Targeting PTEN using small molecule inhibitors

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Abstract

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is well known as a tumour suppressor. It's PI(3,4,5)P_3 lipid phosphatase activity is an important counteracting mechanism in PI3K (phosphoinositide 3-kinase) signalling. Furthermore, PTEN lies upstream of Akt kinase, a key enzyme in insulin signalling regulating glucose uptake and cell growth. Therefore, PTEN has recently gained attention as a valuable drug target for the treatment of diabetes, stroke, cardiac infarct and fertility. This review summarizes the use of small molecules as PTEN inhibitors. Currently available methodologies and techniques for accessing PTEN inhibition *in vitro* and *in cellulo* will be discussed.

Keywords: PTEN, inhibition, Vanadium compounds, OMFP, VO-OHpic

1 Introduction

The phosphatidylinositol (PI) 3-phosphatase PTEN has been initially characterised as tumour suppressor gene with a putative tyrosine phosphatase domain [1], but has since then proven to be a dual specificity phosphatase having inositol lipids as substrates [2]. PTEN is dephosphorylating 3-phosphorylated inositol lipids, counteracting the action of PI3 kinases by removing the "second messengers" created by the action of growth factors, cytokines and cell survival stimulators [2-4]. Chemical intervention tools, such as wortmannin [5], LY294002 [6] or theophylline [7] facilitated the discovery of new chemical inhibitors, with all chemical intervention tools having a tremendous impact on the elucidation of the role of PI 3-kinases in disease and cancer development [8, 9]. PTEN was discovered much later than the PI 3-kinases, and the first PTEN inhibitors were developed in due course a decade ago exploiting the dual specificity nature of the PTEN phosphatase. Bisperoxovanadium compounds were known tyrosine phosphatase inhibitors, which showed a remarkable potency for PTEN *in vitro* and *in vivo* [10]. However, these inhibitors are rather promiscuous targeting PTEN in the nanomolar dosage range as well as other phosphatases containing the CX5R active site motif, whereas the 2nd generation PTEN inhibitor VO-OHpic is fairly specific

for PTEN *in vitro* and *in vivo* [11]. These vanadium-based PTEN inhibitors are currently employed in cellular and animal investigations with some recent applications being listed in Table 1.

Given its role as a tumour suppressor it is not surprising that PTEN has been implicated as a biomarker for certain cancers, in particular those affecting the uterus, brain, skin and prostate [12-14]. A complete loss of this enzyme triggers PTEN induced cellular senescence (PICS) via a p53 dependent mechanism [15]. In contrast, a partial loss of PTEN such as in PTEN (+/-) cells will not be sufficient to counteract the elevated PI 3-kinase activities in cancers, but at the same time will fail to trigger PICS. The consequence of this is a loss of tumour suppression in cancer cells carrying only one active copy of PTEN (haploid insufficiency). Thus, PTEN inhibitors might therefore not just be important research tools, but may be the first steps towards a future drug discovery programmes for PICS based cancer therapy.

Taken these observations together it is important to understand how the properties of the PTEN inhibitors will affect the determination of PTEN activities *in vitro* and *in vivo*. Here, we summarise the current experimental knowledge about the application of PTEN inhibitors, with particular reference to the choice of substrates and the assay conditions.

2 Measuring PTEN inhibition in vitro

2.1 Choices of substrates

PTEN is a dual-specific phosphatase belonging to the family of protein tyrosine phosphatases (PTP) due to its signature active site motif CX5R, yet several studies have indicated that PTEN has rather poor enzymatic activity towards synthetic peptides and para-Nitrophenylphosphate (pNPP). It has therefore been proposed, that PTEN is unlikely to be a classical PTP utilising protein substrates *in vivo*, with the most likely physiological substrate being $PI(3,4,5)P_3$, a key player in cell growth signalling [16]. A comparison of kinetic parameters of PTEN towards various substrates (Table 1) reveals a ~3-fold lower K_M for $PI(3,4,5)P_3$ and a 10 fold higher Vmax than its soluble headgroup IP_4 and ~800-fold lower Vmax than pNPP, demonstrating that *in vitro* $PI(3,4,5)P_3$ is as well the preferred substrate. Another substrate that has been tested is 3-O-Methylfluorescein phosphate (OMFP), an artificial substrate also suitable for phosphatases of the PTP family. The use of OMFP over pNPP has two advantages. Firstly, OMFP can be used both as a fluorogenic or colorimetric substrate, whereby the fluorescence detection is preferred due to higher sensitivity. Secondly, PTEN shows a ~100 times higher affinity towards OMFP (K_M of 216 ± 14 μM) as compared to pNPP (K_M of 25.6 mM).

