NOVEL SMALL MOLECULE INHIBITORS OF 3-PHOSPHOINOSITIDE-DEPENDENT KINASE-1 (PDK1)*

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The PI 3-kinase/PDK1/AKT-signaling pathway plays a key role in cancer cell growth, survival, and tumor angiogenesis and represents a promising target for anti-cancer drugs. Here, we describe three potent PDK1 inhibitors, BX-795, BX-912 and BX-320 (IC50 = 11 - 30 nM) and their initial biological characterization. The inhibitors blocked PDK1/Akt signaling in tumor cells and inhibited the anchorage-dependent growth of a variety of tumor cell lines in culture or induced apoptosis. A number of cancer cell lines with elevated Akt activity were >30 fold more sensitive to growth inhibition by PDK1 inhibitors in soft agar than on tissue culture plastic, consistent with the cell survival function of the PDK1/Akt signaling pathway, which is particularly important for unattached cells. BX-320 inhibited the growth of LOX melanoma tumors in the lungs of nude mice after injection of tumor cells into the tail-vein. The effect of BX-320 on cancer cell growth in vitro and in vivo indicates that PDK1 inhibitors may have clinical utility as anti-cancer agents.

3-Phosphoinositide-dependent kinase 1 (PDK1) is a Ser/Thr protein kinase that can phosphorylate and activate a number of kinases in the AGC kinase super family (named after family members, protein kinase A, protein kinase G and protein kinase C), including Akt/PKB, protein kinase C (PKC), PKC-related kinases (PRK1 and PRK2), p70 ribosomal S6-kinase (S6K1), and serum and glucocorticoid-regulated kinase (SGK) (1). The first identified and best-characterized PDK1 substrate is the proto-oncogene Akt (2). PDK1 phosphorylates the activation loop of Akt (also called the T-loop) on residue Thr308, which promotes the formation of the enzyme conformation with catalytic activity (3). Numerous studies have found a high level of activated Akt in a large percentage (30-60%) of common tumor types, including melanoma and breast, lung, gastric, prostate, hematological and ovarian cancers. When activated in tumor cells, Akt has multiple effects that promote disease progression, including suppression of apoptosis and stimulation of tumor cell proliferation, metabolism, and angiogenesis (4,5). The PDK1/Akt signaling pathway thus represents an attractive target for the development of small molecule inhibitors that may be useful in the treatment of cancer.

Akt comprises a family of Ser/Thr protein kinases containing three highly homologous members (AKT1, AKT2, and AKT3) (2,6). Activation of Akt in cells by PDK-1 requires stimulation of phosphoinositide 3-kinase (PI 3-kinase) whose activity becomes highly elevated in many tumors through the up-regulation or mutation of upstream signaling molecules such as EGF receptors, Ras, Src, and c-ABL or by over-expression of PI 3-kinase itself (2,4). Loss of the tumor suppressor PTEN/MMAC1 is also a common mechanism of Akt activation in tumor cells (7). PTEN has D-3 phosphoinositide phosphatase activity and acts as a negative regulator of PI 3-kinase function by removing its products, PtdIns-3,4-P2 or PtdIns-3,4,5- P3 (8). Phosphoinositides produced by PI 3-kinase bind directly to the regulatory pleckstrin homology (PH) domain of Akt, driving a conformational change in the molecule that enables the activation loop of AKT1 to be phosphorylated by PDK1 at Thr308 (Thr309 for AKT2 and Thr 305 for AKT3) (9,10). Activation of AKT1 is also associated with phosphorylation of Ser473 (Ser474 for AKT2 and Ser472 for AKT3) within a C-terminal hydrophobic...
motif characteristic of kinases in the AGC kinase family. This modification serves to further enhance the kinase activity of Akt (11). While the role of PDK1 in Thr308 phosphorylation is well established, the mechanism of Ser473 phosphorylation is controversial. A number of candidate enzymes responsible for this modification have been put forward, including integrin-linked kinase (ILK) (12), PDK1 when in a complex with the kinase PRK2 (13), Akt itself, through autophosphorylation (14), PKC-α (15), PKCβII (16), DNA-dependent kinase (17), and the rictor-mTOR complex (18).

Besides Akt, many other kinases in the AGC kinase super-family, including PKC, PKA, p70 ribosomal S6 kinase (S6K1), RSK-1, SGK, and PRK-1, have a homologous region of their activation loops containing a consensus substrate recognition site for PDK1 (19). S6K1 is an important regulator of cell growth that controls the synthesis of ribosomal and other proteins in response to growth factors and nutrients (20). Activation of S6K1 requires two PDK1 dependent modifications: phosphorylation of Thr389 (21), which can be mediated by PDK1 through activation of Akt, and the direct phosphorylation of Thr229 within the activation loop of S6K1 by PDK1 (22,23). Promising pre-clinical and clinical studies with rapamycin analogs that block S6K1 through inhibition of mTOR (e.g., CCI-779), also implicate S6K1 as a cancer target (24).

PDK1 can phosphorylate several isoforms of PKC (e.g., PKCδ, PKCζ, and PKCβ2) in vitro or in cells co-transfected with PKC isoforms together with PDK1 (25-27). Consistent with these studies, deletion of both PDK1 alleles in embryonic mouse fibroblast cells results in impaired phosphorylation and activation of Akt, S6K1, and PKCζ (28). Other isoforms of PKC, including PKCα, PKCβ2 and PKCδ showed reduced protein levels (29). The established role of PDK1 in the regulation of Akt, S6K1, SGK, and PKC points to its importance as a critical regulator of cell signaling in cancer cells and tumor progression.

In light of the compelling evidence for the role of the PI 3-kinase/PDK1/Akt signaling in cancer progression, we sought to discover novel small molecule inhibitors that block this pathway for evaluation as anticancer drugs. Our strategy was to screen chemical libraries for lead compounds using a coupled assay measuring phosphatidylinositol (PtdIns) 3,4-P2- and PDK1-mediated activation of AKT2, with the final assay readout being phosphorylation of a peptide substrate by activated AKT2. From our screen, we identified different mechanistic classes of compounds that blocked PDK1 or AKT2, or that interfered with phosphoinositide-dependent activation of AKT2, possibly through inhibition of the PH domain function. In this paper, we report on the biological characterization of optimized compounds that directly inhibit the activity of PDK1 in vitro and in cells, while showing selectivity against a panel of other kinases. These compounds block the growth of a variety of tumor cell lines in culture, in soft agar and in an animal model, supporting their further evaluation as anticancer agents.

