Bisperoxovanadium compounds are potent PTEN inhibitors

Annette C. Schmid[a], Richard D. Byrne[a,1], Ramón Vilar[b], Rüdiger Woscholski[a,*]

[a]Department of Biological Sciences, Imperial College London, Exhibition Road, London SW7 2AZ, UK
[b]Department of Chemistry, Imperial College London, Exhibition Road, London SW7 2AZ, UK

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Abstract The tumour suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN) shares homology with protein tyrosine phosphatases (PTPases). Similarly, bisperoxovanadium (bpV) molecules that are well-established PTPase inhibitors were shown to inhibit PTEN, but at up to 100-fold lower concentrations. The preference and potency of the bpVs towards PTEN was validated in vivo as demonstrated by: (i) an increase of Ser473 phosphorylation of protein kinase B (PKB) at similar low nanomolar doses, (ii) the lack of any effect on the PKB phosphorylation in the PTEN negative cell line UM-UC-3, (iii) the ability to rescue Ly294002-induced phosphoinositide 3-kinase inhibition and (iv) a lack of tyrosine phosphorylation at low nanomolar doses.
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1. Introduction

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a recently identified tumour suppressor that is either mutated or deleted in many cancer cells [1–4]. This protein shows dual phosphatase activity, dephosphorylating both protein [1] and lipid substrates [5], but it displays a higher specificity towards 3-phosphorylated phosphoinositides (PI) such as PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3,4,5)P3 [6]. 3-Phosphorylated lipids are generated by the phosphoinositide 3-kinases (PI 3-kinases) in response to an extracellular stimulation by growth factors. In dephosphorylating intracellular PtdIns(3,4,5)P3, PTEN counteracts the action of PI 3-kinases [7] thereby terminating certain downstream signalling pathways [8]. The major downstream target of PTEN seems to be the protein kinase B (PKB), also referred to as Akt [9,10], which is phosphorylated at two major sites (Thr308 and Ser473) in a PI 3-kinase-dependent fashion [11].

Vanadate is a well-characterised competitive reversible inhibitor for protein tyrosine phosphatases (PTPases) [12–15]. In particular, vanadate derivatives such the bisperoxovanadium (bpV) have been employed as PTPase inhibitors [16,17] and insulin mimetics [18–21]. Since PTEN shares with the PTPases the same active centre, the CX5R motif [22], we hypothesised that bpV compounds should also inhibit PTEN. In this study, we confirmed this hypothesis by characterising the bpVs with respect to their inhibitory potency and specificity, in vitro and in vivo. Our results show that these novel PTEN inhibitors are effective new tools, which will be very useful to further characterise signalling pathways downstream of PTEN.

2. Materials and methods

2.1. Chemical synthesis

Vanadium oxide (V_2O_5) and pyridine-2-carboxylic acid (picolinic acid) were purchased from Sigma–Aldrich and used as received. The synthesis of dipotassium bisperoxo(pyridine-2-carboxyl)oxovanadate (bpV(pic)) was performed as previously described [23], and the purity and quality tested by elemental analysis (performed by the University of North London) as well as ^{51}V NMR and IR spectroscopy.

2.2. Cloning and expression of PTEN

The coding region of the DNA sequence of human PTEN was cloned into a pGEX-4T2 expression vector (Pharmacia). Protein expression was induced overnight in the *Escherichia coli* DH5α strain using 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C. Glutathione *S*-transferase (GST)-fusion protein was purified according to the manufacturer's manual using glutathione–Sepharose 4B (Pharmacia). Protein integrity was confirmed on a Western blot using GST antibody (Novagen).

2.3. Determination of IC_{50} values for PTEN and tyrosine phosphatases

Enzyme activity of recombinant PTEN was measured in 200 mM Tris, pH 7.4, containing 50 ng/μl BSA, 150 μM synthetic dipalmitoyl-PtdIns(3,4,5)P3 (Cell Signals) and 0.25% (w/v) octyl glycoside (Sigma) at 30 °C for 30 min. In order to stop the enzyme reaction, 0.7 vol of colour reagent (2.3 mg/ml malachite green in 3.6 M HCl and 17 mM ammonium molybdate) was added to the assay. The mixture was allowed to develop for 20 min and the absorbance at 625 nm was measured [24,25]. PTPase assays were performed using the synthetic substrate *para*-nitrophenylphosphate (pNPP) and the phosphatases PTP-1B and PTP-β (Upstate Biotechnology) in 25 mM HEPES, pH 7.2, containing 50 mM NaCl, 5 mM dithiothreitol (DTT), 2.5 mM EDTA, 100 μg/ml BSA and 1 mM pNPP (Sigma) for 15 min at 30 °C. The linear increase of absorbance was monitored every 30 s at a wavelength of 410 nm. All experiments were repeated in triplicate. Calculations for IC_{50} values were performed using GraphPad Prism.

