In vitro study of the antidiabetic behavior of vanadium compounds

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Abstract

The paper deals with the so far most efficient antidiabetic transition metal compound family. It focuses on the species distribution of the most frequently studied vanadium(IV,V) compounds in biology: in the gastro-intestinal tract (being important in absorption of the compounds), the blood serum (very likely the main route of their transport), the whole blood (recently the role of the red blood cells are also assumed in their transport) and in the cells (where glutathione and ATP may be the most important redox and complex formation partners of the original vanadium-"insulinomimetics").

The discussed details fit into the general view, but far from a complete and clear understanding of the pharmacodynamics of these antidiabetics. A lot more in vitro and mostly in vivo studies are necessary to justify their real clinical use.

Contents

Abbreviations: **GI**, gastrointestinal; **LMM**, low molecular mass; **HMM**, high molecular mass; **HIV**, human immunodeficiency virus; **DM**, diabetes mellitus; **STZ**, streptozotocin; **BMOV**, bis(maltolato)oxovanadium(IV); **BEOV**, bis(ethylmaltolato)oxovanadium(IV); **acac**, acetylacetone; **mal**, maltol (3-hydroxy-2-methyl-4-pyrone); **imal**, isomaltol [1-(3-hydroxy-2-furanyl) ethanone]; **amal**, allomaltol (3-hydroxy-6-methyl-4-pyrone); **emal**, ethyl maltol (3-hydroxy-2-ethyl-4-pyrone); **ipmal**, isopropyl maltol (3-hydroxy-2-isopropyl-4-pyrone); **alx**, allixin (3-hydroxy-5-methoxy-6-methyl-2-pentyl-4-pyrone); **koj**, kojic acid [5-Hydroxy-2-(hydroxymethyl)-4-pyrone]; **cur**, curcumin; **hpno**, 2-hydroxypyridine *N*-oxide; **dhp**, 3-hydroxy-1,2-dimethyl-4(1*H*)-pyridone; **hopy**, 1-hydroxy-2(1*H*)-pyrimidone; **dmhopy**, 1-hydroxy-4,6-dimethyl-2(1*H*)-pyrimidone; **mhopy**, 1-hydroxy-6-methyl-2(1*H*)-pyrimidone; **pic**, picolinic acid; **6mpic**, 6-methylpicolinic acid; **6etpic**, 6-ethylpicolinic acid; **5ipic**, 5-iodopicolinic acid; **3mpic**, 3-methylpicolinic acid; **3hpic**, 3-hydroxypicolinic acid; **dipic**, 2,6-dipicolinic acid; **biguad**, biguanide; **metf**, metformin (*N'*,*N'*-dimethylbiguanide); **mpno**, 2-mercaptopyridine *N*-oxide; **ROS**, reactive oxygen species; **XANES**, X-ray absorption near edge structure; **Tf**, transferrin(human); **apoTf**, apotransferrin(human); **HSA**, human serum transferrin; **his**, histidine; **asp**, aspartic acid; **tyr**, tyrosine; **gly**, glycine; **NTS**, N-terminal (binding) site; **ATCUN**, amino terminal Cu(II)- and Ni(II)-binding; **MBS**, multiple binding sites; **IgG**, immunoglobulin G; **EPR**, electron paramagnetic resonance; **CD**, circular dichroism; **ICP-MS**, inductively coupled plasma mass spectrometry; $\mathbf{LC_{50}}$, lethal concentration 50%; **Hb**, hemoglobin; **RBC**, red blood cell; **GSH**, glutathione; **GSSG**, glutathione disulfide; **NADH**, nicotinamide adenine dinucleotide (reduced form); **ATP**, adenosine triphosphate;

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1. Introduction

Progress in the bioinorganic chemistry of vanadium and the search in the therapeutic applications have been exponential and numerous reviews have been published in recent years [1-8]. Vanadium compounds have been shown to be potentially effective against diabetes, malign tumors including cancer, endemic tropical diseases (such as trypanosomiasis, leishmaniasis and amoebiasis), bacterial infections (tuberculosis and pneumonia) and HIV infections. Furthermore, vanadium drugs can be operative in cardio- and neuro-protection. So far, vanadium compounds have not yet been approved as pharmaceuticals for clinical use [1]. In this paper we will review the antidiabetic effects of vanadium compounds; other terms as insulin mimetic or insulin-enhancing are also used in the literature. However it is believed that in the complete lack of insulin (how can it be achieved in a living organism?) vanadium is not showing any insulin like effects, accordingly vanadium can only enhance the effects of insulin [2]. For this reason mostly we use the rather neutral antidiabetic expression for the blood glucose lowering effect (as one of the most important role of insulin in biology) of vanadium compounds.

2. Insulin enhancing metal complexes in biological fluids

Diabetes mellitus (‘diabetes’ or DM) is a group of metabolic diseases in which there is high blood sugar level over a prolonged, sometimes nearly a lifetime period. It is induced by a non-existent or insufficient insulin supply, or inadequate response to insulin. In the case of DM type 1 the insulin-producing cells in the body have been destroyed, while in the case of DM type 2 the cells of the body are not responding properly (partial resistance) to the insulin produced. As of 2015, an estimated 415 million people had diabetes worldwide with type 2 DM [9], which usually appears in people over the age of 40, making up about 90% of the cases [10]. Type 1 diabetes can be treated only with insulin, a natural peptide (protein) hormone composed of 51 amino acids, also over 40% of patients with type 2 diabetes require, as part of their treatment, insulin injections. However, peptides currently cannot be taken orally because of their degradation in the GI tract, insulin is given as an injection [11]. This painful and expensive treatment could be replaced by an insulin ’mimicker’ which can be taken orally. Many metal ions such as Cr(III), Mn(II), Mo(VI), Se(V), V(IV/V), W(VI) and Zn(II) have insulin-like effect.