**MATERIALS AND METHODS**

**Materials**- Polyclonal antibodies against phospho-Thr308-Akt, phospho-Ser473-Akt, Akt, phospho-Thr389-p70 S6 kinase, p70 S6 kinase, phospho-Ser241-PDK1, PDK1, phospho-Thr505- PKCδ, PKCζ, Ser21/9-GSK3β, phospho-histone-H3 Ser10, and histone were obtained from Cell Signaling Technologies (Beverly, MA). Recombinant AKT2 and PDK1, containing N-terminal His-tags, were expressed in insect cells and purified by Ni2+-affinity chromatography. Lambda phosphatase was obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were of reagent grade quality. BX-320, BX-795 and BX-912 were synthesized as disclosed previously (Bryant, J., et al., CHK-, PDK-, and AKT-Inhibitory Pyrimidines, Their Production and Use as Pharmaceutical Agents. WO2004048343, 2004).

**Cell culture conditions**- The cell lines MDA-468, MDA-453, HCT-116, U87-MG, U2OS, PC-3, B16 F10, and MiaPaCa were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). LOX amelanotic human melanoma cells were obtained as a gift from the Norwegian Cancer Institute (Oslo, Norway), and HeLa cells were obtained from the Institute of Molecular Biology (Zurich, Switzerland). Growth media and supplements were obtained from Gibco/Invitrogen (Grand Island, NY) and Irvine Scientific (Santa Ana, CA) and were combined following ATCC guidelines. Primary human
mammary epithelial cells (HMEC) and prostate epithelial cells (PrEC) and their respective growth media were purchased from Clonetics/Cambrex (Baltimore, MD).

Kinase Assays – PDK1 was assayed in a direct kinase assay and a coupled assay format measuring PDK1- and (PtdIns) 3,4-P2- mediated activation of AKT2. For the coupled assay, the final assay mixture (60 µL) contained: 15 mM MOPS, pH 7.2, 1 mg/ml bovine serum albumin, 18 mM betaglycerolphosphate, 0.7 mM dithiothreitol, 3 mM EGTA, 10 mM MgOAc, 7.5 µM ATP, 0.2 µCi of \([\gamma^{33}P]ATP\), 7.5 µM biotinylated peptide substrate (biotin-ARRRDGGGAQPFRPRAATF), 0.5 µL of PIP2-containing phospholipid vesicles (3), 60 pg of purified recombinant human PDK1, and 172 ng of purified recombinant human AKT2. Following incubation for 2 h at room temperature, the biotin-labeled peptide was captured from 10 µL of the assay mixture on streptavidin-coated SPA beads, and product formation was measured by scintillation proximity in a Wallac MicroBeta counter. The product formed was proportional to the time of incubation and to the amount of PDK1 and inactive AKT2 added. PDK1 was added at sub-optimal levels so that the assay could sensitively detect inhibitors of AKT2 activation, as well as direct inhibitors of PDK1 or AKT2.

To directly measure PDK1 activity, the final assay mixture (60 µL) contained 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% beta-mercaptoethanol, 1 mg/ml bovine serum albumin, 10 mM MgOAc, 10 µM ATP, 0.2 µCi of \([\gamma^{33}P]ATP\), 7.5 µM of substrate peptide (H2N-ARRRGVTTKTFCGT) and 60 ng of purified recombinant human PDK1. After 4 h at room temperature, we added 25 mM EDTA and spotted a portion of the reaction mixture on Whatman P81 phosphocellulose paper. The filter paper was washed 3 times with 0.75% phosphoric acid and once with acetone. After drying, the filter-bound labeled peptide was quantified using a Fuji Phosphoimager.

Immunoblotting- After treating cells (roughly 80% confluent) as described in the figures, we washed them once with phosphate buffered saline (PBS) and then lysed them with MPER reagent (Pierce Chemicals, Rockford, IL), supplemented with protease and phosphatase inhibitors (Halt protease inhibitor cocktail, Pierce Chemicals; 1 mM sodium fluoride, 1 mM sodium orthovanadate). Lysates were analyzed by Western blotting using the antibodies indicated in the figures.

Growth assays- Cells seeded at a low density (1500-3000 cells per well, 0.1 ml per well, 96-well plates) were incubated overnight. Compound treatments were made by adding 10 µl per well of the compound in 1% DMSO/growth medium (final concentration of DMSO, 0.1%), followed by brief shaking. Treated cells were incubated for 72 h, and viability was measured by the addition of 10 µl of the metabolic dye WST-1 (Roche Applied Science, Indianapolis, IN). The WST-1 signal was read in a plate reader at 450 nm, and a no-cell, or zero-time cell, background was subtracted to calculate the net signal. Results are reported as the average ± sem of two or more replicates.

Apoptosis assays - Cells were seeded and treated identically to cells in the proliferation assay. After 48 h treatment, apoptosis was measured by the addition of 0.1 ml/well of Apo-One reagent (Promega, Madison, WI). Plates were read in a fluorescence plate reader (Wallac Victor2 1420 Multilabel Counter) after 1 h. The fluorescent readout measured the cleavage of a specific caspase 3/caspase7 fluorescent substrate (excitation 485 nm, emission 535 nm), indicating cells that were undergoing apoptosis. Results are reported as the average ± sem of two or more replicates.

