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Development of an automated protein-tyrosine phosphatase 1B inhibition assay and the screening of putative insulin-enhancing vanadium(IV) and zinc(II) complexes

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Abstract

The inhibition of protein-tyrosine phosphatase 1B (PTP1B) is a potential target for treatment of type 2 diabetes. Vanadium and zinc metal coordinated complexes have insulin-enhancing activities, and while vanadium compounds inhibit PTP1B, little is known on the mode of action of zinc compounds. In this study we developed an automated PTP1B inhibition assay that allows for a rapid assessment of the PTP1B inhibition strength of candidate compounds. Synthetic vanadium(IV) and zinc(II) complexes were evaluated: IC_{50} values for vanadium complexes ranged from 0.06 to 0.8 μM whereas for zinc compounds, values were above 10 μM. Vanadium sulfate, a non-conjugated inorganic salt, had stronger inhibition activity than any of the conjugated metal complexes.

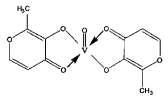
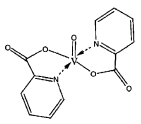
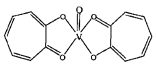
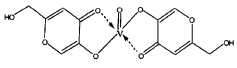
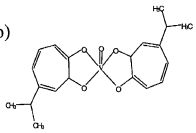
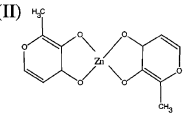
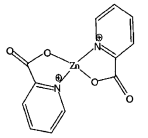
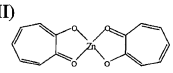
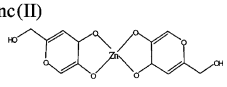
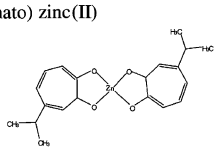
Introduction

Type 2 diabetes, a metabolic disorder characterized by hyperglycemia, is rapidly reaching epidemic proportions in several countries. Type 2 diabetics exhibit impaired insulin action, or insulin resistance. Protein-tyrosine phosphatase 1B (PTP1B) plays a major role in modulating both insulin sensitivity and fuel metabolism and therefore provides a potential pharmaceutical target for the development of treatments for type 2 diabetes (Goldstein 2001). Several groups have confirmed and strengthened the role of PTP1B as a major contributor to insulin resistance and have pursued the development of potent and specific PTP1B inhibitors (Yuen *et al*. 1997, Xie *et al*. 2003).

Vanadium has been shown to have insulin-like effects and can improve the symptoms of diabetes type 2 in a variety of animal models (Rehder 2003, Shechter *et al*. 2003). A major mode of action for vanadium's insulin-sensitizing effects may be associated with PTP1B inhibition (Peters *et al*. 2003). More recently, great attention has been given to the development of vanadium coordination compounds with improved efficacy, such as bis(maltolato) oxovanadium(IV) (BMOV) which can enhance insulin sensitivity (Yuen *et al*. 1997), and is an effective inhibitor of PTP1B activity (Peters *et al*. 2003).

Zinc plays essential structural roles in many proteins and enzymes but also has insulin enhancing activity *in vivo* (Coulston & Dandona 1980). However, unlike vanadium, compounds containing zinc have received less attention towards the development of potential anti-diabetic agents. Our objective therefore, was to develop an automated

Table 1. Full name, structure and elemental analysis for vanadium and zinc compounds synthesized.