2.1. Insulin-enhancing vanadium compounds

Although most foods contain low concentrations of vanadium (0.1 ng g^{-1}), food is the major source of exposure to vanadium for the general population. The absorption of vanadium salts from the gastro-intestinal tract is poor in humans, and the excretion of Vanadium in the kidneys is relatively rapid with a half-life of 20–40 h in urine. Therefore, the toxicity of vanadium compounds is low in general. The estimated vanadium intake of the US population ranges from 10–60 mg V day^{-1}. $VO(IV)SO_4$ is a common supplement used to enhance weight training in athletes at doses of up to 60 mg day^{-1}. In humans, the threshold level for vanadium toxicity is concluded to be approximately 10–20 mg V day^{-1} [4].

Insulin-like effects of vanadium were first demonstrated *in vivo* by McNeill and co-workers [12] by simple adding of sodium orthovanadate (Na_3VO_4) to the drinking water of streptozotocin (STZ)-diabetic rats for several weeks. Later the only partly soluble vanadates were replaced by $VO(IV)SO_4$ and the result was fewer negative side effects [13-15]. However the applied necessary dose of vanadium still was close to the levels at which adverse effects are observed [16]. The question became then, ‘‘is there some way to chemically improve potency of these drug candidate antidiabetic agents?’’. Several vanadium complexes with different coordination mode were synthesized and characterized in order to answer this question. The most frequent basic structures (‘backbones’) are depicted in **Fig. 1**. A key compound in this regard is the bis(maltolato)oxovanadiumV(IV) complex ($VO(IV)(mal)_2$ or BMOV) [17,18], in **Fig. 1**, and its derivative the ethylmaltol complex [2] and allixin [19,20]. The related complex with the pyridinone complex $VO(IV)(dhp)_2$ have also been prospective therapeutics [21]. Several other complexes have been studied, such as $VO(IV)(acac)_2$, $VO(IV)(pic)_2$, $VO(IV)(hpno)_2$, $VO(IV)(mpno)_2$, $VO(IV)(metf)_2$ (the bis complex of metformin an antidiabetics itself) and several picolinato and dipicolinato derivatives. Most of them are neutral *bis* complexes formed with bidentate organic ligands (**Table 1a** and **1b**), where the oxidation state of the vanadium is +4. One exception (**Table 1c**) is the $[VO_2(V)(dipic)]^-$ with negative charge and +5 oxidation state of the central metal ion [22]. All these complexes are labile, and ready to take part in fast ligand exchange reactions.

Table 1a Successful insulin mimetic *in vivo* tests with $VO(IV)L_2$ complexes where the ligands (L) have (O,O) donor set

L	Dose	Duration	Blood glucose level (mM) / animal model							Ref.
	mmol V / kg /day	days	STZ rat o[a]	STZ rat i.p.[b]	STZ mice	ZDF rat o[a]	KKA' mice o[a]	Ref comp.	Ref. value (mM/%)	
mal type:										
imal	0.6	1	17(3)					BMOV	14(2)	[23]
emal	0.6	1		6.5(1.7)				BMOV	7.1(1.6)	[24]
emal	0.1	18				~22				[25]
ipmal	0.1	1	~13					BMOV	~12	[24]
alx	0.06/0.14	30					~10			[26]
alx	0.1/0.06/0.02	6			~10[b]			BMOV	~15[b]	[19]
alx	0.14	9			~12[a]					[20]
hpno type:										
hpno	0.15	20	~10							[27]
hpno	0.05	8		~8						[27]
hopy type:										
mhopy	0.02-0.1	14	~12							[28]
dmhopy	0.1/0.08	14		~11						[29]
acac type:										
cur	0.1	2	no eff.	l.r.[c] (~14%)				BMOV	47%	[30]

[a] Oral gavage

[b] Intraperitoneal injection

[c] Low response (% decrease)

Table 1b Successful insulin mimetic *in vivo* tests with $VO(IV)L_2$ complexes where the ligands (L) have (O,N) or (O,S) or (N,N) donor set

L	Dose mmol V / kg /day	Duration days	Glucose level (mM) / animal model				Ref comp.	Ref. value (mM)	Ref.
			STZ rat		KKA' mice				
			o[a]	i.p.[b]	o[a]	i.p.[b]			
pic type (O,N donors):									
pic	0.48	42	14(2)						[31]
pic	0.06			16(1)					[31]
pic	0.2/0.1	14	8.5/8.1	8.1(1.3)/ 12					[32]
6mpic	0.06/0.02	14		~10					[33]
6mpic	0.2/0.1	100	~10						[33]
6mpic	0.05	12				~14	$VOSO_4$	~22	[34]
6mpic	0.2	14			~17				[34]
6mpic	0.06/0.04/0.02	14		12(3)					[35]
6etpic	0.05/0.04	13				~10			[36]
5ipic	0.1/0.05/0.02	28 /14	8(3)	10.7(6)					[37]
3mpic	0.1/0.05/0.02	14		6.1(2)					[35]
3hpic	0.1/0.05/0.02	14		6.1(1.5)					[35]
mpno type (O,S donors):									
mpno	0.2	20	~8						[27]
mpno	0.05	8		~9					[27]
biguad type (N,N donors):									
metf	0.6	3	< 10				BMOV	~11	[38]
metf	0.12	3		~18			BMOV	~10	[38]

[a] Oral gavage

[b] Intraperitoneal injection

Table 1c Successful insulin mimetic *in vivo* tests with complexes having other than $VO(IV)L_2$ composition (L = ligand)