Soft agar assays - Sterile 35 mm plates were prepared with bottom layers of 0.5% agar in growth medium (1.5 ml per plate, agar temperature 48°C). Trypsinized cells, dispersed into single cells using a 21-gauge needle, were seeded in a 1.5 ml volume of 0.3% agar in growth medium (42°C temperature) added over the bottom agar layer and allowed to incubate overnight. Compound treatments were applied by adding 1ml of 0.3% agar in growth medium (containing the compound) on top of the previously established agar layers. Cells incubated for one week were treated a second time (1 ml/well). After the second week of treatment, colony growth was measured by adding 0.3 ml nitro blue tetrazolium (0.5 mg/ml stock solution in PBS)(Sigma-Aldrich, St. Louis, MO). Viable colonies stained dark blue after 24-48 h of incubation. Digitally scanned plates were assessed for total stained area using ImagePro 4.0 software.
Cell cycle analyses- Subconfluent cells (0.5-2 million cells per 100 mm plate, 5 ml per plate) were allowed to incubate overnight. Compound treatments were applied by adding 50 µl per plate of compound in DMSO (final vehicle concentration, 1%). After 18 h, cells were fixed, stained for DNA with Propidium iodide, and analyzed on a Becton Dickinson FACSCalibur cell sorter. The data were quantitated using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Blood borne metastasis animal model. Athymic (nu/nu) female mice (Simonsen, Gilroy, CA), 6-8 weeks old, were inoculated i.v. (lateral tail vein) on day 0 with 1 x 10^6 LOX cells, suspended at 1 x 10^7 cells/mL in 10 mM glucose in PBS (calcium & magnesium-free). Groups (N=26) were dosed by oral gavage twice daily (12 h apart) with vehicle [20% (w/v) hydroxypropyl-β-cyclodextrin (HPBCD), Cavitron 82005, Cargill, Cedar Rapids, IA] or BX-320 dissolved in vehicle (20 mg/mL) adjusted to pH 4. The dose volume was 10 mL/kg (200 mg/kg), and dosing began 2-4 h before cell inoculation and continued until the end of the study. A small, but equal, number of animals died in the vehicle and BX-320 treated groups during the study, which was related to the dosing regimen and not to compound toxicity. Animals showed no observable side effects of compound treatment. On day 21 (22 days of dosing), mice were euthanized, and the lungs were removed and frozen for future DNA extraction. All experiments were conducted in accordance with the principles and procedures approved by the Berlex Animal Care and Use Committee. Statistical analyses were performed with Statview 5.0 (SAS Institute, Inc, Cary, NC). Significance was determined by the non-parametric Mann Whitney U test. P-values < 0.05 were considered significant.

Quantification of tumor burden by quantitative real time PCR (qPCR). We determined the tumor burden in the mouse lungs by extracting total DNA, then measuring the amount of the human CCR5 gene present by qPCR. To extract and purify total DNA, each lung was homogenized in 1 ml Qiagen ATL buffer (Valencia, CA) with a Mini-Bead Beater-8 (BioSpec) using Zirconia silicate beads (2.5 mm; BioSpec, Bartlesville, OK). We performed DNA extractions using the Qiagen QiaAmp DNA Mini Kit according the manufacturers instructions. For qPCR, we used the following human CCR5 specific primer set and probes: forward primer, 5’ CCTTCTAGTCTTTCTTCCAAAAGAC 3’; reverse primer, 5’ TCGGGAGCCTTTGCTG 3’; and probe, 5’ FAM-CCAAACGCTTCGCAAATGCTTTCTATT-TAMRA 3’. Thermocycling was performed for 40 cycles at 95°C for 15 s and 63°C for 1 min, following initial UNG treatment at 50°C for 2 min with subsequent UNG inactivation and TaqGold activation for 10 min at 95°C. A standard curve was generated using human genomic DNA (Promega) which was used to calculate the number of human cells per mouse lung, assuming 2 copies of the gene per diploid cell.

RESULTS

Identification of novel aminopyrimidine compounds that block PDK1 activity in vitro and in cells – From screening of compound libraries and lead optimization, we identified three potent and selective inhibitors of PDK1 termed BX-795, BX-912 and BX-320 which contain a common aminopyrimidine backbone (Fig. 1). The compounds were identified in a coupled assay measuring PDK1- and PtdIns 3,4-P2-mediated Akt activation, which could detect inhibitors of PDK1, AKT2, or other steps critical for activation of AKT2 (Fig. 2). We also found that the compounds potently inhibited PDK1 enzyme activity in a direct kinase assay format (IC50 values for BX-795, BX-912 and BX-320 were 11 nM, 26 nM and 30 nM, respectively), while failing to block pre-activated AKT2 activity (IC50 > 10 µM) (not shown). These data indicate that BX-795, BX-912 and BX-320 are direct inhibitors of PDK1.

We also performed kinetic studies indicating that BX-912, BX-320 and BX-795 are competitive inhibitors of PDK1 activity with respect to its substrate, ATP, suggesting that they bind to the ATP binding pocket of PDK1 (data not shown). To further investigate how the inhibitors bind to PDK1, we prepared crystals of the PDK1 kinase domain (residues 51-359), as described by Biondi et al. (30). We then introduced compounds
into PDK1 crystals and determined the structures of enzyme-inhibitor complexes by X-ray diffraction methods. These studies confirmed that BX-912 and BX-320 bind to the ATP binding site of PDK1. The structure of BX-320 in the active site of PDK1, shown in Fig. 3, reveals two hydrogen bonds between two aminopyrimidine nitrogens and the amino acid backbone of Ala162, which lies in the hinge region of the molecule. The aminopyrimidine backbone of BX-912 adopts a similar orientation in the active site of PDK1 (data not shown). Several published structures of aminopyrimidines bound to the active site of cyclin-dependent kinases 2 (CDK2) show an analogous mode of binding (31,32).

To examine the kinase selectivity of our PDK-1 inhibitors, we determined their effects on the in vitro activity of ten different Ser/Thr and tyrosine kinases, including the related AGC kinases PKA and PKCα. As shown in Table 1, BX-912, BX-320 and BX-795 displayed greater than 20-fold selectivity for PDK1 relative to the kinases in this panel, with one exception; BX-912 was 9-fold selective for PDK1 relative to PKA. BX-795 and BX-320 displayed significantly higher selectivity against PKA (140 and 35-fold, respectively).

BX-320, BX-795 and BX-912 block PDK1 activity in cells – We next characterized the effect of our compounds on the PI-3K/PDK1/Akt/S6K1 pathway in PC-3 prostate cancer cells. PTEN negative PC-3 cells display constitutive activation of Akt that is reflected in high levels of the PDK1 product, phospho-Thr308-Akt. PC-3 cells also express high levels of phospho-Thr389-S6K1, whose formation is dependent on PI 3-kinase and is thought to result from Akt signaling, although there are conflicting results in some situations (20,33,34).

