

Potential of Adipogenesis and Insulinomimetic Effects of a Novel Vanadium Complex (N'-[(E)-(5-bromo-2-oxophenyl)methylidene]-4-methoxybenzohydrazide)oxido-(1,10-phenanthroline)vanadium(IV) in 3T3-L1 Cells

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Abstract

Recent research on the action of vanadium compounds shows its important effect on adipogenesis processes and adipocyte function. On the basis of previous screening tests in cellular models, the novel vanadium complex (N'-[(E)-(5-bromo-2-oxophenyl)methylidene]-4-methoxybenzohydrazide)oxido-(1,10-phenanthroline)vanadium(IV) was selected for this study. This complex exhibits potent inhibition of tyrosine phosphatases, and differences in the degree of inhibition were observed particularly for phosphatases. A significant increase in intracellular lipid accumulation and proliferative effect on 3T3-L1 preadipocytes confirmed the ability of this complex to enhance adipogenesis. The insulinomimetic activity of the tested complex was also demonstrated in fully differentiated 3T3-L1 adipocytes, in which glucose utilization was potentiated. The obtained results support the hypothesis that vanadium complexes show promising possibilities for use as new therapeutic strategies for the treatment of type 2 diabetes.

Keywords: vanadium complexes, phosphatases inhibition, adipocytes, adipogenesis, glucose utilisation

Introduction

Adipocytes are a key component of adipose tissue that play a significant role in maintaining energy homeostasis, and this role is not limited to simple, direct regulation of glucose and lipid levels. Recent studies suggest that adipose tissue serves as a crucial integrator of glucose homeostasis through endocrine function of adipocytes. Several mechanisms have been identified that are related to cross-talk between the adipose tissue, skeletal muscle, liver, pancreas, brain, and immune system. These mechanisms involve both increased free fatty acid release and altered secretion of numerous proteins from adipose tissue. Secreted proteins such as leptin, adiponectin, resistin, tumor necrosis factor- α and other cytokines, omentin, and visfatin coordinate the metabolic activities of various organs and affect several metabolic and regulatory pathways [1, 2]. In many metabolic disorders, the function of adipose tissue is disturbed, and the role of adipocytes changes significantly; this is not limited to obesity or type 2 diabetes. Furthermore, the targeting of adipose physiology is

a promising approach for the treatment of these conditions [3, 4].

Differentiation of preadipocytes to adipocytes (adipogenesis) is regulated by a set of transcription factors responsible for the expression of key proteins that induce mature adipocyte formation [5]. These proteins include peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer-binding protein (C/EBP) family members, and sterol regulatory element binding protein 1c (SREBP-1c). These factors can modulate the expression of downstream target genes involved in lipogenesis and lipolysis, such as acetyl-CoA carboxylase (ACC) or fatty acid synthase (FAS) [6, 7]. The changes characteristic of adipocyte maturation included also insulin receptor (INS-R), glucose transporter type 1, 3 and 4 (GLUT 1, 3, 4) and glucokinase (GCK) regulated glucose transport and metabolism [8].

A current pharmacological approach to treat insulin resistance and type 2 diabetes is the use of thiazolidinediones (TZDs), which are agonists of PPAR γ and promote the differentiation of preadipocytes into adipocytes, while the sulfonylureas stimulate insulin secretion by blocking ATP-dependent potassium channels of pancreatic β -cells and induce the differentiation of human preadipocytes [9, 10, 11].

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The compounds that modulate the functions of adipocytes and adipose tissue also include vanadium inorganic salts and vanadium complexes, whose insulinomimetic effects have been demonstrated on isolated adipocytes in the 1980s [12]. The mechanisms of metabolism-modifying action of vanadium compounds, including the pronounced antidiabetic effects, are largely associated with the ability of vanadium to inhibit tyrosine phosphatases. The results of previous studies indicate the importance of inhibiting this class of phosphatases in the cellular physiology of adipocytes, which suggest the potential significance of vanadium in the modulation of these processes. Several protein-tyrosine phosphatases have been identified in major insulin-sensitive tissues and also in adipose tissue. These include transmembrane phosphatases such as leukocyte common antigen-related phosphatase (LAR) and CD45 and non-transmembrane phosphatases such as PTP1B, PTPIC, SHP1, SHP2, and PTPRQ. These phosphatases are known as important factors that modulate insulin-related signal transduction and increase insulin sensitivity, glucose uptake, and resistance to obesity [13, 14, 15]. A small number of studies on the effect of vanadium compounds on adipocytes have been carried out; thus, the mechanisms by which vanadium targets preadipocytes and adipocytes are not fully elucidated.