Compounds compositions	L	Dose	Duration	Glucose level (mM) / animal model STZ rat		Ref comp.	Ref. value (mM)	Ref.
		mmol V / kg /day	days	o[a]	i.p[b]			
$VO(IV)L$	*dipic* type, (O,N,O donors)							
	dipic	not given	28	~21		$VO(IV)SO_4$	~15	[39]
$V(III)L_2$	*dipic* type, (O,N,O donors)							
	dipic	not given	28	~18		$VO(IV)SO_4$	~15	[39]
$V(III)L_3$	*mal* type, (O,O donors)							
	imal	0.1	1		17(3)	BMOV	11(4)	[23]
	amal	0.1	1		16(3)	BMOV	14(4)	[23]
	mal	0.6[a] / 0.1[b]	1	15(3)	12(4)	BMOV	16(2)[a] / 8(3)[b]	[40]
	emal	0.6[a] / 0.1[b]	1	19(3)	11(2)	BMOV	16(2)[a] / 8(3)[b]	[40]
	koj	0.1	1		20(1)	BMOV	8(3)	[40]
	dhp type, (O,O donors)							
	dhp	0.1	1		20(1)	BMOV	8(3)	[40]
$VO_2(V)L^-$	*dipic* type, (O,N,O donors)							
	dipic	not given	28	~12.5		$VO(IV)SO_4$	~15	[39]

[a] Oral gavage

[b] Intraperitoneal injection

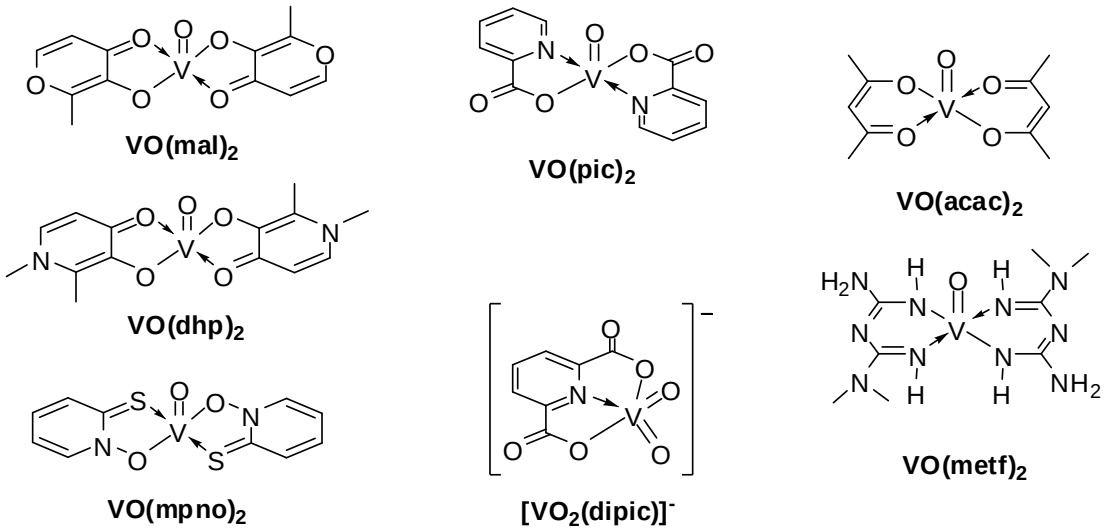

Fig. 1. Basic structure of insulin mimetic vanadium complexes studied in detail. (The water molecules in the coordination sphere are omitted.)

One comparative study of some of these drug candidate vanadium complexes has been made, and it was pointed out that these complexes have 30–70% of the activity of insulin in insulin-depleted mice fibroblast cell culture tests [41]. Based on that publication it would be impossible to differentiate between the effectiveness of the vanadium compounds studied. Similar result, the small efficacy difference between the vanadium compounds can be concluded from **Table 1**. The decreased polarity can increase the insulin mimetic activity, but just until a certain limit [19].

Only one of these compounds were tested in humans: the Phase I clinical trial [16] of *bis*(ethylmaltolato)oxovanadium(IV) (BEOV, the ethyl- derivative of VO(IV)(maltolato)$_2$) was completed in 2000, and the results of the Phase IIa clinical trial were first published in 2009 [2]. The clinical Phase I trials proved that an orally administered 10-90 mg BEOV single dose is safe and tolerable by healthy people, overall bioavailability of vanadium from BEOV was three times higher than that of vanadium from VO(IV)SO_4 and fasted subjects absorbed vanadium thirteen times better than fed subjects [16].

In Phase IIa tests over the course of a 28-day treatment period (daily dose 20 mg complex: 5.8 μmol or 3 mg vanadium), BEOV was consistently well-tolerated. A positive treatment effect was observed in most of the treated subjects, such that reductions in fasting blood glucose were observed when compared to the two placebo subjects [2]. The tests have, however, been abandoned due to renal problems with some of the probands [42].

The orally administered vanadium complex should go first through the GI tract and after the blood serum before arriving to the targeted cell. Biotransformation is possible in the entire route, and the absorption efficacy from the GI tract can be improved by formulation of the drug including various encapsulation techniques [43]. In spite the fact of the promising animal results these did not lead to any clinical tests with humans. However, the original carrier ligand is very likely to be replaced by serum/plasma components or endogenous binding molecules, before the cells would be able to take up the complex.

The active species is vanadate ($H_2VO_4^-$) most likely, one possible final product of the biotransformation of any kind of vanadium compound. The possible mode of action is the inhibition of protein tyrosine phosphatases at the cytosolic site of the cellular insulin receptor and/or the activation of a tyrosine kinase in the signaling pathway [44]. It has also been proposed that cellular redox processes, increases production of ROS is also involved in the antidiabetic effects of the vanadium compounds [45,46].

2.2. Speciation of vanadium complexes in the gastrointestinal (GI) tract

Based on speciation studies [6,8] it is clear that in consequence of the parallel processes of protonation of the metal-binding sites of the coordinating ligands, the neutral *bis* complexes (VO(IV)(mal)$_2$ [47]. VO(IV)(dhp)$_2$ [48], VO(IV)(pic)$_2$ [49], VO(IV)(mpno)$_2$ [50], VO(IV)(acac)$_2$ [51] and their derivatives) will certainly partly decompose in the acidic pH range, *e.g.* at the pH (*ca.* 2) of the gastric juice. The species formed in this way will be charged, and will possess entirely different membrane transport properties. The $[VO_2(V)(dipic)]^-$ complex is stable at pH 2 [22] but it has already a –1 charge.

