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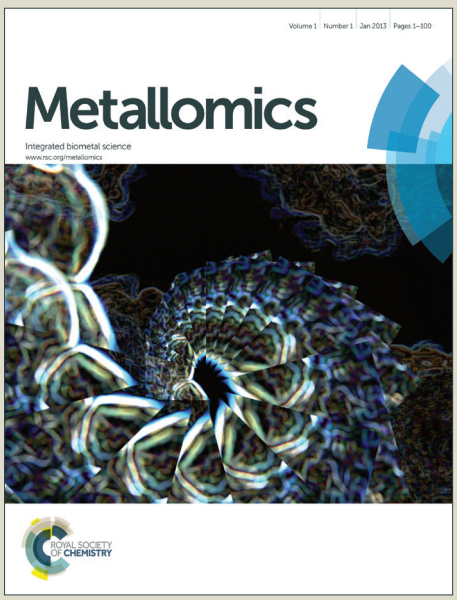

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Antidiabetic potentials of polyoxotungstates: *in vitro* and *in vivo* studies

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Abstract

Diabetes mellitus is a chronic metabolic disorder continuously affecting people all over the world. A common way to treat diabetes mellitus is to limit the conversion of carbohydrates into glucose which is mediated by glucosidase enzymes. Diabetes mellitus is also famous for its life-threatening microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (atherosclerosis) complications. Aldose reductases present in eye lens (ALR1) and kidney (ALR2) are responsible for microvascular complications. The production of advanced glycation end products (AGEs) is involved in the development of atherosclerosis. The present work was aimed at the synthesis and *in vitro/ in vivo* evaluation of different polyoxotungstates against glucosidases (α- and β), aldose reductases (ALR1 and ALR2) and AGEs to discover a new treatment which may limit the complications associated with diabetes mellitus. The compound $Na_{20}[P_6W_{18}O_{79}]\cdot 37H_2O$ was found to be the most potent inhibitor of α-glucosidase (IC_{50} = 1.33 ± 0.41 μM), ALR1 (IC_{50} = 0.4 ± 0.009 μM) and ALR2 (IC_{50} = 0.38 ± 0.02 μM). Animal studies showed that the compound $Na_6[H_2W_{12}O_{40}]\cdot 2H_2O$ was very effective in reducing blood glucose level to 84.25 ± 5.07 mg/dL when compared with standard antidiabetic drug glibenclamide (150.62 ± 9.35 mg/dL) measured after maximum 8 h of dose administration. The data obtained

from *in vitro* and *in vivo* experiments confirm that $Na_{20}[P_6W_{18}O_{79}]\cdot 37H_2O$ and $Na_6[H_2W_{12}O_{40}]\cdot 2H_2O$ could be used as a new treatment of diabetes mellitus.

Introduction:

The prevalence of metabolic syndrome including diabetes, obesity, and dyslipidemia is extraordinary and still rising in world population.[1-4] Diabetes is considered as one of the major causes of premature illness and death particularly in developing countries due to population ageing, expansion and unhealthy lifestyle.[2, 4, 5] The most appropriate way to control type 2 diabetes mellitus is to reduce intestinal carbohydrate absorption mediated by α-amylase and α-glucosidase enzymes.[6] Glucosidases (both α and β) are present at the brush border of small intestine and responsible for the conversion of glycosidic bond into oligosaccharide and finally into monosaccharide.[7] Regardless of similar enzymatic activity there is only one difference between α- and β-glucosidases related to the position of catalytic nucleophile and catalytic proton donor in carbohydrate moiety.[8-10] The chemicals which limit the activity of glucosidases at the brush border of small intestine play a significant role in delaying glucose absorption.[11-13] This delay may prevent rise in postprandial blood glucose level, the most desirable goal to treat type 2 diabetes mellitus.[14] Acarbose [15] is the first α-glucosidase inhibitor and the others include miglitol[16] and voglibose.[17]

