitors, and our results are surprisingly similar considering the possibility of non-specific effects of BX795/BX912.

In conclusion, we propose that PDK1 plays an important role in platelet functional responses. Inhibition of phosphorylation of Thr308 on Akt by BX795 abolishes its activity in an *in vitro* kinase assay and results in inhibition of phosphorylation of two downstream Akt substrates, GSK3 β and PRAS40. Agonist-induced aggregation, ATP secretion, thromboxane generation and clot retraction were also inhibited. Using these pharmacological inhibitors of PDK1 has provided new insights into the role of this important signalling molecule in human platelets. However, care should be taken when assessing potential cancer chemotherapies aimed at the PI3-kinase/Akt pathway.

Acknowledgments

This work is supported by HL118593 and HL93231 from the National Institute of Health to S.P. Kunapuli.

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What is known about this topic?

- 3-phosphoinositide-dependent kinase 1 (PDK1) is a Ser/Thr kinase that can phosphorylate and activate a number of kinases in the AGC kinase superfamily including Akt.
- Full activation of Akt is dependent on Thr308 phosphorylation by PDK1 and Ser473 phosphorylation by mTORC2.

What does this paper add?

- In human platelets, we have shown that blocking Akt Thr308 phosphorylation with pharmacological inhibitors leads to ablation of Akt activity even though Ser473 phosphorylation is unaffected.
- We have shown that inhibition of Akt phosphorylation on Thr308 leads to a reduction in human platelet physiological responses demonstrating the importance of PDK1 in platelet regulation.

Figure 1. BX compounds inhibit agonist-induced Akt, PRAS40 and GSK3β phosphorylation
 Washed, aspirin-treated platelets were incubated with the indicated concentrations of PDK1 inhibitors for 5 min at 37°C prior to stimulation with 200 μM AYPGKF for 2 min at 37°C. All experimental conditions had a final concentration of 0.1 % DMSO. A) Representative Western blot of three independent experiments. The phosphorylation of Akt Thr308 (A), Akt Ser473 (B), PRAS40 Thr246 (C) and GSK3β Ser9 (D) was determined by SDS-PAGE/ Western blotting as described in *Methods* [BX795 (solid lines) or BX912 (hatched lines)]. The graphs represent quantification of phosphorylation (ratio phosphorylated/total)

expressed as a percentage of the signal obtained with AYPGKF without inhibitors, (mean $\pm$ SEM, n=3). *p<0.05.

Figure 2. BX795 inhibits agonist-induced phosphorylation of Akt, GSK3 β and PRAS40
Washed, aspirin-treated platelets were incubated with vehicle or 1 μ M BX795 for 5 min prior to stimulation with 250 μ M AYPGKF. A) Representative Western blot of three independent experiments. The phosphorylation of Akt Thr308 (A), Akt Ser473 (B), GSK3 β Ser9 (C) and PRAS40Thr246 (D) was determined by SOS-PAGE/Western blotting as described in *Methods* (vehicle (solid lines) or 1 μ M BX795 (hatched lines)). Phosphorylation is expressed as a ratio of phosphorylated protein/total Akt. The graphs are representative of three independent experiments.

Figure 3. PDK1-mediated phosphorylation of Akt on Thr308 is essential for its activity
 Washed, aspirin-treated platelets were incubated with vehicle or BX795 for 5 min prior to stimulation with 200 μM AYPGKF for 2 min. Total Akt was immunoprecipitated from detergent lysates, and *in vitro* activity was assessed using a GSK3 β fusion protein as substrate as described in *Methods* (A). Activity is expressed as a percentage of signal obtained with AYPGKF in the absence of BX795. (B) (n=3, mean $\pm$ SEM). * $p < 0.05$.

Figure 4. BX795 inhibits agonist-induced aggregation, ATP secretion and thromboxane formation

Washed platelets without (A) or with (B) aspirin pre-treatment were incubated with BX795 for 5 min prior to activation with 100nM 2-MeSADP. Aggregation and ATP secretion were monitored in a Chronolog lumi-aggregometer. C) Aspirin-treated platelets were pre-incubated with vehicle (-) or 1 μM BX795 (+) for 5 min prior to activation with AYPGKF. Aggregation and ATP secretion were monitored as above. D) Non-aspirin treated platelets were treated as in C and activated with collagen. Aggregation and secretion were monitored as above. Results of all aggregations and secretions are representative of three independent

experiments. E and F) Aspirin-free platelets were treated as in C and activated with 100 nM 2-MeSADP or 5 µg/ml collagen for 3.5 min. Samples were prepared for thromboxane analysis as described in *Methods* (mean ± SEM, n=3). *p<0.05.

Figure 5. BX795 does not inhibit PAR4-induced PKC activity

Washed, aspirin-treated platelets were incubated with vehicle, BX795 or 10 μM GF109203X for 5 min at 37°C prior to stimulation with 200 μM AYPGKF for 2 min. Samples were analysed by SDS-PAGE and Western blotting and probed with a phospho-(Ser) PKC substrate antibody (A) or a phospho-threonine antibody (B) as described in *Methods*. Results are representative of three independent experiments.

Figure 6. BX795 inhibits clot retraction

Washed, aspirin-treated platelets were incubated with vehicle or BX795 for 5 min prior to initiating clot retraction as described in *Methods*. Photographs were taken at the times indicated. Data are representative of three independent experiments.