Recently a useful speciation method, assessment of the chemical states of V, in biological environments was published [52]. Classification can be done on the basis of a three-dimensional diagrams of pre-edge and edge parameters in X-ray absorption near edge structure (XANES) spectra, developed on the basis of a library of model V(V/IV/III) complexes. Based on this method XANES results reported the speciation of four vanadium compounds mimicking oral administration by artificial digestion [53], although the applied vanadium concentration was fairly high, namely 1.0 mM. (Artificial gastric/intestinal juice has been prepared; incubations and commercial liquid semi-synthetic meals were used.)

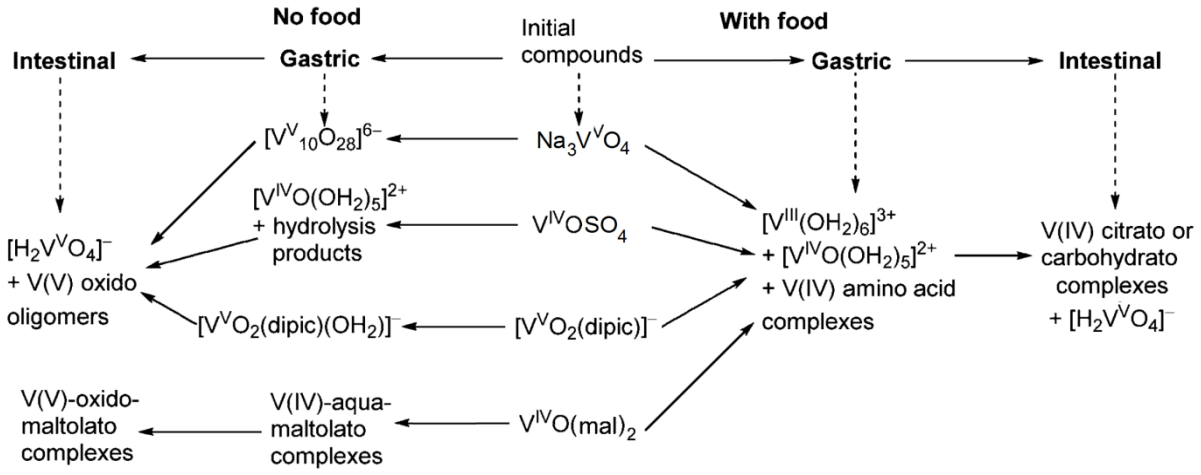

Fig. 2. Proposed biotransformation of the initial vanadium compounds in GI environment based on XANES data taken after artificial digestion. (c_V = 1.0 mM) Taken from Ref. [53].

Typical antidiabetic V(V) and V(IV) complexes undergo profound chemical changes in GI media. The main observation is that in the absence of food V(IV) is oxidized to V(V) only in the intestine and only the dipic dissociates (intestinal), while mal does not. While in the presence of food reduction of V(V) to V(IV) and even to V(III) takes place already in gastric environment together with the total dissociation of the original carrier ligands (Fig. **2.**). The observed significant difference between the presence and absence of food is in a complete coincide with the absorption difference of BEOV between the fasted/fed subjects reported in Phase I trial [16].

As the overall bioavailability of BEOV was three times higher than that of vanadium from $VO(IV)SO_4$ (Phase I[16]) it seems that passive diffusion of neutral species is the most effective absorption process, while the other possibilities *e.g.* vanadate like phosphate or $VO(IV)^{2+}$ species via M^{2+} uptake mechanisms or V(III)/Fe(III) pathways are less important.

All exogenous and endogenous biomolecules being present in the stomach or intestines, where the complexes are absorbed, may play a role in VO(IV) binding. Interactions with these molecules could change the net charge of the complex unfavorably, which will decrease their absorption efficacy. This certainly has to be taken into account during the formulation of the drug (*e.g.* by encapsulation techniques, whereby these problems may well be overcome), results of H. Sakurai et al. [43] support this prediction. In their study $VO(IV)SO_4$ was administered orally in various formulations: in solution, in gelatin capsules and in enteric-coated capsules. It was found that administration of the VO(IV) salt in encapsulated forms improved the metal ion absorption as compared with that associated with the simple solution

form. As far as we know, up to now, no such experiment has been made with vanadium complexes.

These results, together with direct pharmacokinetic measurements [24] reveal that these antidiabetic vanadium compounds dissociate soon after oral administration and absorption and the vanadium part is the active metabolite responsible for the antidiabetic effects. Then what is the role of the carrier ligands? The most obvious answer is to enhance the absorption of the active component and thus to decrease the dose of vanadium.

2.3. Speciation of vanadium with low molecular mass (LMM) constituents of blood serum

After absorption during their transport in the bloodstream, (ternary) complex formation with the serum components, as active vanadium binders has to be considered. From among the LMM constituents the most potent binders e.g. lactate (1,51 mM), phosphate (1,10 mM), citrate (99 μM), and oxalate (9 μM) have been studied. The binding strength of other serum components to vanadium such as amino acids and sulfate are negligible besides these LMM binders. As an illustration the species distribution of the three most studied antidiabetic VO(IV) complex is listed in **Table 2** to show the importance of these LMM serum constituents in VO(IV) binding.

Table 2 Species distribution of three insulin-mimetic VO(IV) compounds (10 μM) in the low molecular mass fraction of blood serum at pH 7.4 (Adapted from Ref. [47])

VO(IV) binder molecule (from serum)	VO(IV)% bound		
	$VO(IV)(mal)_2$	$VO(IV)(pic)_2$	$VO(IV)(6\text{-Me-pic})_2$
In ternary complexes with			
citrate	14	42	30
In binary complexes with			
citrate	73	50	59
lactate	5	3	8
phosphate	8	5	2

It is seen from the data in **Table 2** that of the LMM bioligands, citrate might have a pronounced effect on the serum solution state of vanadium. Lactic acid and phosphate have minor roles and oxalate a negligible role in VO(IV) binding [47].