While it is apparent that the substrate of choice for PTEN inhibition studies is $PI(3,4,5)P_3$, OMFP is a reliable substrate alternative for PTEN inhibition studies [37], which has the advantage of continuously monitoring the progress of the enzyme activity. In contrast, the $PI(3,4,5)_3$ assay is an endpoint determination of the phosphate released by the enzymatic activity of PTEN. OMFP is therefore suitable for high-throughput screening of compound libraries, which can be complemented with assays employing the physiological substrate $PI(3,4,5)P_3$ in order to validate any hits.

2.2 PTEN inhibition assay

As mentioned above, $PI(3,4,5)_3$ is the substrate of choice when determining PTEN activities, but it is also more cumbersome than assaying PTEN using OMFP. To measure PTEN activity using $PI(3,4,5)_3$ as a substrate the release of inorganic phosphate will be determined by adding molybdate under strong acidic conditions (section 4.1), which also denature the enzyme and thus terminate the reaction (endpoint assay). The resulting complex of phosphomolybdate can react with malachite green creating a corresponding colour change. Lipid substrates like $PI(3,4,5)_3$ or other phosphoinositide can be presented as mixed lipid-detergent micelles, with Octyl β-D-glucopyranoside (OG) being a good choice due to its low trace amounts of phosphate. The latter is an important consideration, as some detergents will create considerable backgrounds that impact on the overall suitability of the assay.

As previously mentioned (see Table 1), several vanadium based compounds are currently used to inhibit PTEN. The two main groups of PTEN inhibitors consist of bisperoxovanadium (V) compounds with various ligands and VO-OHpic with vanadium in the oxidation state of IV which were tested for their selectively. Kinetic analysis in the presence of bpVpic (vanadium V) and VO-OHpic were studied using the enzymes PTEN, PTP1B and SopB, which contain the signature motif CX5R characteristic of the PTP family. Although sharing the same active site motif, each enzyme has a unique substrate preference. PTP1B for example, described in the literature as valuable type 2 diabetes target, is a classical PTP acting on proteinaceous substrates [17]. SopB on the other hand, is a bacterial virulence factor with phosphoinositide phosphatase activity which has the capability to hydrolyse all seven natural occurring phosphoinositides [18, 19] . We performed a kinetic study of these three enzymes in the presence of bpVpic and VO-OHpic to obtain IC50 values using OMFP as substrate. As shown in table 3, compound bpVpic inhibits all three tested enzyme with similar potency with IC50s in the range of 30 to 40 nM. In contrast VO-OHpic, shows specificity towards PTEN with an IC50 of 39.6 ± 2.1 nM. The IC50s for PTP1B is ~20-fold higher and ~13-fold higher for SopB as compared to PTEN. Taken all this observations together, VO-OHpic seems to be the choice of compounds, if PTEN is to be selectively inhibited, which is in agreement with our earlier investigation [11].

EDTA has been routinely use in the literature to assay PTP activity as well as probing for reversibility of potent inhibitors [20]. Interestingly, if EDTA is included in the assay solution, the inhibition of all three tested enzymes by VO-OHpic is fully abolished. EDTA is a known for its strong metal chelating characteristics. It is therefore likely, that EDTA chelates the vanadium within the VO-OHpic complex thus destroying the VO-OHpic complex. In contrast, the bisperoxovanadium bpVpic seems to withstand the presence of EDTA as similar IC50s were obtained with and without EDTA. Since EDTA is routinely used in inhibitor screening for PTPs, it is of importance to validate the effect of EDTA especially if metal-based compounds are screened, which can affect compound stability and activity.

3 Measuring PTEN inhibition *in cellulo*

One way to measure PTEN inhibition *in cellulo* is to monitor the status of downstream effectors of PTEN-dependent signalling, such as the protein kinase Akt, also known as protein kinase B. Akt kinase plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration [21]. The activity of Akt is regulated by two

phosphorylation sites, namely serine 473 (Ser473) and threonine 308 (Thr308). Upon activation of PI3 kinase, the phosphoinositide-dependent kinase 1 (PDK1) is translocated to the plasma membrane by binding to $PI(3,4,5)P_3$. PDK1, a serine-threonine kinase, phosphorylates Akt in a $PI(3,4,5)P_3$dependent manner at Thr308. To become fully activated, Akt requires phosphorylation at serine 473 (Ser473), which is phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) [22]. Through counteracting PI3 kinase, the Akt signalling is terminated by the action of PTEN in dephosphorylating $PI(3,4,5)P_3$. Although, the inositol-5-phosphatase SHIP1 and SHIP2 are capable of dephosphorylating $PI(3,4,5)P_3$ at the D5 position, it has been shown that PTEN is mainly responsible for attenuating PI3 kinase signalling, making Akt phosphorylation a *bona fide* readout for PTEN activity *in cellulo* [23] .