We treated PC-3 prostate cancer cells with and without compounds for 18 h and then determined the level of phospho-Thr308-Akt and phospho-Thr389-S6K1 in cell extracts by Western blotting. As shown in Fig. 4A, BX-795 and BX-320 greatly reduced the amount of both phospho-Thr308-Akt and phospho-Thr386-S6K1. BX-compounds were as effective in blocking Akt and S6K1 phosphorylation as the PI 3-kinase inhibitor LY-294002 (100 µM) (Fig. 4A). We also saw similar effects after shorter times of compound treatment (2h or 15 min, not shown). Quantification of Western blot signals revealed that BX-795 inhibited both Thr308-Akt and Thr389-S6K1 phosphorylation with an IC₅₀ value of 300 nM, while BX-320 showed IC₅₀ values of 1-3 µM (Fig. 4B). The more potent cell-based activity of BX-795 is consistent with its lower IC₅₀ for inhibition of PDK1 activity in vitro. BX-912 displayed intermediate potencies (not shown). As a control, we stained Western blots for total Akt and S6K1 proteins. Compound treatment had no significant effect on Akt levels, while we observed a slight decrease in the level of total S6K1 protein that was too small to account for the dramatic loss of phospho-Thr389-S6K1 that we observed.

In addition to the phosphorylation of Thr308 within the activation loop by PDK1, full activation of Akt requires the phosphorylation of Ser473, which is located in the C-terminal hydrophobic motif. The kinase responsible for this modification has not been clearly identified. As shown in Fig. 4A, we analyzed the effect of BX-795 and BX-320 on phospho-Ser473 levels in extracts prepared from compound-treated PC-3 cells. Both compounds reduced the levels of phospho-Ser473, although the potency of this effect appeared to be lower than that for inhibition of phospho-Thr308-Akt levels (Fig. 4A). These data indicate that PDK1 activity is critical for phosphorylation of both Ser473 and Thr308 of Akt, at least in PC-3 cells. We cannot exclude the possibility, however, that the less potent inhibition of Ser473 phosphorylation displayed by BX-compounds resulted from their non-selective action on another kinase.

To further investigate the effect of BX-compounds on phosphorylation of Ser473-Akt, we examined the effect of BX-795 on Akt phosphorylation in IGF-1-stimulated PANC1 cells, which express high levels of AKT2 due to amplification of the Akt2 locus. As shown in Fig. 5, BX-795 effectively blocked IGF-1-stimulated phosphorylation of Akt at Thr308 (Thr309 in AKT2), but failed to inhibit the phosphorylation at Ser473 (Ser474 in AKT2). Indeed, BX-795 promoted a dose dependent stimulation of Ser473-Akt phosphorylation. Taken together, these data indicate that PDK1 can play a key role in Ser473-Akt phosphorylation, depending on the cell type and/or the signaling pathways activated. The phosphorylation of Ser473-Akt in PC-3 cells, which is promoted through loss of PTEN, requires PDK1 activity, while phosphorylation of Ser473-Akt in
PANC1 cells that is stimulated by IGF-1 appears to depend on other factors.

We also found that our PDK1 inhibitors blocked the phosphorylation of GSK-3β, which is another well-characterized target of Akt in cells. As shown in Fig. 6A, treatment of PC-3 cells with BX-320 or BX-795 greatly reduced the levels of phospho-Ser9-GSK3β detected by Western blotting. In contrast, BX-compound treatment caused little or no loss of total GSK3β protein levels in the cells. Treatment of PC-3 cells with LY-294002 also greatly reduced phospho-Ser9-GSK3β levels, consistent with its dependence on PI 3-kinase/PDK1/Akt signaling. Together with the data discussed above, our experiments demonstrate that BX-795 and BX-320 can block PDK1-mediated phosphorylation of Akt in PC-3 cells and inhibit signaling downstream of Akt.

We also investigated the effect of BX-320 and BX-795 on the phosphorylation of PDK1 at Ser241, which is an activation loop residue whose phosphorylation is required for PDK1 catalytic activity (35). Modification of Ser241 has been attributed to PDK1 auto-phosphorylation, since the site conforms to the consensus substrate recognition site for PDK1 and is found to be fully phosphorylated on PDK1 isolated from mammalian, insect, and bacterial cells (35).

Treatment of PC-3 cells with BX-compounds caused a reduction in the levels of phospho-Ser241-PDK1 (Fig. 4A). The potency of this effect was lower than for reduction of phospho-Thr308-Akt or phospho-Thr389-S6K1, however. Compound treatment also appeared to reduce the level of total PDK1 protein in PC-3 cells, although to a smaller extent than for the reduction of phospho-Ser241-PDK1 levels. As expected, LY-294002 had little effect on PDK1 phosphorylation (Fig. 4A), consistent with previous findings that PDK1 activation is not PI 3-kinase dependent. To confirm that the antibody we used was specific for phospho-PDK1 under our Western blotting conditions, we showed that treatment of samples with lambda phosphatase prior to their analysis eliminated the detectable signal for phospho-Ser241-PDK1 (data not shown). These data indicate that PDK1 inhibitors can block phosphorylation of the PDK1-activation loop residue Ser241, providing further support that this modification results from autophosphorylation.

The data also suggest that dephosphorylation of Ser241 in cells may lead to PDK1 instability, although this observation needs further confirmation.