All forms of diabetes are characterized by hyperglycemia and the development of microvascular and macrovascular complications.[18] As a consequence of microvascular pathology, diabetes is a leading cause of blindness, end-stage renal disease and a variety of incapacitating neuropathies.[19-21] Macrovascular complications are associated with increased risk of atherosclerotic plaque, myocardial infarction, stroke and limb amputation due to an increased accumulation of collagen (type IV and VI) and fibronectin in arteries which supply blood to the heart, brain and lower extremities.[22-24] The results obtained from clinical trials confirm a strong relationship between hyperglycemia and insulin resistance in the development of microvascular and macrovascular pathologies in diabetes mellitus.[21, 22, 24] A numerous mechanisms include glucose auto-oxidation, activation of protein kinase C (PKC), polyol pathway, protein glycation and accumulation of advanced glycation end products (AGEs) are triggered by hyperglycemia which may lead to the development of diabetic complications.[25, 26]

Aldose reductases (ALRs) responsible for diabetic microvascular complications are involved in the catalysis of nicotinamide adenine dinucleotide phosphate (NADPH) dependent reduction of aldehyde form of glucose into sorbitol.[27] Depletion of NADPH and accumulation of sorbitol and its metabolite (fructose) in cytosol lead to the progression of osmotic imbalance, altered-membrane-permeability and oxidative stress which cause tissue injury.[28] Accordingly, inhibition of ALR2 represents a potential therapeutic approach in reducing development of diabetic nephropathy.[29-31] An elevated level of ALRs has also been confirmed in other pathological states including inflammation, mood disorder, and human cancers of breast, cervix, liver, ovary and rectum. [25, 32, 33]

Polyoxometalates (POMs) are structurally diverse negatively charged aggregates of transition metal ions (mainly Vanadium, Molybdenum and Tungsten) in high oxidation state.[34-37] Polyoxotungstates (POTs) and the tungsten metal alone have been studied previously in diabetic mice and rats.[38-41] It has been reported that tungstate in small animals regenerates pancreatic beta cell function and stimulates insulin secretion.[38, 42-44] In the present work we have synthesized seven different POTs and tested *in vitro/ in vivo* to evaluate their potential to restore beta cells function and inhibit various enzymes involved in life-threatening complications of diabetes mellitus.

Materials and methods

p-Nitrophenyl α-D-glucopyranoside (pNPG) and Saccharomyces cerevisiae α-glucosidase, β-glucosidase from sweet almond, enzymes substrate (D,L-glyceraldehyde and sodium-D-glucoronate), and nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The materials were used as received without further purification. POTs $Na_6[H_2W_{12}O_{40}]\cdot 2H_2O$ (Na-$W_{12}O_{40}$),[45,46] $Na_{20}[P_6W_{18}O_{79}]\cdot 37H_2O$ (Na-P_6W_{18}),[47] $Na_{33}[H_7P_8W_{48}O_{184}]\cdot 92H_2O$ (Na-P_8W_{48}),[48] $Na_{16}[(O_3POPO_3)_4W_{12}O_{36}]\cdot 38H_2O$ (Na-OP_8W_{12}),[49] $Na_{16}[(O_3PCH_2PO_3)_4W_{12}O_{36}]\cdot 16H_2O$ (Na-OCP_8W_{12}),[49] $Na_{10}[H_2W_{12}O_{42}]\cdot 27H_2O$ (Na-$W_{12}O_{42}$),[50] $Na_6[TeW_6O_{24}]\cdot 22H_2O$ and (Na-TeW_6)[51] used in this study were synthesized according to the previously reported procedures.[52]

Glucosidase isoenzymes inhibition assays

Previously described assay methods of α-glucosidase[53] and β-glucosidase[54] enzymes were followed. Briefly, the solutions of α-glucosidase (2.5 U/ml) and β-glucosidase enzymes (2.0 U/ml) and their substrate *p*-NPG were prepared in 0.07 M phosphate buffer (pH 6.8). The assay was started with pre-incubation of enzyme (10 µL) with test compound (10 µL) at 37°C for 5 min. After pre-incubation, a10 µL of *p*-NPG (10 mM) was added to each well of 96 well plate and further incubated at 37°C for 30 min. The reaction was stopped by adding 80 µL of 0.2 M Na_2CO_3 solution. Negative control wells contain 10 µL of distilled water instead of test compounds, and the standard drug acarbose was used as a positive control. The activity of test compounds against α-glucosidase and β-glucosidase was determined by measuring *p*-nitrophenol at 405 nm wavelength. The percent inhibition was calculated with the following equation:

$$Percent\ Inhibition\ (\%) = \left[1 - \left({absorbance\ of\ sample}/{absorbance\ of\ control}\right) \times 100\right]$$

Dose-response curves of potential inhibitors (≥ 50%) were made and IC_{50} was determined with the help of GraphPad prism 5.0 Software Inc., San Diego California USA.