As can be seen in Figure 1 the phosphorylation levels of Akt at Thr308 as well as Ser473 increases in the presence of PTEN inhibitor VO-OHpic. An activation at a concentration of 50 nM can already be detected which is in good agreement with the known *in vitro* VO-OHpic potency [11, 24], demonstrating the good cell permeability of this inhibitor. The fact that 50 nM of VO-OHpic is sufficient to exert cellular effects also means that the chance of hitting other phosphatases can be reduced to a minimum as the determined IC50s for other Cx5R phosphatases is at least 10-fold higher as shown in table 3.

In order to detect PTEN dependent activation of Akt, it is crucial to starve cells to bring PI3 kinase activity to a very low basal level, without completely blocking it. PI3 kinase generates the substrate for PTEN and thus needs to be active, but too much PI3 kinase activity would overshadow the effect PTEN inhibition might have on the overall dynamic balance between $PI(4,5)P_2$ and $PI(3,4,5)P_3$. Therefore, starved cells are stimulated with a low dose of growth factors in order to activate sufficient amounts of PI3 kinase, which in turn will generate small $PI(3,4,5)P_3$ levels that can be removed by the PTEN, leaving enough $PI(3,4,5)P_3$ to just activate Akt kinase above the baseline. The dose of growth factor (e.g. insulin) needs to be determined prior to experiments on PTEN inhibition in the cellular environment. It is important to use a concentration in the low linear phase of a dose-dependent activation of Akt kinase, since this will provide the necessary dynamic range to detect changes in Akt activation. As shown in figure 1, low insulin will generate a low basal level of Akt activation that can be increased by adding the PTEN inhibitor. Whereas high level of insulin (see Figure 2) will result in such high cellular levels of $PI(3,4,5)P_3$ that will not be significantly affected by inhibiting PTEN. The activation of Akt due to high insulin doses is almost saturated, and thus are not any more limited by PTEN's counteraction against the insulin dependent activation of PI 3kinase. It is therefore of utmost importance to determine the concentration of growth factor that generates an Akt kinase activation well below the saturation phase.

The activity of PTEN *in vivo* can also be measured by quantifying cellular $PI(3,4,5)P_3$ levels upon treatment with PTEN inhibitors. Lipids can be extracted and then analysed using lipid-binding protein domains to detect phosphoinositides [25]. The PLC-δ1 PH domain for example, has been extensively used to detect $PI(4,5)P_2$ in fluorescence microscopy techniques either on live or post-fixation cells.

With respect to $PI(3,4,5)P_3$ binding domains the Akt and GRP1 PH domains are employed the most [26]. The GRP1 PH domain is known to be highly specific for $PI(3,4,5)P_3$ whereas the Akt PH

domain is known to bind PI(3,4)P_2 with similar affinity. The GRPH1 PH domain has been used as a probe to visualize PtdIns(3,4,5)P_3 in cells by live imaging as well as in electron microscopy [27].

The amount of cellular PI(3,4,5)P_3 levels can be detected using the protein-lipid overlay assay once the lipid has been extracted from the cells. Figure 3 shows the result of a protein-lipid overlay assay, after treating NIH3T3 with indicated concentrations of VO-OHpic. As shown the amount of PI(3,4,5)P_3 detected upon treatment with increasing concentration of VO-OHpic is in good agreement with the activation level of phospho-Akt (Figure1), making the protein-lipid overlay assay a good alternative readout when accessing PTEN inhibition in a cellular environment. There are possibilities to use microscopy methods for testing PTEN inhibitors, which would add spatial resolution to the general inhibitory data [28]. While the general precautions concerning the basal activation levels of Akt phosphorylation (see above) will apply also to any cellular imaging technique, the choice of effector being monitored as well as the cell type will have a substantial influence on the optimal conditions for monitoring PTEN dependent effects on cellular function.

The results shown here summarize some recent methodologies and techniques in accessing PTEN inhibition by small molecule *in vitro* and *in vivo*. Two different PTEN activity assays are described to measure PTEN activity/inhibition in vitro using purified enzyme. Each of the two PTEN activity assay described here has their advantage. While the PTEN assay with OMFP substrate offers a simple, on-line recording assay with high-throughput capability, the malachite green assay employs the physiological substrate PI(3,4,5)P_3. In a cellular environment, the inhibitory effect of small molecules on PTEN can be measured by detecting downstream effectors of the PTEN signalling, namely by detecting the activation of the PI(3,4,5)P_3 –dependent protein kinase Akt. Alternatively, the detection of changes in cellular PI(3,4,5)P_3 levels upon PTEN inhibition by the protein-lipid overlay assay can also be employed to measure PTEN inhibition.