2.4. Speciation of vanadium with high molecular mass (HMM) constituents of blood serum

From among the serum proteins transferrin, albumin, immunoglobulins are the most important vanadium binders. In principle the metal ion can bind to these proteins in the oxidation states of +3, +4 and +5. Large numbers of works have been made in this field to clarify the interactions both qualitatively and quantitatively and the results published in numerous review papers [1,3,4,6,8] Binding can occur in metal-protein binary interactions, and in forms of ternary complexes when the carrier ligands also participate in binding. Stability constants of the most likely complexes formed with the HMM components of blood serum are listed in **Table 3**.

Table 3 Stability constants (log β) for the species formed by V(III), $VO(IV)^{2+}$ and V(V) ions with blood proteins.

Human Serum Transferrin (apoTf)			Human Serum Albumin (HSA)			Immunoglobulin G (IgG) or Hemoglobin (Hb)		
Species	log β	Ref.	Species	log β	Ref.	Species	log β	Ref.
V(III)apoTf	20.0±1.5	[54]						
VO(IV)apoTf	13.4±0.2	[6]	VO(IV)HSA	9.1±0.4	[58]	VO(IV)IgG	10.3±1.0	[60]
	13.0±0.5	[55]		9.1±1.0.	[55]	VO(IV)Hb	10.4±1.0	[60]
VO(IV)(mal)apoTf	17.7±0.2	[6]						
			$VO(IV)(mal)_2HSA$	17.2±0.1	[a]			
$(VO(IV))_2apoTf$	25.2±0.4	[6]	$(VO(IV))_2HSA$	20.6±0.4	[58]			
	25.5±0.5	[55]		20.9±1.0	[55]			
V(V)apoTf	6.0±0.1	[56]	V(V)HSA	1.8±0.3	[56]			
	7.5±0.2	[57]		3.0	[59]			
$V(V)_2apoTf$	11.5±0.3	[56]						
	14.1±0.5	[57]						

[a] Calculated using data from Ref. [48,61]

The order of affinity of the three vanadium oxidation states towards human apoTF is therefore: V(III)°>°VO(IV) > V(V), in the presence of carbonate and V(III) ~ VO(IV) > V(V) in its absence [3]. In one type of complexes vanadium is assumed to bind at the Fe(III) binding site [61] and in the bis- metal complex a closed conformation is assumed for strong vanadium binding. Carbonate or hydrogen-carbonate is needed as synergistic anion in the oxidation state of +3 and +4 for strong binding [3]. Other anions as lactate can also behave as

synergistic in some cases [55]. A much weaker binding of the metal ion is assumed primarily in oxidation state of +4 through the surface His imidazole-N donor. This binding mode is assumed mostly in the case of the ternary VO(IV)-apoTf-carrier ligand complexes [55] (**Fig. 3**). The actual donor atoms of the Fe-binding site coordinated to VO(IV) may be two of the four residues Asp63, Tyr95, Tyr188 and His249 of the N-terminal lobe of human apoTF, and/or two of residues His585, Asp392, Tyr426 and Tyr517 of the C-terminal lobe 2. In the case of Type 2 binding, histidine residue may be His14, His289, His349, His350, His473, His606 and His642.

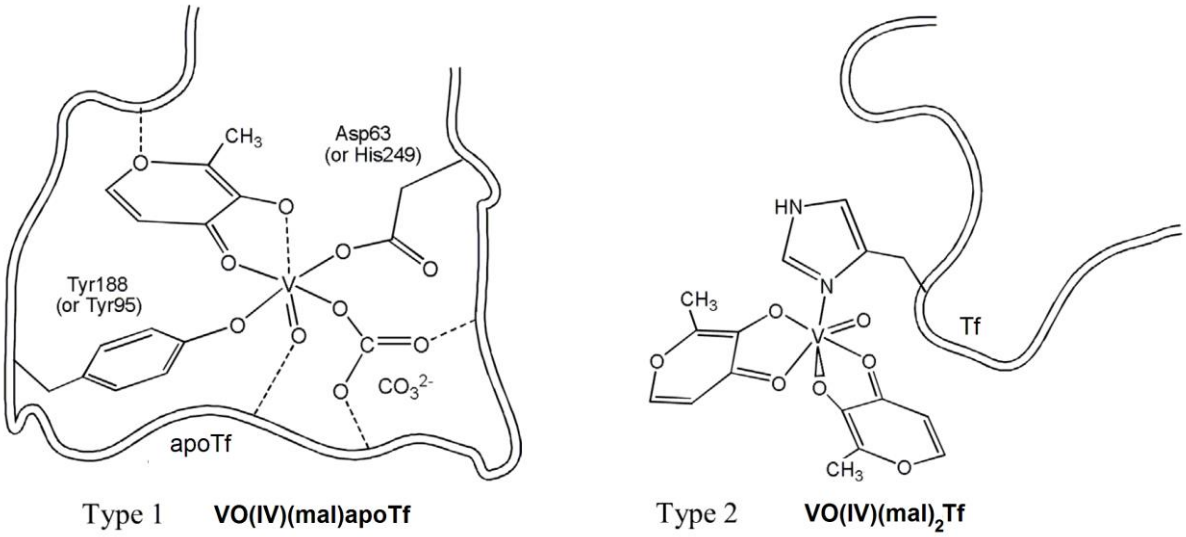

Fig. 3. Binding mode for VO(IV)-complexes to transferrins; example of BMOV. In the case of Type 1 binding, the VO(IV) : carrier : apoTf stoichiometry is 1 : 1 : 1 or 2 : 2 : 1. Adapted from Ref. [3] and partly modified.

HSA, a globular protein is the other important metal binding serum protein. It contains two strong binding sites, one at the N-terminus (NTS/ATCUN), which is specific among others for Cu(II) and Ni(II), and the other is the multi-metal binding site (MBS) which is specific among others for Zn(II), Cd(II) and Co(II). Detailed studies in the binary VO(IV)-HSA system revealed two different metal ion binding a strong one, later assigned to the MBS site and a weak one, later assigned binding to side chain carboxylate of Asp/Glu or imidazole-N of His [58].