Isolation, purification and assay method of aldose reductases

A previously described extraction method for ALR1 and ALR2 was followed.[55] Briefly, calf eyes, and kidneys were obtained from a local abattoir and lenses were removed immediately. The obtained kidneys and lenses were separately homogenized for 20 min in 3 volumes of 10 mM sodium phosphate buffer (pH 7.2), contains sucrose (0.25 M), EDTA dipotassium salt (2 mM) and β-mercaptoethanol (2.5 mM). The homogenate was centrifuged at 10,000 rpm for 15 min to remove insoluble material. Supernatant was collected, centrifuged and dissolved in ammonium sulfate (40% w/v). This procedure was repeated twice with increasing concentration of ammonium sulfate up to 75% w/v where pure enzymes (ALR1 and ALR2) were precipitated, redissolved in NaCl (50 mM) and dialyzed overnight. After dialysis, sample volume was recorded and stored at -80 ºC until used.

A previously described method was adopted to evaluate the activity of test compounds against ALR1 and ALR2 isoenzymes which is based on measuring the consumption of NADPH

at a certain wavelength of 340 nm.[56] Briefly, the solution of enzyme, substrate (D,L-glyceraldehyde) and test compounds were prepared. The reaction was intiated by the addition of substrate (10 mM) after pre-incubation at 37 ºC for 10 min. Appropriate blanks were employed for the correction of oxidation associated with NADPH.

Antiglycation assay

The compounds were subjected to evaluate antiglycation activity by a previously described method.[57] Concisely, a 500 µL of albumin (1 mg/mL final concentration) was incubated with 400 µL of glucose (500 mM) in the presence of 100 µL of test compound after making 3-fold serial dilutions. The reaction was allowed to proceed at 60°C for 24 h and then stopped by adding 10 µL pure trichloroacetic acid (TCA). The mixture was kept at 4°C for 10 min and centrifuged at 10,000 rpm for 10 min. The precipitates were re-dissolved in 500 µL PBS (pH 7.4) and immediately quantified for relative amount of glycated BSA based on fluorescence intensity at excitation (370 nm) and emission (440 nm) wavelengths.

In vivo Hypoglycemic Activity of Compounds

Animals and maintenance

Healthy adult 8-10 weeks old male BALB/c mice (n = 20, average body weight = 30 g) were obtained from National Institute of Health, Islamabad, Pakistan and maintained in the animal house of pharmaceutical sciences department, COMSATS institute of information technology, Abbottabad- Pakistan. The animals were kept at 24°C on a 12 h light/dark cycle and adapted to standard rodent food with free access to water. To prevent stress due to over-crowding, five animals were housed per cage (5" × 9" × 11" made of steel mesh). The animal handling was done according to the guidelines provided by the "Ethics Committee on Care and Use of Animals for Scientific Research" of the Department of Animal Sciences.

Induction of Diabetes

An intra-peritoneal (IP) injection of alloxan monohydrate (150 mg/kg) was given to induce diabetes in mice.[58] Being small rodents, the mice have highly variable plasma glucose (75-100 mg/dL) levels.[59] Therefore, the mice with fasting blood glucose level > 200 mg/dL were considered diabetic and used for the current study.[60-64]

Experimental design

Animals were divided into four groups (n = 5) include positive control group (diabetic mice treated with glebanclamide (10 mg/kg)), negative control group (diabetic untreated mice), normal control group (non-diabetic normal mice treated with distilled water) and treatment group (diabetic mice treated with test compound). All groups were studied at the same time. For better understanding the control and treatment groups are described in Table 1.

Glucose Determination

After each dose of the test compound, the blood samples were drawn in a wakeful state through caudal venipuncture using a 26-gauge butterfly cannula at 0 h followed by 2, 4, 6 and 8 h. Blood glucose level was checked with a dextrostix using a hand-held glucometer (Accu-Check Active, Roche) with detection sensitivity varying from 50-700 mg/dL.

