Electronic Supplementary Information (ESI)

Exploring organic oxidovanadium(IV) complexes as YopH inhibitors: mechanism of action and modeling studies

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1. Methods

1.1. Recombinant protein expression and purification

Previously described methodologies were used to perform the expression and purification of recombinant PTPs YopH, PTP1B, PtpA and PtpB. LYP and PTP-PEST were expressed in Escherichia coli BL21(DE3) induced with 0.5 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubated for 20 h at room temperature. The following methodology was used to purify the hexahistidine tagged LYP and PEST: the protein was purified from lysates of isopropyl-beta-D-thiogalactopyranoside (IPTG) induced E. coli BL21(DE3) under native conditions using HisTrap HP 1 mL columns (GE Healthcare BioSciences) connected to an Äkta System (Amersham Biosciences). The protein were eluted at 1 mL/min using a 4 steps program and a mix of two different buffers, buffer A (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10% glycerol) and buffer B (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 0.5 mM imidazole, 10% glycerol, 1 mM β-mercaptoethanol). The first 3 steps were performed with an isocratic elution with 2%, 12% and 20 % of buffer B respectively. The last step was a linear gradient from 20 to 100% of buffer B. The purity of the protein preparations was assessed by SDS-PAGE under reducing conditions. Fractions containing purified recombinant protein were dialyzed overnight at 4°C, using the dialysis buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, 5 mM dithiothreitol (DTT). The protein was then stored at -80°C for activity assays.

1.2. Oxidovanadium (IV) complexes

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C, H, N and S analyses were carried out with a Thermo Scientific Flash 2000 elemental analyzer. A 500-MS Varian Ion Trap Mass Spectrometer was used to measure ESI-MS of methanolic solutions of the oxidovanadium(IV) semicarbazone complexes in the positive mode. The FTIR absorption spectra (4000-400 cm\(^{-1}\)) were measured as KBr pellets with a Shimadzu IRPrestige-21 instrument. \(^1\)H NMR spectrum of L4 were recorded on a Bruker DPX-400 instrument (at 400 MHz) at 30°C in DMSO-d\(_6\).

L4

Salicylaldehyde semicarbazone ligand L4 was synthesized from an equimolar mixture of 2-hydroxybenzaldehyde (salicylaldehyde) and semicarbazide as previously published. The mixture was stirred with p-TsOH (catalytic amounts) in dry toluene as solvent at room temperature until no more carbonyl compound was detected by TLC (SiO\(_2\), 1 % MeOH in CH\(_2\)Cl\(_2\)). The resulting solid was collected by filtration. The compound was recrystallized from chloroform and MeOH.

\[^1\]H NMR (d): 6.81 (m, 1H), 6.87 (m, 1H), 7.16 (t, 1 H), 7.72 (d, 1 H), 6.53 (br. s, 2 H), 8.14 (br. s, 1 H), 10.19 (br. s, 2 H), 9.85 (br, 1H) ppm.
Compounds 1 and 2

$[\text{V}^{IV}\text{O} (L4-2H)(\text{NN})]$ complexes 1 and 2, where L4 = salicylaldehyde semicarbazone and NN = 5-amine-1,10-phenanthroline (aminophen) or 1,10-phenanthroline (phen), were synthesized by suspending 0.375 mmol of L4 (67 mg) and 0.375 mmol of NN (73 mg aminophen or 68 mg phen) in 15 mL of absolute ethanol previously purged with nitrogen for 10 min, as previously shown. $[\text{V}^{IV}\text{O}(\text{aacac})_2]$ (0.375 mmol, 100 mg), where acac = acetylacetonate, was suspended in 6 mL of absolute ethanol, previously purged with nitrogen, and was added to the previous mixture. This was then heated at reflux under nitrogen for 4 h. The reddish brown solids formed were filtered off from the hot mixture, and washed three times with 2 mL portions of EtOH:Et$_2$O (1:1) [2,3].

$[\text{V}^{IV}\text{O}(\text{L}1-2\text{H})(\text{aminophen})]$. 1. Anal. Calc. for C$_{39}$H$_{33}$N$_5$O$_3$V: C, 54.68; H, 3.67; N, 19.13. Found: C, 54.55; H, 3.68; N, 19.20. ESI-MS (MeOH) m/z [Found (Calcd)]: 441.1 (441.06) (10%) [M+H$^+$]. IR (KBr): 1609, $\nu$(C=O); 1501, $\nu$(C=N); 959, $\nu$(VO) cm$^{-1}$.

$[\text{V}^{IV}\text{O}(\text{L}1-2\text{H})(\text{phen})]$. 2. Anal (%) calc. for C$_{50}$H$_{43}$N$_7$O$_3$V: C, 56.61; H, 3.56; N, 16.51. Found: C, 56.58; H, 3.54; N, 16.55. ESI-MS (MeOH) m/z [Found (Calcd)]: 425.1 (425.1) (100%) (M+H$^+$). IR (KBr): 1612, $\nu$(C=O); 1600, $\nu$(C=N); 960, $\nu$(VO) cm$^{-1}$.

**Compound 3**

Bis(pyridine-2-thiol $N$-oxide) oxovanadium(IV) was synthesized according to a previously reported procedure by reaction of the sodium salt of pyridine-2-thiol $N$-oxide and VOSO$_4$·5H$_2$O in 2:1 molar ratio under nitrogen atmosphere in ethanol medium. The compound was isolated as a purple solid, washed first with several portions of water and then with ethanol and dried. Anal (%) calc. for C$_{10}$H$_{18}$N$_5$O$_5$S$_2$V: C, 37.62; H, 2.53; N, 8.77; S, 20.09. Found: C, 37.65; H, 2.55; N, 8.80; S, 20.11. IR (KBr) (cm$^{-1}$): 1240, $\nu$(NO); 966, $\nu$(VO).