4 Experimental procedures for measure PTEN inhibition *in vitro* and *in cellulo*

Methods

Materials:

- Recombinant active PTEN phosphatase (Expressed as GST-fusion in E.coli)
- Tris-HCl (#93363, Sigma-Aldrich)
- DL-Dithiothreitol (DTT) (#43815, Sigma-Aldrich)
- PI(3,4,5)P_3, diC16-Na (#208, CellSignals)
- I(1,3,4,5)P_4, (#501, CellSignals)
- 4-nitrophenyl phosphate (pNPP), (#71768, Sigma-Aldrich)
- 3-O-Methylfluorescein phosphate (OMFP), (M2629, Sigma Aldrich)
- 3-O-Methylfluorescein (OMF), (BIM0143, Apollo Scientific)

- Octyl β-D-glucopyranoside (OG), (#494459, Calbiochem)
- Malachite Green oxalate salt, (#M6880, Sigma-Aldrich)
- Ammonium molybdate tetrahydrate, (#09878, Sigma-Aldrich)
- Bismuth(III) citrate, (#480746, Sigma-Aldrich)
- Hydrochloric acid 37%, (#320331, Sigma-Aldrich)

4.1 Inhibition assay of PTEN using PI(3,4,5)P_3 as substrate

1. Malachite green dye preparation: A 2x concentrated malachite green reagent is prepared by dissolving 5 mM malachite green, 17 mM ammonium heptamolybdate, 77 mM bismuth citrate and 1.7 M HCl in distilled water. The mixture is stirred overnight in the dark and filtered next day. A calibration curve for inorganic phosphate can be obtained using KH_2PO_4.
2. PI(3,4,5)P_3 substrate preparation: To prepare a 300 µM(10x stock) substrate solution, PIP3 is dissolved in MilliQ grade distilled water with 1% (w/v) Octyl β-D-glucopyranoside. The solution is mixed briefly and sonicated in a bench top bath sonicator for 10 min. After sonication, the substrate solution is kept on ice until use.
3. VO-OHpic inhibitor preparation. For inhibition studies, VO-OHpic is prepared as a 10x stock solution in 10% DMSO at concentration ranging between 10 nM to 10 µM.
4. Enzyme activity of PTEN is measured in 100 mM Tris (pH 7.4) containing 0.5 mM DTT. For a 100 µL reaction, 80 µL of the PTEN mixture containing 100 mM Tris and 0.5 mM DTT is pre-incubated with 10 µL of the 10x stock solution of VO-OHpic into a 96-well plate. For the control reaction. Pre-incubate at room temperature for 10min. After 10 min, add 10 µL of the 10x substrate stock solution to the control and reaction wells. Incubate the reaction for 20min at 37°C.
5. After reaction time, stop the reaction by adding 100 µL of malachite green dye to the reaction and the control well. Add 80 µL of PTEN mixture to the control well. Let the dye to develop for 10 min at room temperature. Read the absorbance at 625 nm.
6. Calculate the PTEN activity by subtracting the relevant blanks.

7. Use standard curves to transform activities from OD units to molar turnover.

4.2 PTEN inhibition assay with OMFP as substrate

1. OMFP substrate preparation: The cyclohexylammonium salt of 3-O-Methylfluorescein phosphate (OMFP) is dissolved in DMSO to a concentration of 20 mM. Sonication might be required to accelerate the process. DMSO stock solutions of OMFP can be stored at -20°C in the dark. For inhibition assays, a 10x stock solution is prepared by diluting OMFP to a concentration of 2 mM containing 10% DMSO.
2. The enzyme activity of PTEN is measured in 100 mM Tris (pH 7.4) containing 0.5 mM DTT. For a 100 µL reaction, 80 µL of the PTEN mixture containing 100 mM Tris and 0.5 mM DTT is pre-incubated at room temperature with 10 µL of the 10x stock solution of PTEN inhibitor using a 96-well plate. After 10 min, reactions were initialised by adding 10 µL of the 10x substrate stock solution (OMFP) to the PTEN-buffer mixture. The conversion of OMFP to OMF by PTEN will cause a corresponding change in fluorescence units (FU) in the 96-well microtiter plate (excitation at 485 nm and emission at 525 nm), which can be used to monitor PTEN activity continuously.