* Corresponding author. Fax: +44-20-7594-5207.
E-mail address: r.woscholski@imperial.ac.uk (R. Woscholski).

[1] Present address: Cell Biophysics Laboratory, CRUK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX, UK.

Abbreviations: bpV, bisperoxovanadate; bpV(HOpic), dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate; bpV(bipy), potassium bisperoxo(bipyridine)oxovanadate; bpV(phen), potassium bisperoxo(1,10-phenanthroline)oxovanadate; bpV(pic), dipotassium bisperoxo(pyridine-2-carboxyl)oxovanadate; DTT, dithiothreitol; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; NCS, newborn calf serum; PI 3-kinase, phosphoinositide 3-kinase; PtdIns(3,4,5)P3dC16, L-α-D-myo-phosphatidylinositol 3,4,5-triphosphate 3-*O*-phospho-linked, D(+)-*sn*-1,2-di-*O*-hexadecanoylglyceryl; PKB, protein kinase B; pNPP, *para*-nitrophenylphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; PTPase, protein tyrosine phosphatase

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2.4. Tissue culture

NIH3T3 cells (passage 5–20) were grown in 10% newborn calf serum (NCS) DMEM (Invitrogen) in 6-well plates at 37 °C and 5% CO_2. Starvation of the cells was carried out over 72 h in DMEM containing 0.5% NCS. Prior stimulation of the cells medium was changed to 0% DMEM. UM-UC-3 (passage 5–20), which is a $PTEN^-$ bladder tumour cell line [26,27] was grown in 10% MEM (Invitrogen), starved with 0.5% MEM also for three days and finally incubated with serum free MEM.

2.5. Western Blot analysis

Cell lysates (NIH 3T3 and UM-UC3) were loaded on 10% SDS–PAGE and transferred to PVDF membranes for PKB analysis and nitrocellulose for phosphotyrosine detection. The membranes were then blocked (5% milk powder in TBST) and subsequently analysed for PKB phosphorylation using an anti-phospho-PKB (Ser473) antibody (NEB) or tyrosine phosphorylation employing an anti phosphotyrosine antibody (4G10) according to the manufacturer's instructions. To control for equal loading, the amount of tubulin and PKB was analysed in parallel.

2.6. Phosphorylation of tyrosine residues in NIH3T3 cells

NIH3T3 cells were grown and starved as described earlier. After 72 h starvation, cells were incubated for 15 min with 10% NCS, 0.5 μg/ml insulin, 10, 1, 0.1 and 0.01 μM bpV(pic) and potassium bisperoxo(1,10-phenanthroline)oxovanadate (bpV(phen)), respectively. In addition, cells were co-treated with the lowest dose of bpV and 0.5 μg/ml insulin. Cell lysates were analysed on Western Blots using an anti phosphotyrosine antibody (Upstate).

2.7. Cytotoxicity assay

Cytotoxicity of bpV compounds was measured with MTT assays. NIH3T3 cells were resuspended in serum-free media and exposed to concentrations of all four bpV compounds between 1 mM and 0.1 nM for 2 h. MTT solution (final concentration of 5 mg/ml) (Lancaster Synthesis Ltd.) was added to the cells which were further incubated for 4 h. Cell pellets were resuspended in DMSO containing 0.1 M HCl and measured at 570 nm.

3. Results and discussion

3.1. PTEN is inhibited by bpV compounds, in vitro

PTEN has been described as a tumour suppressor, which is mutated in many cancer tissues [1,4,28,29] and exerts this role mainly by the negative regulation of the PI 3-kinase/PKB signalling pathway [7,9,10]. Many researchers who are currently characterising the role of PTEN in signalling and disease rely on the employment of PTEN null cell lines, such as glioblastoma [30] or UM-UC cells [31], PTEN knockout of cells [10], which are not accessible in fully differentiated systems such as muscle cells or neurons. Such efforts could be substantially boosted if specific inhibitors were available, which in conjunction with knockout models could be used to distinguish between scaffolding effects and catalytic function of the deleted PTEN as well as opening up new biological systems.