Immunoglobulins are various glycoproteins (divided into different classes); among them only IgG occurs in high enough concentration (an average of 84 μM) to be a potential metal ion binder. EPR studies in the VO(IV)-IgG system revealed that the binding mode resemble to that of the weak binding of VO(IV)-HSA [3].

Many authors revealed ternary complex formation in VO(IV)-serum protein-carrier ligand systems by detailed EPR and CD studies. One example of the most likely binding modes of these complexes is depicted in **Fig. 4**.

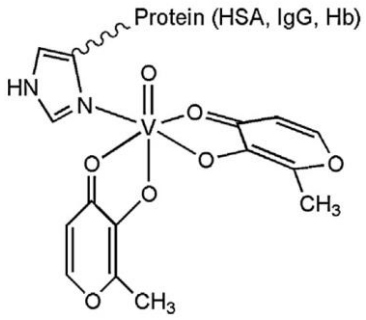

Fig. 4. Mixed species VO(IV)$(mal)_2$(Protein) formed in aqueous solution at pH 7.4, Protein indicates human serum albumin (HSA), immunoglobulin G (IgG) and hemoglobin(Hb). Adapted from Ref. [3,55].

2.5 Speciation of vanadium(IV) and vanadium(V) in blood serum

The maximum daily oral dose in the Phase I clinical trial was 95 mg BMOV, equivalent to 15 mg or 0.22 mg/kg vanadium for a 70 kg person [16]. For an absorption efficacy of 30% [62] and an overall blood content of 5 L, if all of this vanadium enters the blood at the same time, the maximum concentration attainable would be ~20 μM. However, this is only a rough estimation, but it clearly shows a well-defined limit. In animal studies involving much higher doses up to 12 mg/kg vanadium, two independent research groups determined the maximum vanadium concentration in the blood to be 2–3 μg/mL, *i.e. ca.* 40–60 μM. The maximum value of the vanadium concentration in the human blood during treatment (Phase I-IIa) was not published [2,16]. Modeling calculations were performed in order to explore the potential biotransformation processes in serum [6,8].

The speciation [63,64] of the metal ion among the LMM and HMM components of blood serum, and the original carrier ligands, including mixed ligand species was calculated based on stability constants and concentration data at three concentration levels of antidiabetic compounds (1, 10 and 100 μM), the results are summarized in **Fig. 5**.

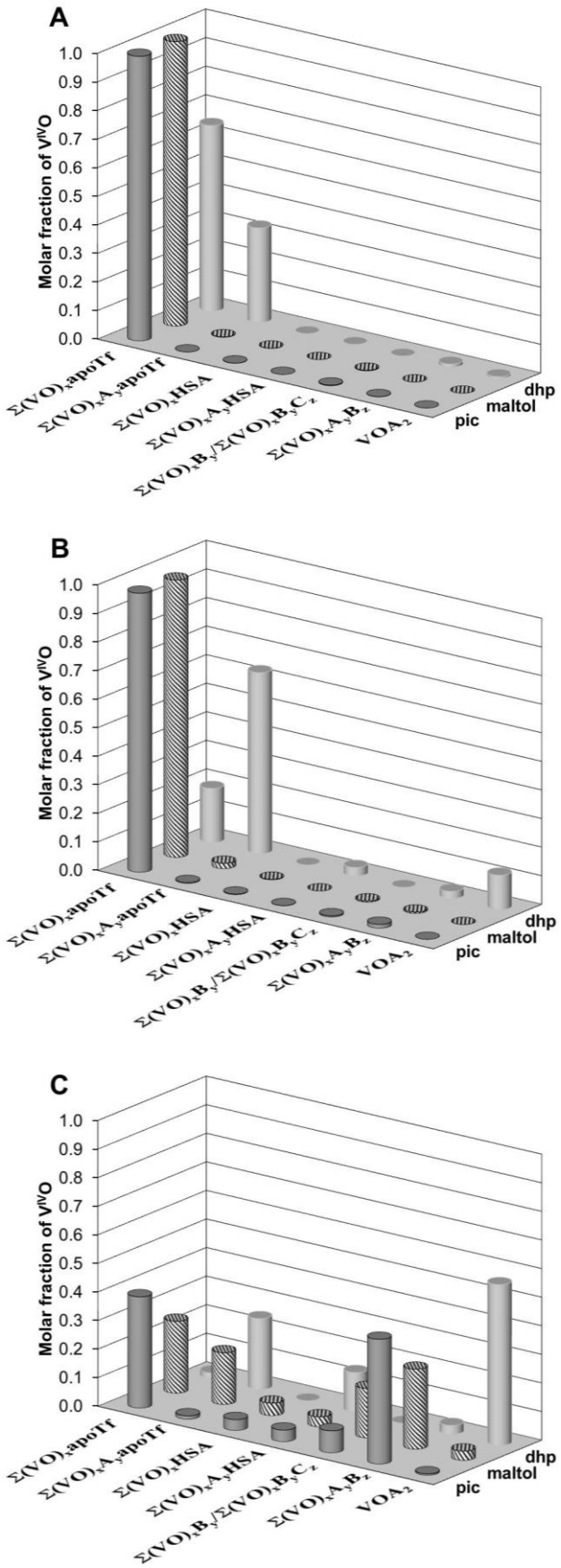
A
B
C
Molar fraction of V^IVO
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Σ(VO)xapoTf
Σ(VO)xAyapoTf
Σ(VO)xHSA
Σ(VO)xAyHSA
Σ(VO)xBy/Σ(VO)xByCz
Σ(VO)xAyBz
VOA2
dhp
maltol
pic

Fig. 5. Speciation of various potentially antidiabetic VO(IV) compounds (**A**: 1 μM, **B**: 10 μM, **C**: 100 μM) in serum at pH 7.4. A carrier ligands, pic, mal, dhp; B and C: LMM components of the serum: citric acid, lactic acid, phosphate. "B" taken from Ref. [63] "A" and "C" calculated similarly based on the published data in Ref. [63].