Studies in vitro and with cells co-transfected with PKC-δ and PDK1 show that PDK1 can phosphorylate PKC-δ at Thr505 (19,25). This residue lies within the activation loop of PKC-δ, and its phosphorylation is required for formation of the catalytically active enzyme. We therefore examined whether our PDK1 inhibitors could reduce the levels of phospho-Thr505-PKC-δ in PC-3 cells. As shown in Fig. 6, phospho-Thr505-PKC-δ is detectable in non-stimulated PC-3 cells by Western blot using a phosho-specific antibody. Treatment with lambda phosphatase, however, eliminated most, but not all, of the phospho-Thr505-PKC-δ staining, while completely eliminating the detection of phospho-Thr398-S6K1 on Western blots. These data indicate that part of the phospho-Thr505-PKC-δ signal was not phospho-specific. Treatment of PC-3 cells with BX-795 and BX-320 reduced the amount of phospho-Thr505-PKC-δ we detected to roughly the background level established by phosphatase treatment, while not significantly affecting the total level of PKC-δ protein. The data show, therefore, that BX-compounds can inhibit the phosphorylation of Thr505-PKC-δ in PC-3 cells. We also observed a similar degree of inhibition in PC-3 cells after stimulation of Thr505-PKC-δ phosphorylation with TPA treatment (data not shown). It also appears that higher concentrations of BX-320 are needed to block PKC-δ phosphorylation than S6K1 phosphorylation. Further studies are needed to confirm this, however. Taken together, our data indicate PDK1 is responsible for the phosphorylation of Thr505-PKC-δ and the activation of this kinase in PC-3 cells.

PDK1 inhibitors selectively inhibit growth and/or promote apoptosis in tumor cells versus normal epithelial cells – As shown in Fig. 7, BX-320 blocked the growth of MDA-468 cells (IC\textsubscript{50} = 0.6 µM), which are PTEN negative breast tumor cells expressing high levels of activated Akt. The compound also promoted a 12-fold induction of caspase-3/7 activity after 48 h of treatment (IC\textsubscript{50} = 0.5 µM), indicating a strong pro-apoptotic response. Indeed, most of the treated cells were dead by 72 h. In contrast, treatment of normal
primary human mammary epithelial cells (HMEC) or primary prostate epithelial cells (PrEC) with BX-320 did not result in a detectable increase in caspase 3/7 activation. Consistent with these data, BX-320 had only weak inhibitory effects on HMEC and PrEC growth (IC<sub>50</sub> ~ 10 µM) that were roughly 20-30-fold less potent than that observed with MDA-468 cells. Similar results were seen with BX-912 (not shown). These experiments demonstrate that BX-compounds potently inhibit the growth of MDA-468 cells and promote a dramatic apoptotic response while showing selectivity against normal primary epithelial cells.

To further understand how BX-compounds inhibit MDA-468 cell growth, we measured the distribution of cells at different points in the cell cycle after treating asynchronously growing cultures of tumor cells for 18 h with 10 µM BX-912 or BX-320. Both compounds promoted a pronounced increase in the population of MDA-468 cells with 4N DNA content, indicative of a block at the G2/M phase of the cell cycle (Fig. 8).

As shown in Fig. 9A, BX-320 also potently inhibited the growth of HCT-116 colon cancer cells in culture (IC<sub>50</sub> = 0.37 µM). Like MDA-468 cells, HCT-116 cells are PTEN negative and constitutively activate the PI 3-kinase/PDK1/Akt signaling pathway. Unlike MDA-468 cells, however, treatment of HCT-116 colon cancer cells with BX-320 for up to 72 h blocked the growth of cells without inducing visual signs of apoptosis or an increase in caspase 3/7 activity. BX-320 blocked the growth of HCT-116 in soft agar with a potency (IC<sub>50</sub> = 0.28 µM) similar to that for inhibition of anchorage-dependent growth (Fig. 9B,C). BX-912 also potently inhibited the growth of HCT-116 cells in soft agar, showing a 96% inhibitory effect at a dose of 1 µM (data not shown).

**BX-compounds inhibit the growth of other cancer cell lines** – We examined the effect of BX-compounds on the anchorage-dependent and -independent growth of tumor cell lines derived from a variety of different cancers, including melanoma, glioblastoma and breast, prostate, cervical and pancreatic cancers, as shown in Table 2. Based on the studies discussed above, we focused on BX-320, which had the added advantage of being orally available in rodents (not shown). BX-320 displayed a wide range of potencies for inhibition of anchorage-dependent growth. In total, BX-compounds potently inhibited growth on plastic of six of the nine cell lines we tested (IC<sub>50</sub> < 2 µM). Two cell lines were resistant to growth inhibition by BX-compounds, namely, PC-3 prostate (IC<sub>50</sub> BX-320 = 11 µM) and U87-MG glioblastoma cells (no growth inhibition at 10 µM BX-320). MDA-453 cells showed intermediate sensitivity to the inhibition of anchorage-dependent growth by BX-320 (i.e., IC<sub>50</sub> = 3.6 µM).

We then used the same panel of 9 cell lines to examine the effect of BX-320 on the growth of tumor cell colonies in soft agar. BX-320 potently inhibited anchorage-independent growth of the entire panel (Table 2), including U87-MG and PC-3 cells, which were relatively insensitive to anchorage-dependent growth inhibition, as discussed above. For example, BX-320 blocked the growth of MDA-453, U87-MG and PC-3 cells in soft agar with IC<sub>50</sub> values of 0.093 µM, 0.3 µM and 0.4 µM, respectively. These potencies were >30-40 fold higher than what we observed for inhibition of growth on plastic. Similarly, BX-795 and BX-912 potently inhibited the growth of PC-3 cells in soft agar, displaying IC<sub>50</sub> values of 0.25 µM and 0.32 µM, respectively. Taken together, these studies demonstrate that compounds potently inhibited the growth of tumor cell lines in soft agar, while displaying a wide range of potencies for inhibition of anchorage-dependent growth of tumor cell lines. Three cell lines, PC-3 and U87-MG and MDA-453, which all express high levels of activated Akt, were strikingly more sensitive to growth inhibition in soft agar than on plastic. This observation is consistent with the PDK1 inhibitory activity of our compounds and the role established for the PDK1/Akt signaling pathway in producing critical survival signals in tumor cells that can prevent anoikis (36).

**BX-320 shows efficacy in a blood borne metastasis model** – We selected BX-320 for testing in an in vivo tumor model. Two h before injection of LOX melanoma cells into the tail vein of nude mice, we began oral dosing of animals with 200 mg/kg of BX-320, twice a day. After 21 days, we measured the tumor burden in the lung. As shown in Fig. 10, BX-320 significantly inhibited the growth of lung tumors in this model. These data indicate that BX-320 has efficacy in an in vivo tumor model, which may reflect an inhibition of
productive implantation of tumor cells in the lung, or an inhibition of subsequent tumor growth.