4.3 Cellular PTEN inhibition assay

Materials

1. Cell line: NIH 3T3 mouse fibroblasts (or other PTEN expressing cell lines)
2. Dulbecco's Modified Eagle's Medium, high glucose (DMEM) (D5671, Sigma-Aldrich) supplemented with 10% newborn calf serum (ATCC)
3. Dulbecco's Modified Eagle's Medium, low glucose (DMEM) (D6046, Sigma-Aldrich)
4. Phosphate buffer saline (PBS) (Gibco, Life Technologies)
5. Trypsin-EDTA solution (T4174, Sigma-Aldrich)

6. Insulin (I6634, Sigma-Aldrich). Prepare a 1mg/mL solution in 0.02 M HCl, filter-sterilised and store in aliquots at -20°C.
7. PTEN inhibitor VO-OHpic at final concentration ranging between 50 nM and 1 μM.
8. 1 x SDS sample buffer: 62.5 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 5% β-mercaptoethanol, 10 % glycerol
9. Phospho-Akt (Ser473) antibody (Cell Signaling)
10. Phospho-Akt (Thr308) antibody (Cell Signaling)
11. Akt antibody (Cell Signaling)
12. Tris-buffered saline with 0.1% Tween 20 (TBST): 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20 (v/v).

Method

1. NIH 3T3 mouse fibroblast are grown in DMEM, high glucose with 10% newborn calf serum and passaged when required (around 80-90% confluency) using trypsin/EDTA to maintain cultures. For PTEN inhibition experiment passage cells 1 in 10 into 60 mm dishes. Grow cells until 70-80% confluency.
2. Wash cells twice with PBS and starved in low glucose DMEM without serum for 16 hours.
3. After starvation, wash dishes with PBS and add fresh starvation medium.
4. Add PTEN inhibitor and solvent control to dishes and incubate in a CO_2 incubator for 30 min.
5. Add insulin to a final concentration of 0.02 μg/mL and incubate for 10 min.
6. Remove media and wash cells twice with ice-cold PBS.
7. Add 300 μL of 1x SDS lysis buffer to each dish and scrape cells of into a 1.5 mL centrifuge tube.
8. Boil sample 94°C for 5 min and cool on ice.
9. Run SDS gel electrophoresis and transfer the protein onto a nitrocellulose membrane according to manufacturer's instructions.

10. Block the membrane using a 5% milk powder solution in TBST for 1 hour at room temperature.
11. Wash the membrane twice with TBST for 5 min each.
12. Incubate the membrane with a 1:1000 dilution of phospho-Akt antibody or pan-Akt antibody in TBST containing 5% BSA at 4°C for 16 hours.
13. Wash membrane 3 times for 10 min with TBST.
14. Incubate membrane with appropriate secondary antibody according to manufacturer's instructions.
15. Wash as in step 13. Visualise western blot using ECL solution according to manufacture specification.

4.4 Protein-lipid overlay assay for detecting cellular PI(3,4,5)P3 levels upon PTEN inhibition

Materials

1. Cell line: NIH 3T3 mouse fibroblasts (or other PTEN expressing cell lines)
2. Dulbecco's Modified Eagle's Medium, high glucose (DMEM) (D5671, Sigma-Aldrich) supplemented with 10% newborn calf serum (ATCC)
3. Dulbecco's Modified Eagle's Medium, low glucose (DMEM) (D6046, Sigma-Aldrich)
4. Phosphate buffer saline (PBS) (Gibco, Life Technologies)
5. Trypsin-EDTA solution (T4174, Sigma-Aldrich)
6. Insulin (I6634, Sigma-Aldrich). Prepare a 1mg/mL solution in 0.02 M HCl, filter-sterilised and store in aliquots at -20°C.
7. PTEN inhibitor VO-OHpic
8. Recombinant GRP1 PH domain (Expressed as GST-fusion protein in our laboratory)
9. Anti-GST HRP conjugated antibody
10. ECL reagent or similar