Results and discussion

The significance of tungsten metal containing compounds in biochemical diseases including diabetes, obesity and liver cirrhosis has been studied previously.[65-67] Here we tested POTs both *in vitro*/ *in vivo* to evaluate their potential to treat diabetes and its complications. Seven different POTs (Figure 1) were tested against α- glucosidase enzyme. Upon initial screening, compounds Na-$W_{12}O_{40}$, Na-P_6W_{18}, Na-OP_8W_{12} and Na-$W_{12}O_{42}$ showed ≥50% inhibition while compounds Na-P_8W_{48}, Na-OCP_8W_{12} and Na-TeW_6 were found inactive with percent inhibition of 19 ± 6%, 3 ± 1% and 13 ± 8%; respectively. The active compounds were further tested to determine IC_{50} value (see Figure 2). The compound Na-P_6W_{18} proved to be the most potent inhibitor among tested compounds with IC_{50} value of 1.33 ± 0.41 μM while compounds Na-$W_{12}O_{40}$, Na-$W_{12}O_{42}$ and Na-OP_8W_{12} showed less inhibition with IC_{50} of 3.24 ± 0.76, 3.93 ± 0.28 and 6.07 ± 0.91 μM; respectively (see Table 2). These compounds showed no activity when tested against β-glucosidase enzyme (see Table 2).

The test compounds showed good inhibition when tested initially against ALR1 and ALR2 except Na-P_8W_{48} with 13 ± 6% and 27.4% inhibition respectively. The compound Na-OP_8W_{12} was found inactive against ALR1 (see Table 2). The highest inhibition against ALR1 and ALR2

was seen by compound Na-P_6W_{18} with IC_{50} value 0.4 ± 0.009 and 0.38 ± 0.02 μM; respectively. (see Figure 3 and 4). The compound Na-TeW_6 was proved to be the least potent against ALR1 and ALR2 (IC_{50} = 1.12 ± 0.09 and 1.95 ± 0.84 μM; respectively), this could be attributed to the presence of tellurium which was absent in other tested compounds.

In vivo experiments in mice were conducted to determine whether these compounds were equally effective in lowering blood glucose level. Alloxan monohydrate was used to induce diabetes and further evaluate beta cells regeneration capability of tungstate present in POTs.[38, 68] The compounds Na-$W_{12}O_{40}$, Na-P_6W_{18}, and Na-OP_8W_{12} were chosen in the study and blood glucose level was checked after every 2 h and compared with standard anti-diabetic drug glibenclamide (see Table 3). The compound Na-$W_{12}O_{40}$ which has been proved to be the most potent inhibitor of α-glucosidase and aldose reductases was also active in lowering blood glucose level up to 84.25 ± 5.07 mg/dL after 8 h of dose administration.

Conclusions

Polyoxotungstates (POTs) were proven to be effective towards hyperglycemic control by inhibiting the activity of carbohydrate hydrolyzing enzymes like α-glucosidase. POTs also showed hypoglycemic potency against the enzymes ALR1 and ALR2 which are involved in the polylol pathway. POTs were employed to inhibit the formation of advanced glycation products. *In vivo* hypoglycemic activity of the POTs was also evaluated, resulting in good results. A comparison between *in vivo* and *in vitro* hypoglycemic activity of POTs was also conducted and illustrated interesting results. Epalrestat which is a potent inhibitor of ALR2 with K*i* value 7.7 nM is only effective in preventing diabetic complications without controlling blood glucose levels of diabetic patient.[69] In contrast our results showed that the compound Na-P_6W_{18} has proved to be the most potent inhibitor among all tested POTs which is capable of reducing blood glucose levels and also preventing diabetes induced complications. Thus Na-P_6W_{18} could be an ideal candidate for the management of hyperglycemia and diabetes associated complications.