**DISCUSSION**

Signaling through a wide variety of cytokines, growth factors and adhesion molecules converges on the PI 3-kinase/PDK1/Akt signaling pathway, which plays a key role in regulating cancer cell growth, invasion, apoptosis, and tumor angiogenesis. Furthermore, the PI 3-kinase/PDK1/Akt signaling pathway is found to be highly activated in common cancers, including melanoma and hematological, breast, colon, pancreatic, gastric, prostate and ovarian cancers. These findings have sparked a keen interest in the development and testing of PI 3-kinase/PDK1/Akt pathway inhibitors as anticancer drugs. In this paper, we report the discovery and biological characterization of three potent small molecule inhibitors of PDK1: BX-912, BX-320, and BX-795. In addition to activation of Akt, PDK1 phosphorylates and promotes the activity of other AGC family kinases important in cancer progression, including PKC and S6K1. BX-compounds effectively blocked PDK-1 activity in PC-3 cells, as shown by their ability to block phosphorylation of S6K1, Akt, PKCδ, and GSK3β. The compounds also inhibited the proliferation of a wide variety of tumor cell lines. The compounds were particularly effective at blocking the growth of tumor cell colonies in soft agar. We also demonstrated that BX-320 is efficacious in blocking the growth of tumors in the lungs of nude mice after introduction of LOX tumor cells via the tail vein. These data indicate that PDK1 inhibitors may be clinically useful anticancer drugs.

We performed a number of studies investigating the effect of BX-compounds on targets of PDK1 action in cells. BX-treatment of PC-3 cells greatly lowered levels of phospho-Thr308-Akt, which is the best-characterized cellular product of PDK1. BX-treatment also blocked the activity of Akt, as reflected by lower levels of phospho-GSK3β, which is a downstream target of Akt. Phosphorylation of Thr389-S6K1 was also inhibited by BX-compounds, consistent with studies indicating that PDK1/Akt can promote phosphorylation of Thr389 through the TSC1/2/Rheb/mTOR signaling pathway (21). PDK1 is also thought to phosphorylate the activation loop of PKC family members, including conventional, novel and atypical PKCs, based on in vitro phosphorylation studies and experiments involving co-transfection of PDK1 with PKC isoforms in cells. Our studies showing that BX-compounds blocked the phosphorylation of Thr505-PKC-δ in PC-3 cells demonstrate that endogenous levels of PDK1 can be critical for PKC activation. Further studies are warranted to further delineate the role of PDK1 in the activation of various PKC isoforms in other cell types. While staurosporine and its analog UCN-01 have previously been shown to inhibit PDK1 (37), these compounds display low kinase selectivity. Therefore, our BX-compounds represent the first potent small molecule inhibitors of PDK1 that can be considered to be selective. Besides their potential use as anticancer drugs, our studies show that the compounds are useful tools to understand the role of PDK1 in normal and tumor cell biology.

BX-compounds block the growth of a wide range of tumor cell lines. We observed that some cell lines, such as U87 MG, PC-3 and MDA-453, were relatively resistant to growth inhibition on plastic, but were potently inhibited in soft agar, showing > 30 fold higher sensitivity to BX-320. These cells are PTEN negative and display high levels of Akt activation. Thus, their increased sensitivity to BX-320 when grown in soft agar may be caused by inhibition of the PDK1/Akt signaling pathway, which provides anti-apoptotic signals important for cell survival or growth, particularly in unattached cells. For example, activation of Akt was shown to be necessary to allow cells transfected with mutated and oncogenic Ras or BCR-Abl to grow as transformed colonies in soft agar (36,38). PTEN expression also induced anoikis in breast cancer cell lines (39).

Interestingly, we also found that our compounds reduced levels of phospho-Ser473-Akt in PC-3 cells, although at higher concentrations than needed to lower phospho-Thr308-Akt levels. These data suggest that PDK1, or a kinase activated through PDK1, can play a dominant role in Ser473-Akt phosphorylation in PC-3 cells. We cannot exclude the possibility, however, that BX-compounds inhibit other kinases in addition to PDK-1 that regulate Ser473 phosphorylation. This inhibition of Ser473 phosphorylation by BX-compounds was cell line dependent, since the BX-
compounds not only failed to inhibit phosphorylation of Ser473-Akt in IGF-stimulated PANC1 cells, but promoted a significant stimulation. Consistent with these results, we found that staurosporine, which potently inhibits PDK1 and many other kinases, reduced levels of both phospho-Thr308-Akt and phospho-Ser473-Akt in PC-3 cells, while promoting a dose dependent 5-fold stimulation of Ser473 phosphorylation in IGF-1-stimulated PANC1 cells (J. Wu and R. I. Feldman, unpublished data). Staurosporine was also reported to stimulate Ser473-Akt phosphorylation in HEK-293 cells (40). These results suggest that different kinases may be used to phosphorylate Ser473-Akt in different cell lines.

Such variation is also suggested from a number of seemingly conflicting studies reported in the literature. For example, PDK1-null embryonic fibroblasts demonstrate greatly reduced levels of Thr308-Akt phosphorylation, but exhibit wild-type levels of phospho-Ser473-Akt (28), while knockdown of PDK1 expression in U87-MG glioma cells by antisense oligonucleotide molecules efficiently blocked both Ser473 and Thr308-Akt phosphorylation (41). A number of different mechanisms for Ser473-Akt phosphorylation have been proposed that would depend on PDK1 activity (12-16,19,28), including Akt autophosphorylation, phosphorylation of Ser473-Akt by PDK-1 when in a complex with PRK2 in cells, and by PKC-α or PKCβII. In contrast, recent data indicated that the staurosporine-independent activity in HEK-293 cells that phosphorylates Ser473-Akt is DNA-dependent protein kinase (17). In this regard, we have found that our BX-compounds are not potent inhibitors of DNA-dependent kinase (D. Zhu and R. I. Feldman, unpublished observations). Clearly, further experiments are needed to address the mechanism of Ser473-Akt phosphorylation in different cell types and the direct or indirect role of PDK1 in this process. Our selective PDK1 inhibitors can be useful for these studies.