Compound (name, abbreviation, structure)	Found (Calc.)	UV-vis data	IR data (cm^{-1})	NMR
1 Bis(maltolato) oxovanadium(IV) BMOV 	for $C_{12}H_{10}O_7V$ C, 42.37 (45.45) H, 3.63 (3.18) O, 33.52 (35.31)	(H_2O) λ max = 204.77 nm (ε = 8.19 × 10^4 $M^{-1}cm^{-1}$), 283 (ε = 1.85 × 104)	1610, 1550, 1485 ν(C=O, C=C); 995 ν(V=O)	
2 Bis(picolinato) oxovanadium(IV) $VO(pic)_2.H_2O$ 	for $C_{12}H_{10}N_2O_6V$ C, 43.33 (43.79) H, 3.44 (3.06) N, 8.18 (8.51) O, 23.08 (29.16)	(H_2O) λ max = 207.69nm (ε = 6.91 × 10^4 $M^{-1}cm^{-1}$)	3500 br ν(H-O) ; 1640, 1630, 1600, 1570 ν(C=N and C=C); 980, 970 ν(V=O)	
3 Bis(tropolonato) oxovanadium(IV) $VO(trop)_2$ 	for $C_{14}H_{10}O_5V$ C, 36.77 (54.39) H, 2.47 (3.26) O, 24.36 (25.87)	(methanol) λ max = 250.95 nm (ε = 7.75 × 10^4 $M^{-1}cm^{-1}$)	900-1000 ν(V=O), 1500-1600 ν(C=O, C=C)	
4 Bis(kojato) oxovanadium(IV) $VO(koj)_2$ 	for $C_{12}H_{10}O_9V$ C, 26.17 (41.28) H, 2.50 (2.89) O, 40.67 (41.24)	(DMSO) λ max = 273.16 nm (ε = 4.03 × 10^3 $M^{-1}cm^{-1}$), 325.77 (7.47 × 10^3)	3500 br ν(O-H), 1610, 1550, 1500 ν(C=O); 1470 ν(C=C) ; 1270, 1240, 1200, 1180, 1150, 1075, 1060, 980 ν(V=O) ; 940, 800, 760, 580	
5 Bis(hinokitolonato) oxovanadium(IV) $VO(hino)_2$ 	for $C_{20}H_{22}O_5V$ C, 60.06 (61.07) H, 7.00 (5.64) O, 20.62 (20.34)	(DMSO) λ max = 274.91 nm (ε = 1.31 × 10^4 $M^{-1}cm^{-1}$), 349.74 (1.48 × 10^4), 404.03 (8.89 × 10^3)	1576 ν(C=O); 1514 ν(C=C)	
6 Bis(maltolato) zinc(II) $Zn(ma)_2$ 	for $C_{12}H_{14}O_8Zn$ C, 40.99 (40.10) H, 4.01 (4.74) O, 36.40 (34.05)	(H_2O) λ max = 205.93 nm (ε = 6.61 × 10^4 $M^{-1}cm^{-1}$), 225.23 (5.80 × 10^4), 273.16 (2.74 × 10^4)	3500 broad ν(H-O-H), 1614, 1578 ν(C=O); 1517 ν(C=C) ; 1277, 1202 ν(C-O)	^{1}H NMR [δ (ppm)]: 2.339[3H, s, CH3]: 6.543 [1H, d, 6H; J = 5 Hz]: 8.113 [1H, d, 5H; J = 5 Hz]. ^{13}C-NMR [δ (ppm)]: 15.24 [C1]: 110.30 [C5]: 150.18 [C6]: 152.39 [C3]: 154.14 [C2]: 178.16 [C4]