**1.3. Phosphatase activity and inhibition**

The screening, IC$_{50}$ and kinetic assays were carried out in 96-well plates and the final reaction volume was 200 µL. The activity of PTPs was evaluated using pNPP as substrate and the formation of $p$-nitrophenol was monitored spectrophotometrically using TECAN Magellan Infinite M200 at 410 nm for 10 minutes (with readings every minute). The negative control was considered the absence of enzyme and compound; the positive control the presence of enzyme and the final concentration of 4% of dimethylsulfoxide (DMSO). To perform the IC$_{50}$ values were also determined to the ligands L1-L4 of these complexes (concentrations ranging from 0.1 µM to 100 µM), and to VOSO$_4$ (concentrations ranging from 0.1 µM to 2 µM). VOSO$_4$ was dissolved in water, but 4% DMSO was added to the enzymatic reaction, as described above. The GraphPadPrism 5.0 was used to analyze the experimental data and the IC$_{50}$ values were calculated by linear regression (Percentage of activity x log of inhibitor concentration). The already described methodology was used to determine the mechanism of inhibition and $K_i$ values. The IC$_{50}$ and $K_i$ value of sodium orthovanadate (a general PTP inhibitor) were also estimated as internal control using the same conditions used to the other compounds. We obtained to sodium orthovanadate the IC$_{50}$ of 0.81 ± 0.05 µM and $K_i$ value of 1.58 ± 0.59 µM.

**1.4. Selectivity assay**

The selectivity of vanadium complexes was performed by determining the IC$_{50}$ values for Mycobacterium tuberculosis PtpA (final concentration of 85 nM) and PtpB (22 nM), and human PTPs LYP (113 nM), PTP-PEST (103 nM) and PTP1B (25 nM). All the enzymes were also incubated 10 minutes at 37 °C with complexes before adding 20 mM of pNPP (except for PtpA, in this case it was pre-incubated at room temperature). The selective index (SI) was calculated by the division IC$_{50}$ of the complex being tested to the IC$_{50}$ of PtpA.
1.5. Reversibility assay

Reversibility was determined by measuring the recovery of enzyme activity after a rapid and high dilution of the enzyme-inhibitor complex. To address this issue, the method used was incubating the enzyme at a concentration 100x the amount needed for the activity assays (1.3 µM) and the concentration of the inhibitors was 10x its IC$_{50}$. After 30 min at 37 °C the mixture was diluted 100x in the enzymatic reaction, thus the enzyme concentration becomes the same concentration used in the inhibition assays (13 nM), but the inhibitor concentration changes from 10x IC$_{50}$ to 0.1x IC$_{50}$. The curve from each vanadium complex was measured and compared with that in the absence of compound (4% DMSO) (Figure S1). As observed in the Figure S1, the inhibitor 3, after dilution, has 83% of the activity relative to the control, indicating a reversible inhibition. Since the vanadium complexes 1 and 2 presented 57% and 69% of activity, respectively, suggesting a mechanism slowly reversible.

![Vanadium complexes](image)

**Figure S1.** Reversibility of YopH inhibition by vanadium complexes 1-3.

1.6. Computational modeling

The 3D structure of small molecules were sketched in Maestro and quickly relaxed with the OPLS force field. Geometry optimization of Vanadium complexes was performed at the DFT theory level with the B3LYP method and using the 6-311G** basis set. RESP charges were further calculated on the optimized geometry. All quantum mechanics computations were performed using the Gaussian03 program. Non-complexed fragments were not submitted to the quantum mechanics optimization. Molecular docking was performed with three different docking programs, using custom settings described hereafter. For docking with AutoDock 4.2, receptor grids were generated within a box of 70x 70x 70 points centred on Cys403 sulphur atom and having a grid spacing of 0.375 Å. Fifty Genetic Algorithm runs were performed for each ligand, and the maximum number of energy evaluations was increased to 2500000 while all other parameters were kept at their default value. The 10 most stable conformations generated by the DFT optimization were docked with FRED (version 3.0.1), The receptor was built with the make_receptor utility and the ligand binding site had a total volume of 1958Å$^3$. Docking with GOLD (version 5.2.2) was carried out by using thePLP, Chemscore and Goldscore functions. In all these cases, the receptor was centred on Cys403 sulphur atom and had a radius of 16 Å. Docking accuracy was improved up to 200% and all other parameters were kept at their default value.
**Figure S2.** Alternative binding mode of compound 2 towards the catalytic active site of YopH. The ligand is shown as cyan sticks, the protein as green cartoon and lines. Residues described in the text as crucial for binding to Vanadium complexes are shown as orange sticks and are labeled. In this pose, complex 2 does not interact with Gln446, but performs an H-bond interaction with Arg404. Moreover, this binding mode is not reinforced by cation-π interactions with Lys447 and Arg409 and is associated to a lower chemscore Fitness value (Fitness = 20.83).

**Figure S3.** Predicted binding mode of non-complexed fragments L1 (A), L2 (B), L3 (C) and L4 (D) within the catalytic pocket of YopH. Small molecules are shown as cyan sticks, the protein as green transparent surface. Ligand non-polar H atoms were omitted.

For benchmarking purposes, the crystallographic YopH inhibitor pNCS was docked towards YopH catalytic site using GOLD, and the relative affinity was estimated by the Chemscore function. The scoring value of pNCS, which is known to inhibit YopH with a $K_i$ of about 25 µM, was 17.21, in line with the trend observed for the test set.\(^{18}\)
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