In summary, we have developed potent and selective inhibitors that block PDK1. These compounds inhibit the growth of a wide variety of tumor cell lines while showing distinctly lower potency for inhibition of normal breast and prostate epithelial cell growth. The most broadly characterized compound, BX-320, which displays oral availability in rodents, blocked growth of tumors in the lungs of nude mice at well tolerated doses, measured 4 weeks after the injection of LOX melanoma cells in the tail vein. These data support further pre-clinical studies evaluating the potential of PDK1 inhibitors as anticancer drugs.

REFERENCES


**FOOTNOTES**

* We gratefully acknowledge Angela Riffel for her analysis of lung tumor burden in our *in vivo* efficacy studies, Rhonda Humm and Jean McRobbie for assistance in cell culture and Wei Xia and Ronald Cobb for their production of recombinant proteins used in our studies. We also thank Gary Phillips for his helpful discussions and support.
Table 1 – Potencies of compounds for inhibition of PDK1 and other kinases in vitro

<table>
<thead>
<tr>
<th>Kinase</th>
<th>BX-912 IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>BX-95 IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>BX-320 IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold Selective</td>
<td>Fold Selective</td>
<td>Fold Selective</td>
</tr>
<tr>
<td>PDK-1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.012</td>
<td>0.006</td>
<td>0.039</td>
</tr>
<tr>
<td>Chck-1</td>
<td>0.83</td>
<td>0.51</td>
<td>0.82</td>
</tr>
<tr>
<td>PKA</td>
<td>0.11</td>
<td>0.84</td>
<td>1.4</td>
</tr>
<tr>
<td>PKC</td>
<td>1.25</td>
<td>9.3</td>
<td>5.7</td>
</tr>
<tr>
<td>GSK3β</td>
<td>7.4</td>
<td>0.62</td>
<td>4.0</td>
</tr>
<tr>
<td>CDK2/Cyclin E</td>
<td>0.65</td>
<td>0.43</td>
<td>1.5</td>
</tr>
<tr>
<td>EGFR</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Insulin Receptor</td>
<td>6.1</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>C-Kit</td>
<td>0.85</td>
<td>0.32</td>
<td>0.89</td>
</tr>
<tr>
<td>T-Fyn</td>
<td>2.1</td>
<td>6.4</td>
<td>&gt;10</td>
</tr>
<tr>
<td>KDR</td>
<td>0.41</td>
<td>1.1</td>
<td>1.4</td>
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</table>

<sup>1</sup>Coupled AKT2 activation assays were performed as described under “Materials and Methods.” Other kinase assays with purified recombinant enzymes used an ATP concentration at or below its Km value.

Table 2 – Potencies of compounds for inhibition of tumor cell growth on plastic or in soft agar

Growth assays were performed as described under Materials and Methods. IC<sub>50</sub> values are in µM.

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>BX-320 Soft Agar IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>BX-795 Soft Agar IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>BX-912 Soft Agar IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth (plastic) IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Growth (plastic) IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Growth (plastic) IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>MDA-468 (breast)</td>
<td>0.12</td>
<td>0.72</td>
<td>0.32</td>
</tr>
<tr>
<td>PC-3 (prostate)</td>
<td>0.40</td>
<td>0.25</td>
<td>0.32</td>
</tr>
<tr>
<td>U87-MG (glioblastoma)</td>
<td>0.30</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>MDA-453 (breast)</td>
<td>0.093</td>
<td></td>
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</tr>
<tr>
<td>HeLa (cervical)</td>
<td>1.2</td>
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<tr>
<td>HCT-116 (colon)</td>
<td>0.28</td>
<td>1.4</td>
<td>0.59</td>
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<tr>
<td>B16F10 (mu-melanoma)</td>
<td>0.55</td>
<td>1.8</td>
<td></td>
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<tr>
<td>LOX (melanoma)</td>
<td>0.31</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>MiaPaCa (pancreatic)</td>
<td>0.33</td>
<td>0.85</td>
<td>1.9</td>
</tr>
</tbody>
</table>
FIGURE LEGEND

Fig. 1. Compound structures for BX-795, BX-912, and BX-320.

Fig. 2. Potencies of compounds for inhibiting PDK1 kinase activity in vitro. BX-320 (○), BX-795 (□), and BX-912 (△) were assayed for inhibition of PDK1 mediated AKT2 activation and the direct inhibition of PDK1 as described under Materials and Methods. Representative assay data is shown in the figure. The IC₅₀ values for inhibition of the coupled PDK1/ AKT2 assay, averaged from N independent experiments, are BX-320, 39 nM (N= 12); BX-795, 6 nM (N= 2); BX-912, 12 nM (N= 14). The average IC₅₀ values for inhibition of the direct PDK1 assay are BX-320, 30 nM (N = 5); BX-795, 11 nM (N = 2); BX-912, 26 nM (N = 6).

Fig. 3. Structure of BX-320 bound to the catalytic domain of PDK1. We crystallized the PDK1 catalytic domain (residues 51-359) as described by Biondi et al. (30). BX-320 was introduced into PDK1 crystals and the structure determined from X-ray diffraction data to a resolution 2.17 angstroms. As shown, BX-320 binds in the ATP binding pocket, making two hydrogen bonds through aminopryimidine nitrogens to Ala162 located in the hinge region.

Fig. 4. BX-compounds block phosphorylation of PDK1 targets in PC-3 cells, including phospho-Thr308-Akt and phospho-Thr389-S6K1. We treated PC-3 cells with BX-795 and BX-320 for 18 h and analyzed their effects on a number of PDK1 targets using Western blotting, as described under Materials and Methods. A, Shown are levels of phospho-Thr308-Akt, phospho-Ser473-Akt, phospho-Thr389-S6K1, and phospho-Ser241-PDK1. Also shown, as controls, are levels of total (non-phosphorylated) Akt, S6K1, and PDK1. B, We quantified the effects of BX-795 and BX-320 on levels of phospho-Thr308-Akt and phospho-Thr389-S6K1 as described under Materials and Methods. Shown are the averages from six experiments (± standard error of the mean).