7 Bis(picolinato) zinc(II) $Zn(pic)_2.H_2O$ 	for $C_{12}H_{10}N_2O_5Zn$ C, 37.68 (43.99) H, 4.75 (3.08) N, 7.14 (8.55) O, 30.27 (24.42)	(H_2O) λ max = 204.77 nm (ε = 3.13 × 10^4 $M^{-1}cm^{-1}$), 272.58 (1.11 × 10^4), 315.25 (2.84 × 10^3)	3500 br ν(H-O) ; 1632, 1594, 1566 ν(C=N and C=C)	^{1}H NMR [δ (ppm)]: 7.689 [1H], 8.136 [t, 1H, J = 7.5 Hz], 8.196 [d, 1H, J = 7.5Hz], 8.416 [s, 1H]. ^{13}C NMR [δ (ppm)]: 124.399[C3], 127.309[C5], 141.095[C4], 146.911[C2], 151.512[C6], 165.898[C1]
8 Bis(tropolonato) zinc(II) $Zn(trop)_2$ 	for $C_{14}H_{10}O_4Zn$ C, 54.56 (54.66) H, 3.76(3.28) O, 19.87 (20.80)	(DMSO) λ max = 271.41 nm (ε = 6.85 × 10^3 $M^{-1}cm^{-1}$), 339.80 (1.75 × 10^4), 384.63 (1.17 × 10^4)	1595, 1514 ν(C=O), 1415, 1330, 1224 ν(C=C)	^{1}H NMR [δ (ppm)]: 6.910 [t, 1H;J = 9.5 Hz]; 7.229 [d, 1H,J = 11 Hz]; 7.441 [t, 1H,J = 10.5 Hz]. ^{13}C NMR [δ (ppm)]: 178.72, 172.66, 170.80, 137.99, 124.32, 124.50, 123. 57, 121.07
9 Bis(kojato) zinc(II) $Zn(koj)_2$ 	for $C_{12}H_{10}O_8Zn$ C, 41.18 (41.46) H, 3.19 (2.90) O, 33.39 (36.82)	(DMSO) λ max = 273.75 nm (ε = 1.18 × 10^4 $M^{-1}cm^{-1}$), 340.39 (1.75 × 10^4), 383.38 (1.25 × 10^4)	3500 broad ν(O-H), 1624, 1578 ν(C=O) ; 1517 ν(C=C); 1277, 1202 ν(C-O)	^{1}H NMR [δ (ppm)]: 4.360[d, 2H, J = 6 Hz], 5.666[t, 1H, J = 6 Hz], 6.555[s, 1H] 7.879[s, 1H]. ^{13}C NMR [δ (ppm)]: 60.588[C1], 106.463[C3], 138.923[C6], 154.426[C5], 168.795[C2], 180.735[C4]
10 Bis(hinokitolonato) zinc(II) $Zn(hino)_2$ 	for $C_{20}H_{22}O_4Zn$ C, 59.08 (61.31) H, 5.87 (5.66) O, 16.58 (16.34)	(DMSO) λ max = 273.75 nm (ε = 1.18 × 10^4 $M^{-1}cm^{-1}$), 340.39 (1.75 × 10^4), 384.63 (1.25 × 10^4)	1597, 1576 ν(C=O) ; 1504 ν(C=C)	^{1}H NMR [δ (ppm)]: 7.359 (dd, 1H, H(4); J = 10.5 Hz), 7.197 (s, 1H, H(7)), 7.067 (d, 1H, H(5); J = 11 Hz), 6.877 (d, 1H, H(3); J = 10 Hz), 2.866 (m, 1H, H(8)), 1.212 (d, 6H, CH3; J = 7 Hz). ^{13}C NMR [δ (ppm)]: 24.362 [C9]; 38.863[C8]; 122.770, 123.786, 123.900[C4,5,7]; 138.335[C3]; 160.167[C6];178.434,178.876[C1,2]