In Fig. 5. the sum of the concentration of the similar type of species are depicted: $VO(IV)A_2$: VO(IV) bound in the binary *bis* complex, $(VO(IV))_xB_y$: binary species formed with the LMM components of the serum, $(VO(IV))_xB_yC_z$: ternary species of the LMM components of the serum, $(VO(IV))_xA_yB_z$: ternary species of an antidiabetic complex with LMM components of the serum, $(VO(IV))_x$apoTf: binary species of VO(IV) with apoTf, $(VO(IV))_xA_y$apoTf: ternary species of an antidiabetic complex with apoTf, $(VO(IV))_x$HSA: binary species of VO(IV) formed with HSA, $(VO(IV))_xA_y$HSA: ternary species of an antidiabetic complex with HSA.

The following conclusions could be drawn: (i) It is clear that apoTf, one of the two important HMM binders, is much more efficient than HSA and will displace 90-95% (1 μM and 10 μM VO(IV) compound concentration levels) of the original carrier from the complex or will form ternary complexes with them. (ii) Only the hydroxypyridinone derivative dhp is a strong enough carrier to preserve a significant proportion of the VO(IV) in the original complex or still bound to VO(IV) in a ternary complex with apoTf. In the other two cases (pic, mal), the carrier ligands are completely displaced. Similar behavior can be expected from the $VO(IV)(acac)_2$, as the acac ligand is much weaker metal ion binder than dhp, mal and pic. (iii) Among the LMM binders, citrate is the main 'active' component, able to influence the solution state of these antidiabetics but only at 100 μM vanadium compound ($VO(IV)(pic)_2$,$VO(IV)(mal)_2$) level, when there is no Tf enough to bind all the metal ions. Among the LMM components the ternary complex(es) with the original carrier ligands dominate(s). (iv) The HSA containing fraction is negligible at 1 μM and 10 μM VO(IV) compound concentration level, similarly to citrate, it is able to bind the metal ion (or complex) only when the apoTf is already saturated with VO(IV). The total vanadium containing HSA fraction is lower than 20% in all three cases even at 100 μM vanadium compound level. (v) The speciation is strongly concentration dependent in the 10-100 μM VO(IV) concentration range.

The dominance of apoTf in VO(IV) binding was confirmed with the use of native blood serum measurements by ultrafiltration, separation through a 10 kDa membrane, the LMM and the HMM fraction bound VO(IV) was measured by atomic absorption spectroscopy method [8,65]. Similarly, the protein bound VO(IV) was separated by anion exchange

chromatography and determined by ICP-MS. Only Tf was able to bind VO(IV), the binding ability of the other important serum protein HSA was negligible [6].

Accordingly, the most important role of the carrier ligand seems to facilitate the absorption of VO(IV) from the GI tract, but the complexes fall apart at last in the serum. Pharmacokinetic investigations proved this prediction by using labeled $VO(IV)(mal)_2$ complexes [24].

It should be mentioned, that even at higher concentrations of the VO(IV) complexes, such as mM level, which exceed the serum level of the strongest VO(IV) binder protein Tf, HSA also becomes an important binder of the VO(IV) species in serum, due to its significantly higher concentration [63]. However, there is no real clinical importance of this observation, as for example in the whole blood the LC_{50} value of vanadate is in the 2-5 mM range (estimated based on [66]).

Among several drug candidate ligands (hpno/mpno/pic/dhp), the hpno (2-hydroxypyridine-N-oxide, the O derivate of mpno) forms the highest stability complex with acidic solution [56]. Based on modeling calculation it is clear that at biologically relevant concentrations, c(V(V)) < 10 μM, the Tf is the only V(V) binder in the blood serum. Under such conditions, neither the carrier ligands, nor HSA nor the LMM biomolecules present in the serum (lactate, citrate, phosphate, Gly or His) form sufficiently strong complexes to compete with apoTf, even though *ca.* 5% of the V(V) exists as free $H_2VO_4^-$ ion in solution [56]. As an illustration species distribution of VO_2(V)-mal is depicted in Figure 6.

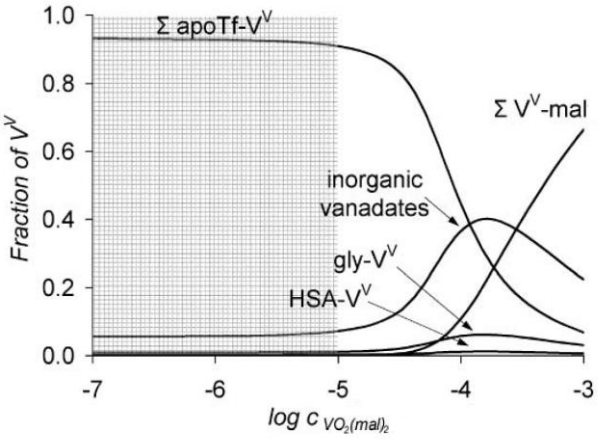

Fig. 6. Calculated speciation of $[VO_2(V)mal)_2]^-$ in blood serum. The textured area represent the concentration range relevant to oral administration of V(V) (≤ 10 mM).Taken from Ref. [56].

2.6. Interactions of vanadium in the whole blood

The interaction of VO(IV) ion with hemoglobin (Hb) was studied with spectroscopic (EPR and UV−vis) techniques. Binding of Hb to VO(IV) in vitro was proved, and three unspecific sites were characterized, with the probable coordination of His−N, Asp−O^-, and Glu−O^- donors. The value of log β for VO(IV)Hb is 10.4, significantly lower than for human serum apoTf. In the systems with potential antidiabetic VO(IV) compounds, mixed species cis-VO(IV)L_2(Hb) (L =mal or dhp are observed with equatorial binding of an accessible His residue, whereas no ternary complexes are observed with acac. The experiments of uptake of [VO(IV)$(mal)_2$], [VO(IV)$(dhp)_2$] by red blood cells indicate that the neutral compounds penetrate the erythrocyte membrane through passive diffusion, and percent amounts higher than 50% are found in the intracellular medium. The biotransformation of [VO(IV)$(mal)_2$], [VO(IV)$(dhp)_2$] inside the red blood cells was proved [60].