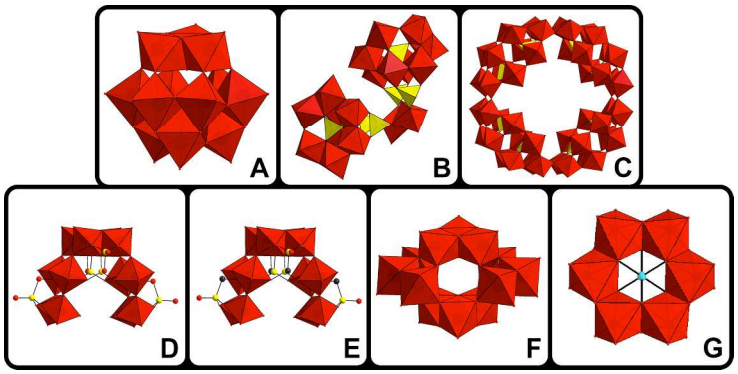

Figure 1: Polyhedral/ball-and-stick representation of Na-$W_{12}O_{40}$ (A), Na-P_6W_{18} (B), Na-P_8W_{48} (C), Na-OP_8W_{12} (D), Na-OCP_8W_{12} (E), Na-$W_{12}O_{42}$ (F), Na-TeW_6 (G). Color code; octahedra: WO_6 (red), PO_4 (yellow); balls: W (black), P (yellow), O (red), C (gray), Te (blue).

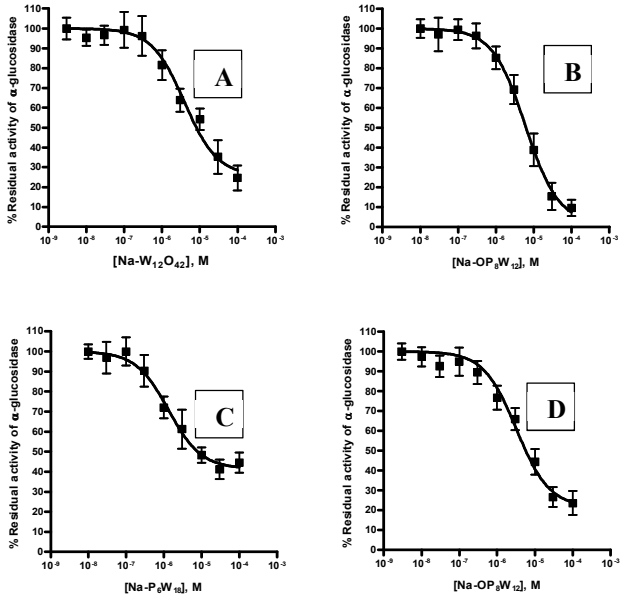

Figure 2. Concentration-dependent inhibition for selected, potent α-glucosidase inhibitors by polyoxotungstates (A) Na-$W_{12}O_{42}$ (B) Na-OP_8W_{12} (C) Na-P_6W_{18} and (D) Na-OCP_8W_{12}. Data points represent means ± SD from three separate experiments, each run in duplicates.