11. Hybond-C extra nitrocellulose membrane (GE Healthcare)
12. Chloroform
13. Methanol

Methods

1. NIH 3T3 mouse fibroblast are grown in DMEM, high glucose with 10% newborn calf serum and passaged when required (around 80-90% confluency) using trypsin/EDTA to maintain cultures. For PTEN inhibition experiment passage cells 1 in 10 into 100 mm dishes. Grow cells until 70-80% confluency.
2. Wash cells twice with PBS and starved in low glucose DMEM without serum for 16 hours.
3. After starvation, wash dishes with PBS and add fresh starvation medium.
4. Add PTEN inhibitor and solvent control to dishes and incubate
5. Add insulin to a final concentration of 0.05 µg/mL and incubate in a CO_2 incubator for 30 min.
6. Remove media and wash cells twice with ice-cold PBS.
7. Add 5 mL of 0.5 M ice-cold TCA to the dish and scrape cells of into a 15 mL centrifuge tube.
8. Centrifuge sample at 1500 rpm for 10 min and discard the supernatant.
9. Wash the pellet with 2 mL of 5% TCA/ 1 mM EDTA. Centrifuge at 1500 RPM for 10 min and discard the supernatant.
10. To extract PIP3 add 750 µL of Methanol:Chloroform:H2O (80:40:1) and incubate at room temperature for 15min. Vortex in between.
11. Centrifuge for 10 min at 1500 rpm and transfer supernatant in a 1.5 mL centrifuge tube.
12. Add 250 µL of Chloroform and 450 of 0.1 M HCl to induce phase split. Incubate sample for 10 min at room temperature and vortex every 2 min. Centrifuge at 1500 rpm for 10 min and collect lower phase into a new centrifuge tube. Dry down lipids and re-suspend into 20 µL of a solution of methanol:chloroform:water (2:1:0.8) and vortex briefly.

13. Spot a total of 5 µL of lipid extracts for every studied condition onto a Hybond C nitrocellulose membrane. Spot 1 µL at a time, let the membrane dry and add another 1 µL up to a total of 5 µL.
14. Let the membrane dry at room temperature for 1 hour.
15. Block the membrane with 2mg/mL of fatty acid free BSA in TBST for 1 hour at room temperature.
16. Incubate the membrane with 10-50 nM of GRPH1 PH domain for 16 hours at 4°C in blocking buffer.
17. Wash the membrane 5 times 10min with TBST.
18. Incubate the membrane with a 1:5000 dilution of anti-GST-HRP conjugated antibody for 1 hour at room temperature in blocking buffer.
19. Wash as in 17.
20. Visualise the membrane using ECL reagent and chemiluminescence reader.

Figure captions

Figure 1 NIH 3T3 cells were starved overnight, incubated with indicated concentration of VO-OHpic for 30 min, followed by insulin stimulation as indicated. Cells were collected and analysed by western blotting using phospho-Akt (Thr308 and Ser473) and total Akt antibodies. The blot in Figure 1 (top panel) is representative of two independent repeats. The resulting blot intensities were quantified using ImageJ where high insulin stimulation (10 µg/mL) was set to 100% (bottom panel). Error bars shown are the standard error from the two independent experiments.

Figure 2 NIH 3T3 cells were starved overnight, incubated with indicated concentration of VO-OHpic for 30 min, followed by insulin stimulation as indicated. Cells were collected and analysed by western blotting using phospho-Akt (Thr308 and Ser473) and total Akt antibodies. The blot in Figure 1A was quantified using ImageJ (http://imagej.nih.gov/ij/) where high insulin stimulation (10 µg/mL) was set to 100%. Blot shown is representative of two independent experiment.

Figure 3 Protein-lipid Overlay assay to detect PI(3,4,5)P_3 from VO-OHpic treated NIH3T3 cells. NIH 3T3 cells were starved for 16 hours and then treated with VO-OHpic at concentration as indicated. Cells were stimulated with 0.05 μg/mL insulin for 30 min. Lipids were extracted and detected with GST-tagged GRP1 domain. Blot shown is representative of three independent experiment.

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Tables:

Compound	Dose	Cell tissue/Organ	Effect upon PTEN inhibition	Ref
bpV(phen)	0.4 μmol/mouse	C57BL/6 Mice	Increased lung tissue levels of phospho-Akt and ERK	[29]
bpV(pic)	5 μM	Skin-derived precursors	Enhancing the PI3K-Akt path-way through PTEN inhibition improved self-renewal of human foreskin	[30]
bpV(pic)	20μg/100 g	Wistar rats	Prevents ischemic brain injury by activating the mTOR signaling pathway	[31]
bpV(pic)	2mg/1000g	Wistar rats	Attenuation of hippocampal apoptosis	[32]
bpV(pic)	10 – 90 nM	SH-SY5Y cells	Reduction of okadaic acid-induced tau phosphorylation	[33]
bpV(pic)	200 μg/kg	Adult female Sprague-Dawley rats	Injury-induced neuronal soma atrophy was reversed by PTEN inhibition	[34]
bpV(OHpic)	1 μM	Neonatal mouse ovaries	Demonstrated the use of PTEN inhibitor to generate mature human oocytes for use in novel IVF techniques	[35]
bpV(OHpic)	1 μM	Ovarian cortical tissue	Affects human ovarian follicle development by promoting the initiation of folliclegrowth and development to the secondary stage	[36]
VO-OHpic	500 nM	Human xenograft	Induced senescence and inhibits tumorigenesis in vivo	[15]
VO-OHpic	500 nM	Carcinoid cell line BON	Decreased secretion and synthesis of serotonin	[37]
VO-OHpic	100 nM	Mouse adrenal chromaffin cells	Abolished the effect of compound IC87114, a potent inhibitor of PI3Kδ in promoting potentiation of Ca2 + -stimulated catecholamine release	[38]
VO-OHpic	10 μg/kg	Mice	Induced cardioprotection against ischemia–reperfusion injury	[39]
VO-OHpic	500nM	Breast cancer cells MDA-MB-231	Abolished the effect of PI3 kinase inhibitor IC87114 on Akt inhibition	[40]
VO-OHpic	0.1-5μM	Mouse ventricular cardiomyocytes	Highly protective against cell death induced by ischemia and reperfusion	[41]