Given that PTPases and PTEN share considerable homology in their active site, we reasoned that the previously characterised PTPase inhibitors [16,17], bpV compounds, could be targeting the phosphatidylinositol 3-phosphatase PTEN as well [6]. Testing several different compounds in vitro (Table 1) revealed that bpVs with polar *N*,*O* ligands [dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate (bpV(HOpic)) and bpV(pic)] had a strong preference towards PTEN (IC_{50}: 20–40 nM), while bpVs with the neutral *N*,*N* ligands [potassium bisperoxo(bipyridine)oxovanadate (bpV(bipy)) and bpV(phen)] seemed to be more promiscuous targeting both PTPases (PTP-1B and PTP-β) and PTEN, although with distinct affinities. With respect to PTEN, it can be concluded that all bpVs inhibit PTEN with 10- to 100-fold lower concentrations than PTPases. While PTEN binds all four molecules in a similar fashion, PTPases seem to distinguish in terms of size and charge, which is probably due to the differential spatial arrangement in the catalytic site of the two targets, since the active site of PTEN [32] is much wider than the corresponding tyrosine phosphatase structure [33].

Although bpVs have been investigated towards their ability to target tyrosine phosphatases in the past, to our knowledge the four bpVs employed here have not been comprehensively analysed in their effectiveness on the two PTPases, PTP-1B and PTP-β. Recently, IC_{50} values for bpVs were obtained for the alkaline phosphatase [34] and for insulin receptor dephosphorylation [16], the latter being linked to tyrosine phosphatase inhibition, revealing no significant differences between the *N*,*N* and *N*,*O* compounds. Thus, the observed preference of the tyrosine phosphatases, PTP-1B and PTP-β, for the *N*,*N* ligand containing bpVs might be a characteristic for the particular enzymes employed here.

3.2. bpVs stimulated PKB phosphorylation at nanomolar concentrations

Guided by the above-described in vitro characteristics, we investigated whether the four bpVs could inhibit PTEN in vivo. Any loss of PTEN activity will increase PtdIns(3,4,5)P3 levels if PI 3-kinases are sufficiently active. Therefore, one would expect a dose-dependent activation of PKB, a well characterised downstream target of PTEN-dependent signalling [35], after treatment with bpVs. Thus, we used PKB phosphorylation as a monitor for the cellular PtdIns(3,4,5)P3 level in NIH3T3 cells. Since starved fibroblasts need a certain dose of growth factors in order to show drug dependent changes in PKB phosphorylation (R.D. Byrne and R. Woscholski, unpublished observation), all samples were stimulated with 0.5 μg/ml insulin.

As shown in Fig. 1(a), the tested compounds induced the phosphorylation of PKB in a dose-dependent manner. No activation could be established with concentrations up to 10 nM, however, at higher concentrations a significant enhancement of the phosphorylated PKB signal was observed. Three

Table 1
IC_{50} values for bpV compounds for the phosphoinositide 3-phosphatase PTEN and the protein tyrosine phosphatases PTP-β and PTP-1B

Enzyme	*N*,*N* ligand		*N*,*O* ligand	
	bpV(bipy)	bpV(phen)	bpV(HOpic)	bpV(pic)
PTEN	18 nM ± 0.8	38 nM ± 2.4	14 nM ± 2.3	31 nM ± 1.7
PTP-β	60.3 nM ± 9.6	343 nM ± 88.5	4.9 μM ± 0.9	12.7 μM ± 3.2
PTP-1B	164 nM ± 22.6	920 nM ± 45.2	25.3 μM ± 2.9	61 μM ± 10.5

Phosphatase assays were performed as described in Section 2. The IC_{50} values are shown as the means ± S.E. of triplicate experiments.