UV–Vis spectra were recorded on a SINCO 2001 spectrophotometer; infrared spectra were recorded as KBr pellets using FT-IR-NIR, IFS-66/S (Bruker) spectrophotometer; elemental analysis were performed using Flash EA 1112 Series analyzer operating at 500 °C (Thermo Quest). ^{1}H and ^{13}C NMR spectra were obtained by 500 MHz Unity Inova 500NB (Varian) spectrophotometer, all using DMSO-*d*6 solvents (Aldrich) unless indicated otherwise.

standard screening procedure to compare the efficacy of vanadium and zinc compounds on their ability to inhibit PTP1B. We synthesized a total of 10 vanadium and zinc coordination complexes (Table 1) and calculated their IC_{50} values. With the results, the relative PTP1B inhibition potencies of vanadium and zinc compounds, generated under the same set of experimental conditions, were compared.

Materials and methods

Preparation of vanadium and zinc compounds

All the solvents, except water, were purchased from Aldrich (reagent grade) and distilled prior to use. The starting materials including organic ligands such as maltol, $ZnSO_4$ and $VOSO_4$ were purchased from Aldrich and used as received. Most compounds were synthesized by following or slightly modifying procedures described previously (McNeill *et al.* 1992, Yuen *et al.* 1997, Barret *et al.* 2001, Melchior *et al.* 2001) and all elemental analysis for synthesized compounds are included in Table 1.

Protein-tyrosine phosphatase 1B inhibition assay

PTP1B was assayed using the fluorogenic substrate, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP, Molecular Probes Inc.), and recombinant human PTP1B (Merck-Calbiochem). Serial dilution of all inhibitors and assays were performed with an automated liquid handling system (Multiprobe II Plus, Perkin Elmer) in a two-step procedure. Inhibitors diluted with reaction buffer (25 mM MOPS, 50 mM NaCl, 1 mM dithiothreitol, 0.05% Tween 20, pH 7) at 52.5 μl were mixed with 15 μl PTP1B (13.3 nM) in a 96-well, non-transparent assay plate, shaken for 30 min at 37 °C , after which 7.5 μl DiFMUP (10 μM) substrate was added. Fluorescence was measured at 358/455 nm (Ex/Em) using a plate spectrophotometer (Victor 3, Wallac), after 30 min at 37 °C, under constant shaking.

Results and discussion

The development of automated and high-throughput assays has greatly facilitated the screening process of candidate compounds for a wide variety of applications in the medical and biotechnology fields. In the present work we developed an automated PTP1B inhibition-based assay that can be used for the initial screening of candidate insulin-enhancing compounds. We compared the inhibitory effects of 11 vanadium and zinc compounds, part of which have been previously described to have anti-diabetic effects (McNeill *et al.* 1992, Melchior *et al.* 2001, Kojima *et al.* 2003, Rehder 2003, Shechter *et al.* 2003).

A phosphatase inhibition assay employing DiFMUP as a substrate has been recently used to study the inhibition kinetics of BMOV (Peters *et al.* 2003). In the present study, we developed an automated DiFMUP-based PTP1B assay which can produce up to 12 dose–response curves, ranging from 10^{-4} to 10^{-9} M inhibitor, per 96-well plate, in 90 min. To minimize vanadium's oxidative effects on disulfide groups and avoid enzyme aggregation, the reducing agent, DTT, and the surfactant, Tween 20, respectively, were added to the assay buffer.

We compared the inhibition potencies of 11 vanadium and zinc compounds and calculated their IC_{50} values based on the means of three replicate experiments (Figure 1). Vanadium compounds had greater PTP1B inhibition potencies than zinc compounds and, of all compounds, $VOSO_4$ had the lowest IC_{50} value. Of the coordination complexes, compounds 5 and 1, respectively, were the strongest inhibitors of PTP1B. Kinetic analysis of the four strongest inhibitors showed a pattern of classical competitive inhibition, as exemplified by $VOSO_4$ (Figure 2). The K_i values of these four compounds were 0.11, 0.15, 0.63 and 0.7 μM for $VOSO_4$, 5, 1 and 2, respectively. In the case of BMOV, for example, the calculated K_i of 0.63 μM was lower than the previously reported value of 0.9 μM (Peters *et al.* 2003), possibly reflecting differences in assay conditions such as incubation times and buffer composition. Nevertheless, besides increased efficiency, one advantage of utilizing an automated system for many candidate compounds in one assay is the increased power of comparison between IC_{50} or K_i values generated under identical conditions. The fact that $VOSO_4$ had greater inhibition potency than the coordination complexes is consistent with the recent finding that uncomplexed vanadate ion (VO_4) is what actually binds to the active site of PTP1B (Peters *et al.* 2003).