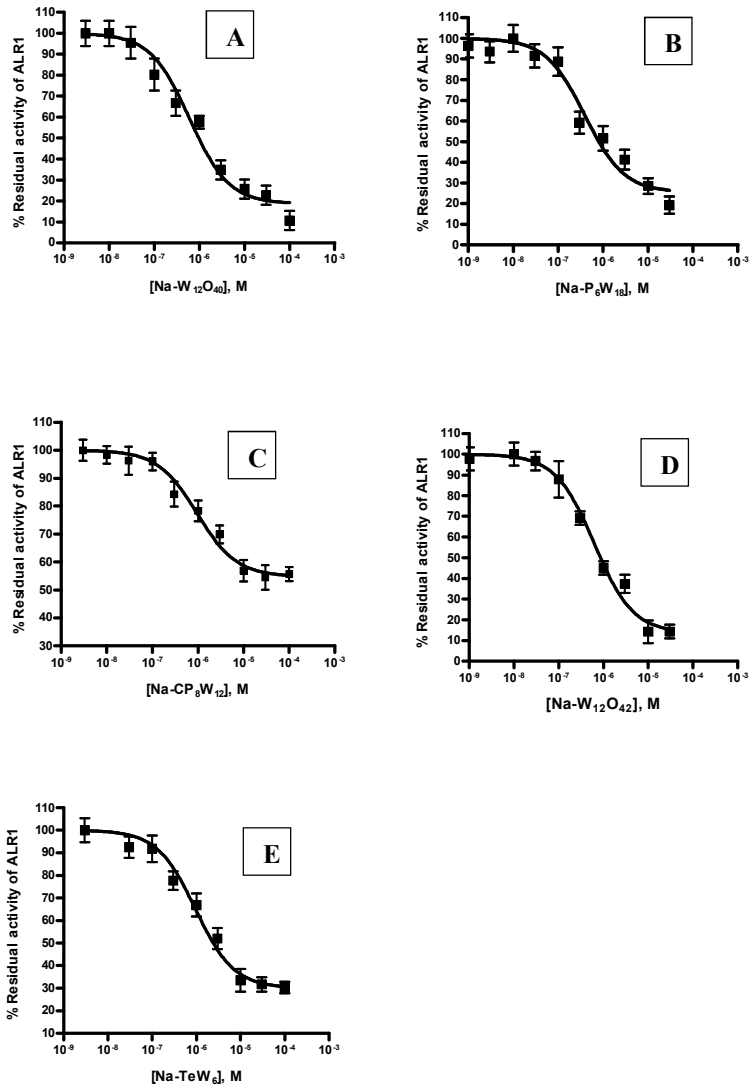

Figure 3. Concentration-response curves for selected, potent ALR1 inhibitors (A) Na-$W_{12}O_{40}$ (B) Na-P_6W_{18} (C) Na-CP_8W_{12} (D) Na-$W_{12}O_{42}$ (E) Na-TeW_6.

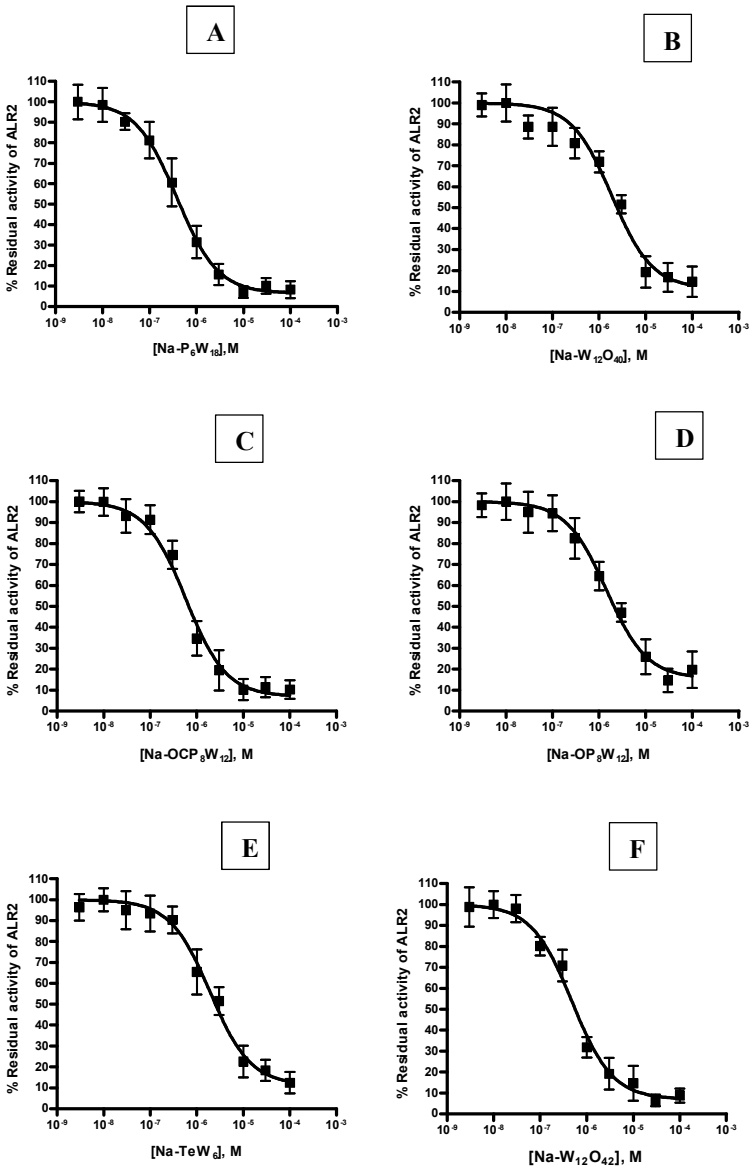

Figure 4. Concentration-response curves for selected, potent ALR2 inhibitors (A) Na-P_6W_{18} (B) Na-$W_{12}O_{40}$ (C) Na-OCP_8W_{12} (D) Na-OP_8W_{12} (E) Na-TeW_6 (F) Na-$W_{12}O_{42}$.