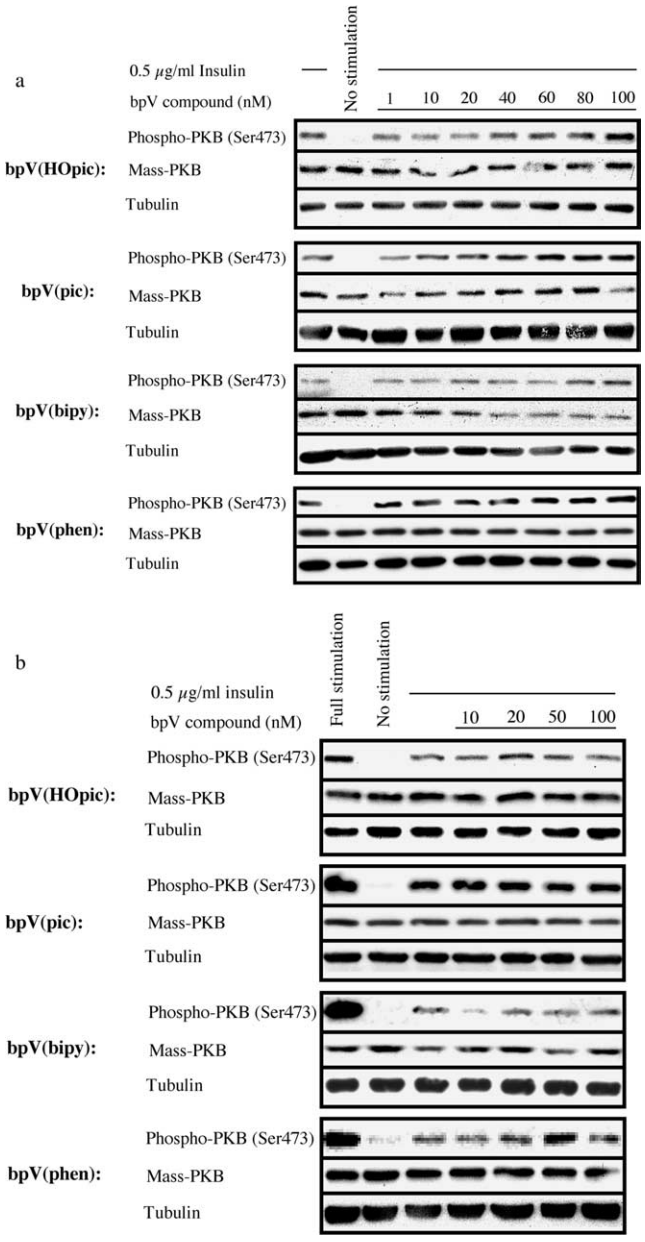

Fig. 1. Dose-dependent inhibition of PTEN by bpV compounds, in vivo. Starved NIH3T3 (a) and UM-UC-3 (b) cells were incubated with the indicated concentrations of bpVs for 5 min and stimulated for 15 min with 0.5 μg/ml insulin. For comparison, full stimulation of the cells was carried out by adding 10% NCS. Cell lysates were analysed by Western blotting for PKB phosphorylation and equal loading (tubulin and mass-PKB) as described in Section 2.

independent blots were quantified by optical densitometry (NIH Image) and the calculated IC_{50} value for bpV(pic) was 48 nM (±8.5). This indicates that PTEN can also be inhibited with low nanomolar concentrations of bpV compounds, in vivo. To further validate whether bpVs target PTEN similar experiments were performed employing the PTEN negative tumour cell line UM-UC-3. As shown in Fig. 1(b), no significant PKB phosphorylation could be observed. Taking all these observations together, we suggest that the activation of the pathways downstream of PI 3-kinase is due to PTEN inhibition. Exposure of the bpVs in media overnight did not alter their potency towards PTEN inhibition (data not shown), indicating that these molecules are highly stable in the applied assay conditions.

3.3. PTEN inhibitor bpV(pic) can partly abrogate Ly294002 induced PKB inhibition

Ly294002 is a commonly used PI 3-kinase inhibitor which reduces PKB phosphorylation [36]. Since PTEN is the natural inhibitor of PI 3-kinases, we examined whether the bpV(pic) is able to rescue Ly294002-induced PKB inhibition. Indeed, a shift in the IC_{50} of Ly294002 could be clearly detected (Fig. 2), providing further proof that bpVs act as PTEN inhibitors.

3.4. The bpV inhibitors increased phosphorylation of tyrosine residues in vivo

As pointed out above bpVs are well-characterised PTPase inhibitors in the micromolar concentration range. In order to confirm the in vivo potency of the bpVs on PTPases, the tyrosine phosphorylation of total cell lysates was examined. Western blot analysis (Fig. 3) for phosphorylated tyrosine residues revealed the typical pattern of protein bands of stimulated cells [37]. Although untreated cells showed a similar pattern of detectable bands, these had much weaker intensity. The highest degree of tyrosine phosphorylation could be detected in cells treated with 10 μM bpV(pic), which is in agreement with earlier investigation revealing that phosphorylation of tyrosine residues was induced by 10 μM sodium orthovanadate [37] or 0.5 mM pervanadate [38], and is more potent than 10% NCS [39]. In contrast, 10 μM bpV(phen) induced a weaker stimulation supporting the results presented above (Table 1). Interestingly, low nanomolar concentrations of the compounds that were effective for PTEN inhibition did not induce any significant changes when compared to unstimulated cells, with or without co-treatment with 0.5 μg/ml insulin. In conclusion, bpV compounds will target PTPases in vivo only at micromolar concentrations, further corroborating that these compounds are specific for inhibitors for PTEN in the nanomolar concentration range.