3T3-L1 preadipocytes are the widely used cell model in research on adipocyte biology and the pharmacological action of compounds with potential applications in pharmacotherapy. 3T3-L1 cells are suitable models for assessing preadipocyte differentiation and for studying lipid and glucose metabolism [16]; furthermore, several studies on the effect of vanadium compounds on this cell model have been conducted. Bis(allixinato)oxovanadium(IV) stimulates the translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes following the activation of tyrosine phosphorylation of the insulin receptor beta-subunit (IR) and insulin receptor substrate (IRS) as well as Akt kinase [17]. Similar targets were identified for bis(alpha-furancarboxylato)oxovanadium(IV) (BFOV)-enhanced expression of IRS-1 and GLUT4 mRNA, which can explain the improvement of dexamethasone-induced insulin resistance in 3T3-L1 adipocytes [18]. Bis(acetylacetonato)-oxovanadium(IV) (VO(acac)), bis(maltolato)-oxovanadium(IV) (BMOV), and sodium metavanadate (NaVO_3) attenuated lipolysis, and both Akt and ERK pathways were activated by these compounds; however, only Akt activation contributes to the antilipolytic effect of the vanadium compounds, without the involvement of ERK activation [19]. Other factors are also affected by vanadium. Vanadyl sulfate (VOSO_4) increased adiponectin cell content and release from 3T3-L1 adipocytes through a PKB-dependent transduction pathway [20].

Most studies on the effect of vanadium compound on 3T3-L1 cell model have focused on the effects of vanadium on matured, fully differentiated adipocytes; however, recent research in the adipogenic action of vanadium compounds, show limited but important influence on this process.

Three novel synthesized binary vanadium (V)-Schiff base complexes induce differentiation of 3T3-L1 preadipocytes into mature adipocytes, and this effect is comparable to the differentiation effect induced by insulin; further, in combination with insulin, vanadium showed potential synergistic interactions. Differentiated mature adipocytes showed strong increase in GLUT4 as compared to undifferentiated pre-adipocytes; this indicates a possible mechanism for the observed effects [21]. Interestingly, vanadium(IV)-chlorodipicolinate (VOdipic-Cl) showed the opposite effect on adipogenesis in 3T3-L1 preadipocytes and inhibited preadipocyte differentiation and adipogenesis by activating the LKB1/AMPK-dependent signaling pathway [22]. These findings indicate the potential use of vanadium complexes as promising agents in the treatment of metabolic diseases such as obesity or diabetes type 2, where inhibition or activation of adipogenesis is an important pathogenetic and therapeutic mechanism.

In the present study, a new vanadium complex *N'*-[(*E*)-(5-bromo-2-oxophenyl)methylidene]-4-methoxybenzohydrazide) oxido(1,10-phenanthroline)vanadium(IV) (complex (1)) was selected on the basis of results of screening tests in three cell-based models [23].

The aim of this study was assessment of this vanadium complex on adipogenesis and differentiation of 3T3-L1 cells.

Methods

Inhibition of human PTP1B, SHP1, SHP2 and LAR phosphatases

Test and reference compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and further diluted in phosphate buffered saline (PBS). The final concentrations of compounds were 10 μM (0.1% DMSO) and 1 μM (0.01% DMSO). All reactions were carried out at room temperature in black 384-well plates (Perkin Elmer). To the solution of the test compounds an equal volume of a test solution of phosphatase (50 ng/mL PTP1B, 400 ng/mL SHP1, 50 ng/mL SHP2, and 5 ng/mL LAR) in a reaction buffer: 25 mM of 3-(*N*-morpholino) propanesulfonic acid (MOPS), 50 mM NaCl, 1 mM dithiothreitol (DTT), and 0.05% Tween-20, pH 7.0. After 10 minutes, a solution of phosphate 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) was added until its final concentration was 0.1 mM. After 20 minutes of incubation at room temperature, the fluorescence intensity (355 nm excitation and 560 nm emission) was measured on a multifunction plate reader (POLARstar Omega, BMG Labtech, Germany) [24, 25]. Assays were conducted in triplicates. The results were normalized to the untreated control (phosphatase with solvent only), wherein the intensity of fluorescence was taken as 100%.