Table 1. Different control groups of mice which are used for in vivo experiment.

Group	Type of treatment
Positive control group	Diabetic mice treated with Glibenclamide
Negative control group	Untreated diabetic mice
Normal control group	Normal mice treated with distilled water
Treatment group	
• Group A	Compound Na-$W_{12}O_{40}$ treated
• Group B	Compound Na-P_6W_{18} treated
• Group C	Compound Na-OP_8W_{12} treated

Table 2. Potential activities of POTs against different enzymes associated with diabetic complications.

Code	Structural formula	β-glucosidase (%)	α-glucosidase IC_{50} [μM]	ALR1 IC_{50} [μM]	ALR2 IC_{50} [μM]	Antiglycation IC_{50} [μM]
Na-$W_{12}O_{40}$	$Na_6[H_2W_{12}O_{40}]\cdot 2H_2O$	12 ± 5	3.24 ± 0.76	0.5 ± 0.06	1.89 ± 0.34	86.4 ± 4.9
Na-P_6W_{18}	$Na_{20}[P_6W_{18}O_{79}]\cdot 37H_2O$	16 ± 8	1.33 ± 0.41	0.4 ± 0.009	0.38 ± 0.02	211.6 ± 8.5
Na-P_8W_{48}	$Na_{33}[H_7P_8W_{48}O_{184}]\cdot 92H_2O$	33 ± 11	19 ± 6	13 ± 6	27 ± 9	14.80 ± 2.36
Na-OP_8W_{12}	$Na_{16}[(O_3POPO_3)_4W_{12}O_{36}]\cdot 38H_2O$	23 ± 9	6.07 ± 0.91	21 ± 10	1.44 ± 0.19	246.46 ± 5.54
Na-OCP_8W_{12}	$Na_{16}[(O_3PCH_2PO_3)_4W_{12}O_{36}]\cdot 16H_2O$	12 ± 4	3 ± 1	0.99 ± 0.08	0.58 ± 0.07	251 ± 5
Na-$W_{12}O_{42}$	$Na_{10}[H_2W_{12}O_{42}]\cdot 27H_2O$	20 ± 7	3.93 ± 0.28	0.56 ± 0.10	0.48 ± 0.02	624 ± 9
Na-TeW_6	$Na_6[TeW_6O_{24}]\cdot 22H_2O$	28 ± 12	13 ± 8	1.12 ± 0.09	1.95 ± 0.84	419 ± 4

Table 3. Blood glucose concentration (mg/dL) in diabetic mice treated with standard drug and selected polyoxotungstates.

Time (hours)	Non-diabetic mice treated with distilled water (mg/dL ± SEM)	Compound (Treatment group)			Standard drug (Positive control)
		Na-$W_{12}O_{40}$ (mg/dL ± SEM)	Na-P_6W_{18} (mg/dL ± SEM)	Na-OP_8W_{12} (mg/dL ± SEM)	Glibenclamide (mg/dL ± SEM)
0	92.63 ± 6.71	225.21 ± 3.65	211.61 ± 4.78	266.81± 3.14	245.27 ± 8.94
2	95.47 ± 4.64	165.84 ± 5.15	190.05 ± 4.97	252.81 ± 3.40	229.48 ± 10.17
4	99.82 ± 5.98	128.27 ± 3.21	194.23 ± 5.28	241.65 ± 3.89	210.07 ± 4.30
6	87.35 ± 9.62	94.83 ± 3.59	194.47 ± 5.10	232.42 ± 4.08	170.68 ± 7.07
8	94.21 ± 3.89	84.25 ± 5.07	190.62 ± 3.90	223.26 ± 5.02	150.62 ± 9.35

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