Although much more is known on the anti-diabetic effects and possible modes of action of

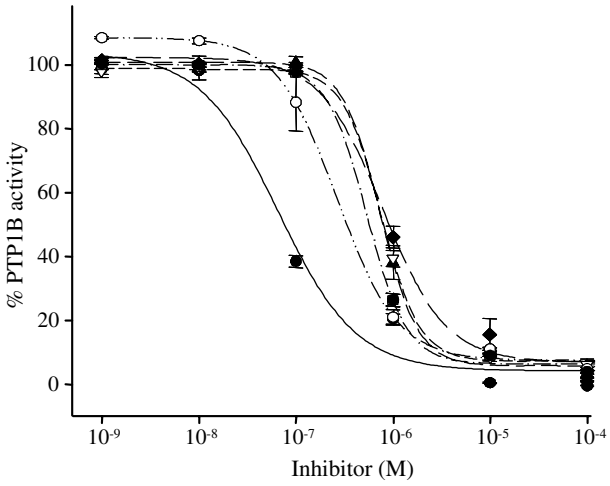


Fig. 1. Dose effect of six distinct vanadium compounds on percent PTP1B activity. Zinc compounds did not inhibit PTP1B activity at doses lower than 10 μM. IC_{50} values calculated from the regression curves fit for each inhibitor are: 0.06, 0.54, 0.75, 0.76, 0.80, 0.27 for ●, $VOSO_4$; and compounds ■, 1; ▲, 2; ▽, 3; ♦, 4; and ○, 5; respectively, and 10, 30, 30, 60 and 60 for compounds 6, 7, 8, 9 and 10, respectively (regression curves not shown for zinc compounds). Symbols represent mean percent activity ± SEM, $n = 3$ replicate experiments.

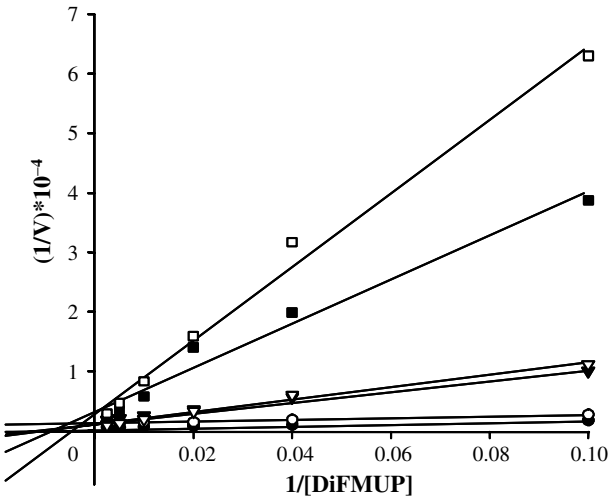


Fig. 2. Lineweaver–Burk plot of $VOSO_4$ at ●, 0 μM; ○, 0.1 μM; ▼, 1 μM; ▽,5 μM; ■, 10 μM; and □, 100 μM, over a range of substrate concentrations. Velocity (μmol min^{-1}) was calculated by using the molar extinction coefficient of 14 000 min^{-1} M^{-1} for DiFMUP. The enzyme kinetics pattern observed for PTP1B inhibition by $VOSO_4$ was similar to that observed for compounds 1, 2 and 5 (data not shown).

vanadium, zinc compounds have also begun to receive attention as potential organometallic anti-diabetic agents (Sakurai *et al.* 2002, Kojima *et al.* 2003). Zinc coordination complexes were shown to suppress free fatty acid release from rat adipocytes, and to exhibit *in vivo* blood glucose lowering effects (Sakurai *et al.* 2002), but zinc's possible involvement in PTP1B inhibition has not been examined to date. In the present study, zinc compounds were only effective at inhibiting PTP1B at concentrations greater than 10 μM, at least one order of magnitude greater than that required for vanadium compounds. Taken together, these results suggest that zinc and vanadium's anti-diabetic effects may occur through different prevailing modes of action. Further

studies will be required to understand the mechanisms of zinc action and expand our knowledge on the applications of organometallic compounds in the prevention and/or treatment of diabetes type 2.

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