3.5. Cytotoxicity of bpV compounds

The effects of the bpVs on cell viability were carried out by employing MTT assays, a means of measuring the activity of

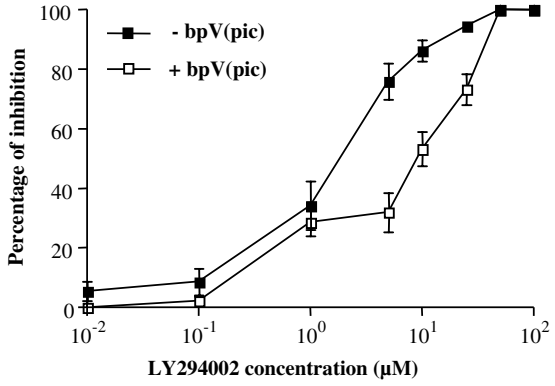

Fig. 2. Counteraction of Ly294002-induced inhibition of PKB phosphorylation by bpV(pic). Quiescent NIH3T3 cells were pre-treated with indicated concentrations of Ly294002 and incubated with or without 200 nM bpV(pic) for 5 min, followed by a stimulation with 10 μg/ml insulin for 15 min. Phosphorylation of PKB in the cell samples was analysed by Western blotting and optical densitometry (NIH Image). The values were presented as the means ± S.E. taken from three independent experiments.

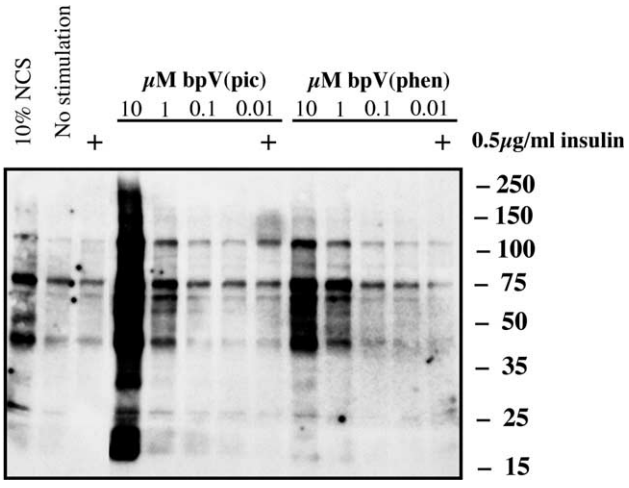

Fig. 3. Phosphorylation of tyrosine residues induced by growth factors, bpV(pic) and bpV(phen). Indicated concentrations of bpV(pic) and bpV(phen) were applied to starved fibroblasts for 15 min (see Section 2). Molecular weight markers are indicated in kDa. The figure shows a representative blot out of three.

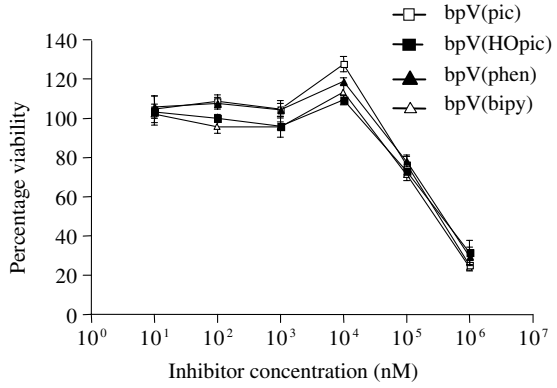

Fig. 4. Cytotoxicity of bpV compounds: NIH3T3 cells were treated with concentrations of up to 1 mM bpV(pic), bpV(HOpic), bpV(bipy) and bpV(phen) and MTT assays were undertaken as described in Section 2. The results were presented as the means ± S.E. of triplicate determinations.

living cells via mitochondrial dehydrogenases. As shown in Fig. 4, all bpVs showed similar cytoxicity significantly killing cells at concentrations equal or higher than 100 μM. This indicated that nanomolar doses, which can inhibit PTEN phosphatase activity in vitro and in vivo, do not affect cell viability.

In summary, newly discovered PTEN inhibitors, bpVs, were characterised with respect to their properties and their role in insulin mimetic signalling. We clearly show that bpVs preferentially inhibit PTEN at nanomolar concentrations using four independent approaches: (i) IC_{50} assays for PTEN and PTPases in vitro, (ii) phospho-PKB Western blot analyses in NIH3T3 and PTEN negative UM-UC-3 cells, (iii) counteraction of Ly294002-induced PI 3-kinase inhibition and (iv) phospho-tyrosine Western blot analyses in NIH3T3 cells. Since the bpVs inhibit PTEN at concentrations where no significant cytotoxicity could be observed, these compounds will be useful research tools for investigators analysing PI 3-kinase-mediated signalling.

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