These results suggest that interactions with the red blood cells cannot be neglected in the transport process or the pharmacological action of the antidiabetic vanadium compounds. The vanadium entered the red blood cells can either be stored there and considered as a pool, or be deactivated by biotransformation reactions in the cytosol. This will depend on the nature and the extent of these biotransformation reactions [60].

XANES speciation studies of antidiabetic vanadium complexes in the whole blood [67] were carried out using the same classification method on 3D diagrams of pre-edge and edge parameters similarly to GI speciation (Section 2.1). The outcome in agreement with the above results suggests an important role of the red blood cells in the biospeciation of vanadium. Although it should be noted, that the applied concentration of vanadium was 1 mM, which is therapeutically usually not relevant, we cannot ignore the main conclusion of these two papers that “the role of RBCs in the metabolism of metal-containing drugs in the blood is no less important than that of serum proteins, such as albumin, immunoglobulins, or transferrin [67]

2.7. Speciation of vanadium in the cells

Vanadium, either in oxidation state IV or V, mainly binds to Tf in human serum (Section 2.4.). Accordingly, vanadium may be assumed to enter the cell through the Tf receptor following the iron pathway. Neutral antidiabetic complexes bound to certain proteins may cross membranes by passive diffusion too [60].

In the intracellular medium, reducing agents can redox-interact with vanadate(V). A frequently discussed candidate for the reduction is GSH [60,68-69]. A high intracellular excess of GSH increases the possibility of formation of VO(IV) and its complexation with

either GSH or GSSG. Both have been shown to be reasonably potent binders for VO(IV) [68,70-71]. Other effective reducing agents, such as NADH or ascorbate, may cause the formation of even V(III) species [72-73]. Hydrolytic degradation of VO(IV) may be responsible for the reoxidation to vanadate(V).

Among the LMM binders, the widely distributed ATP may also be of importance [60, 69], as it efficiently binds VO(IV) and is also present in mM concentration in cells. Comparing the VO(IV) complex-forming properties of ATP and GSH, it can be concluded that in the whole pH range ATP is a more efficient VO(IV) binder. When ATP and GSH are simultaneously considered as potential VO(IV) binders, GSH is not expected to be able to compete with ATP for binding to VO(IV). Since ATP is a strong VO(IV) binder, ATP will chelate the metal ion, forming binary and/or ternary complexes, and thus might somehow be involved in the antidiabetic action of the VO(IV) compounds.

XANES spectroscopic studies [74] on vanadium uptake and speciation in mammalian cells and cell culture media were carried out also by P. Lay and coworkers, similarly to the other two XANES studies (GI tract/blood). In this work they experimentally proved the earlier criticism of some of the authors of this paper: (Section 2.1.) namely the 1.0 mM vanadium concentration is therapeutically irrelevant. Such conditions were toxic for the cells at >8 h treatments, but the authors cannot use lower vanadium concentration because of the X-ray fluorescence detection limit [75]. However, the conclusions of this study are that the easy interconversions of V(IV) and V(V) species in the cells, *i.e.* the antidiabetic V(V) and V(IV) complexes undergo profound transformations in cell culture media, and the resultant products are further metabolized by cultured mammalian cells.

After vanadium enters the body and undergoes several biotransformations finally it may be excreted or accumulated by different tissues. Direct comparison studies between ^{48}V-BMOV and $^{48}VO(IV)SO_4$ demonstrated a similar pattern of biodistribution to that of inorganic vanadium salts observed earlier: the order of relative accumulation is bone > kidney > liver. The absorption level is low, the bones retaining only the *ca.* 0.1% of an oral dose/g tissue 24 h after an oral dose of $VO(IV)SO_4$, however half-life of elimination is quite long (>10 d for ^{48}V in bone after a single dose 10 μM by oral gavage in rats) [16].

3. Conclusions

There has been significant progress concerning speciation of antidiabetic vanadium compounds in biology (in GI tract, in blood serum, in the whole blood and in the cells),

although there are still unknown details concerning the translocation of the complexes from one place to another. A recent review about the speciation of these antidiabetic vanadium compounds in cell culture media gives a somewhat more realistic picture the possible solution state of these prodrugs in biological fluids [76], however the applied concentration of vanadium in the most referred experimental studies (XANES: 1 mM, EPR/NMR: 0,5 mM-1mM) is significantly higher than the therapeutically relevant concentration.

Further questions to clarify in order to overcome the difficulties in the clinical use of vanadium complexes in diabetes:

(i) In vivo studies for a deeper understanding of the mechanistic details of transport, targeting and mode of action of the vanadium compounds at therapeutically relevant concentration range.

(ii) Further studies on the kinetics on these processes may give important information on the better understanding of their biological action.

(iii) Detailed, long term toxicity measurements are necessary in order to clarify the accumulation, excretion process of vanadium in the body.

As a summary we can say that vanadium compounds, if the toxic side effects can be overcome, and the stable, well absorbable vanadium compounds can be targeted to the specific tissues, when they can exert their biological actions without significant negative side effects, they may have a clinical application in the future. Although, we must admit that a rigorous analysis of the question by medical experts has led to an opposite conclusion [77].

Complexation of vanadium is useful only to enhance the absorption of the (pro-)drug. The active metabolite is the vanadium itself, probable in the oxidation state of +5, as the compounds dissociate before exert their antidiabetic effect(s), which is not necessarily sufficient or specific enough in the target cell. Our opinion is that to make a vanadium compound to a more applicable drug new chemical ideas are necessary to be found to increase the main biological effect (or the specificity) which seems to be a hard challenge.

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