Fig. 5. BX-320 inhibits phosphorylation of Thr308-Akt and Thr389-S6K1 in IGF-1-stimulated PANC1 cells, while promoting Ser473-Akt phosphorylation. PANC1 cells were serum-starved overnight and treated with vehicle or various concentrations of BX-320, followed by stimulation of the cells with IGF-1. After 15 minutes, cell extracts were prepared and analyzed for levels of phospho-Thr308-Akt (○), Thr389-S6K1 (△), or Ser473-Akt (□) by Western blotting. LY294002 (100 µM) inhibited all three phosphorylation products to baseline levels (not shown). Displayed in the Figure are relative levels of phosphoproteins quantified by Western blotting as described under Materials and Methods.

Fig. 6. PDK1 inhibitors inhibit the phosphorylation of Thr505-PKCδ and Ser9/21-GSK3β in PC-3 cells. A, We treated PC-3 cells with BX-795 and BX-320 for 18 h and analyzed their effects on phospho-Thr505-PKCδ and total PKCδ, and phospho-Ser9/21-GSK3β and total GSK3β levels by Western blotting, as described under Materials and Methods. Also shown, as a control, is the effect of compounds on phospho-Thr389-S6K1. The sample in lane 2 (labeled Ptase) was treated with lambda-phosphatase prior to analysis to demonstrate the specificity of phosphorylation-specific antibodies. B, The effect of compound treatments on phosphoThr505-PKCδ levels (from A) were quantified as described under Materials and Methods. The data show that the BX-compounds lowered phosphoThr505-PKCδ levels to that of the phosphatase treated controls.

Fig. 7. BX-320 inhibits the growth of MDA-468 breast cancer cells and induces apoptosis. A, The proliferation of MDA-468 and primary epithelial cells (HMEC and PrEC cells) was measured after treatment with BX-320 at the concentrations indicated for 72 h, as described under Materials and Methods. B, We measured the induction of apoptosis in MDA-468, HMEC, and PrEC cells treated with BX-320 for 48 h, as described under Materials and Methods. Results are the averages of duplicate measurements ± standard error.
Fig. 8. PDK1 inhibitors promote a block at G2/M phase of the cell cycle. A, Proliferating MDA-468 breast cancer cells were treated for 18 h with 10 µM BX-912 or BX-320 and subjected to cell cycle analysis by FACS as described under Materials and Methods.

Fig. 9. BX-320 inhibits the growth of HCT-116 colon cancer cells. A, BX-320 inhibits the growth of HCT-116 colon cancer cells on plastic and induces apoptosis. B and C, BX-320 inhibits the growth of HCT-116 colonies in soft agar. Cells were treated with compound for 14 days as described under Materials and Methods. In C, the effects of BX-320 on the growth of HCT-116 in soft agar were quantified as described under Materials and Methods. Results are the averages of duplicate measurements ± standard error.

Fig. 10. BX-320 blocks the growth of LOX melanoma tumors in the lungs of nude mice. LOX melanoma cells were introduced into the tail vein of nude mice, and tumors were measured as described under Materials and Methods. The results represent the average of 26 animals per group +/- the standard deviation. The P value (0.0016) was determined by the non-parametric Mann-Whitney U test.
Figure 1

BX-795  BX-912  BX-320
Figure 3
Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>BX-795</th>
<th>BX-320</th>
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<tbody>
<tr>
<td>μM:</td>
<td>0</td>
<td>.1</td>
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<tr>
<td>P-Thr308-Akt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-Ser473-Akt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Akt</td>
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<tr>
<td>P-Thr389-S6K1</td>
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<tr>
<td>Total S6K1</td>
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<td>P-Ser241-PDK-1</td>
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<tr>
<td>Total PDK-1</td>
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</table>

B

[Graphs showing the percent of control level vs. Log [BX-795], M and Log [BX-320], M for P-Thr389-S6K1 and P-Thr308-Akt]
Figure 5

![Graph showing relative phosphoprotein level vs. IGF-1 + increasing BX-320 (μM). Lines represent P-S473-Akt, P-T389-S6K1, and P-T308-Akt.]
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>Veh</th>
<th>Ptase</th>
<th>LY</th>
<th>BX-795</th>
<th>BX-320</th>
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<tr>
<td><strong>μM</strong></td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>30</td>
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<tr>
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<tr>
<td>P-Thr389-S6K1</td>
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<tr>
<td>P-Ser9-GSK3-β</td>
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<td>GSK3-β</td>
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<td></td>
</tr>
</tbody>
</table>

B

![Graph showing P-Thr505-PKC-δ (as % of control) for different conditions](image-url)

- Vehicle
- Phosphatase treated
- LY-294002
- BX-795
- BX-320
Figure 7

A

% growth inhibition

Cont  -7.5  -7  -6.5  -6  -5.5  -5  -4.5

log [BX-320], M

- MDA-468
- HMEC
- PrEC

B

Fold apoptosis induction

Cont  -8  -7  -6  -5  -4

log [BX-320], M

- MDA-468
- HMEC
- PrEC
Figure 8

MDA-468

vehicle

10 μM BX-912

10 μM BX-320

2N  4N
Figure 9

A

Growth Inhibition

% Inhibition of growth

Cont -8 -7 -6 -5

log [BX-320], M

Fold-induction Apoptosis

B

Vehicle

0.3 μM BX-320

0.1 μM BX-320

0.3 μM BX-320

1.0 μM BX-320

C

% Inhibition of growth

Cont -7 -6 -5

log [BX-320], M
Figure 10

LOX cells in the lung (X 100,000)

Vehicle

BX-320

**P Value 0.0016
Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1 (PDK1)
Richard I. Feldman, James M. Wu, Mark A. Polokoff, Monica J. Kochanny, Harald Dinter,
Daguang Zhu, Sandra L. Biroc, Bruno Alicke, Judi Bryant, Shendong Yuan, Brad O.
Buckman, Dao Lentz, Mike Ferrer, Marc Whitlow, Marc Adler, Silke Finster, Zheng Chang
and Damian Arnaiz

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