Table 1: Recent applications of vanadium-based PTEN inhibitors *in cellulo* and *in vivo.*

Substrate	K_M (μM)	Vmax (nmoles*mg^{-1}*min^{-1})
$PI(3,4,5)P_3$	30 ± 4	47.5 ± 1.8
$I(1,3,4,5)P_4$	88 ± 9	9.1 ± 0.4
pNPP	25600 [16]	-
OMFP	216 ± 14	0.4 ± 0.1

Table 2: Kinetic parameters of PTEN phosphatase activity measured with various substrates. Please refer to methods section for experimental details.

	PTP1B bpVpic	PTP1B VO-OHpic	PTEN bpVpic	PTEN VO-OHpic	SopB bpVpic	SopB VO-OHpic
HEPES	37.5 ± 1.6	790.3 ± 41.8	29.3 ± 3.1	39.6 ± 2.1	42.3 ± 6.2	513.6 ±3.2
HEPES/EDTA	60.5 ± 2.2	> 20000	36.3 ± 4.3	> 20000	50.3 ±5.3	> 20000

Table 3. IC50 values (nM) of PTEN, PTP1B and SopB inhibition by vanadium inhibitors bpVpic and VO-OHpic. Assays were performed using OMFP as substrate (see methods). The values shown are the mean ± standard deviation of three independent experiments.

Figures:

Figure 1:

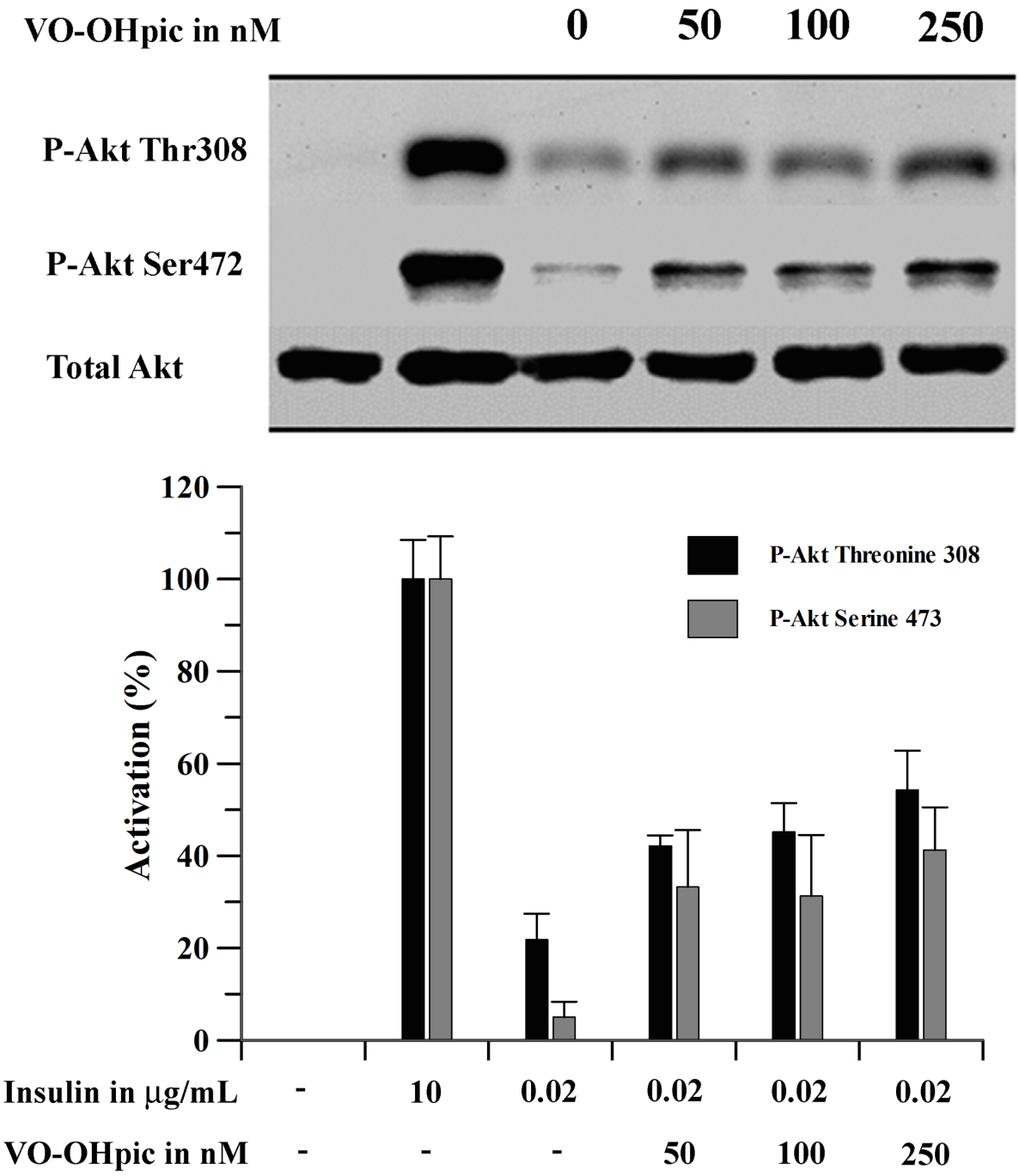

VO-OHpic in nM
0
50
100
250
P-Akt Thr308
P-Akt Ser472
Total Akt
120
100
80
60
40
20
0
Activation (%)
P-Akt Threonine 308
P-Akt Serine 473
Insulin in µg/mL
-
10
0.02
0.02
0.02
0.02
VO-OHpic in nM
-
-
-
50
100
250

Figure 2:

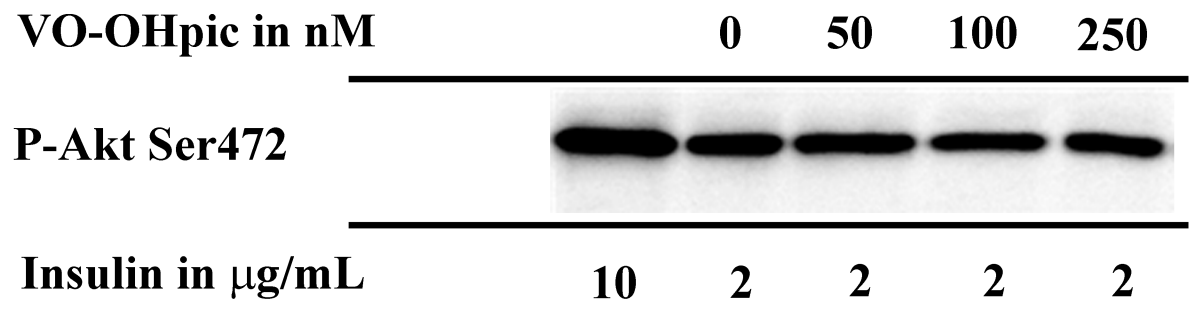
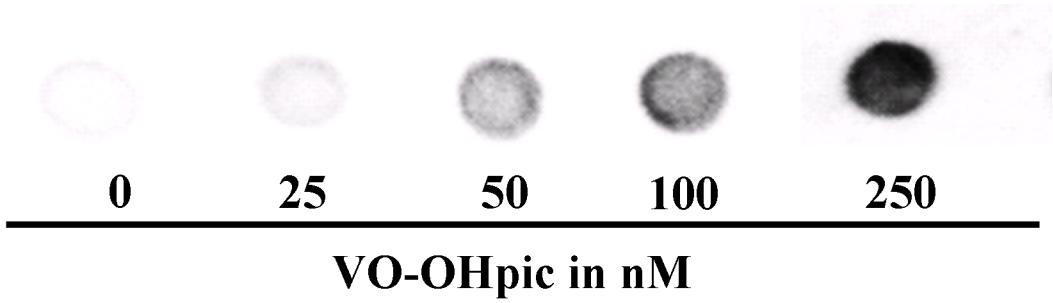

VO-OHpic in nM
0
50
100
250
P-Akt Ser472
Insulin in μg/mL
10
2
2
2
2

Figure 3:


0
25
50
100
250
VO-OHpic in nM