Cell models

3T3-L1 cell line (ATCC CL-173) derived from fibroblasts of mouse embryo tissue was cultured according to a standard pro-

toloc in DMEM medium supplemented with 10% bovine calf serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂.

For differentiation experiments, the cells were seeded in 96-well poly-D-lysine-coated plates and cultured to reach confluency. The medium was then (differentiation day 0) switched to differentiation medium I (DMEM, 10% fetal bovine serum, 25 nM 3-isobutyl-1-methylxanthine (IBMX), 500 µM dexamethasone, and 670 nM insulin). After 48 hours of incubation (differentiation day 2), the medium was changed to differentiation medium II (DMEM, 10% fetal bovine serum, and 670 nM insulin), and tested reference compounds and vanadium complexes were added. After 24 hours of incubation (differentiation day 3), cell number, intracellular lipid accumulation, and glucose concentration were determined. To assess the influence of vanadium complexes on the later phase of differentiation process, 3T3-L1 cells were treated as above but without the addition of tested compounds, and differentiation medium II was replaced with DMEM with 10% fetal bovine serum. In this medium, the cells were maintained until the seventh day of differentiation, and then tested reference compounds and vanadium complexes were added for 24 hours, and cell number, intracellular lipid accumulation, and glucose concentration were determined (differentiation day 8). Incubation of cells with vanadium complexes was performed in triplicates, and each compound was tested in two to three independent experiments [26, 27]. Lipid accumulation, glucose utilization and cell number were determined in each well.

Lipid accumulation

After incubation with tested compounds 3T3-L1 cells were washed with PBS with calcium and magnesium and 5 µl AdipoRed reagent (Lonza) in 200 µl PBS was added and incubated for 10 minutes in 22°C. Fluorescence signal proportional to lipids content in cells was measured at excitation in 530 nm and emission in 550 nm using multimodal microplate reader POLARStar Omega (BMG Labtech). The results were normalized to the untreated control (cells with solvent only), wherein the intensity of fluorescence was taken as 100%.

Glucose utilisation

After incubation, supernatants from 3T3-L1 cells were collected and glucose concentration was determined based on enzymatic reaction with glucose oxidase. End product of the reaction was determined fluorometrically using Amplex Red Glucose/Glucose Oxidase Kit (Invitrogen) according to manufacturer protocol. 10 µl supernatant diluted in 50 mM PBS pH 7.4 and 10 µl reagents contained 4 U/ml glucose oxidase, 0.4 U/ml horseradish peroxidase and 200 µM 10-acetyl-3,7-dihydroxyphenoxazine in 50 mM PBS pH 7.4 was added to 384-well black microplate and incubated for 30 minutes at 37°C. All assays were conducted in triplicates. Fluorescence signal was measured at excitation in 530 nm and emission in 580 nm using multimodal microplate reader POLARStar Omega (BMG Labtech) and glucose concentration in samples was calculated in MARS Data Analysis Software based on glucose standards. Glucose utilisation was

Table 1. 3T3-L1 preadipocytes differentiation method and experiments scheme

Experiment day	Treatment of the cells in early phase of differentiation	Experiment day	Treatment of the cells in late phase of differentiation
0	IBMX + dexamethasone + insulin in DMEM + FBS	0	IBMX + dexamethasone + insulin in DMEM + FBS
2	Tested and control compounds 50 µM + insulin in DMEM + FBS	2 - 7	DMEM + FBS alone
3	Experiment readout and assays		
7	-	7	Tested and control compounds 50 µM in DMEM + FBS
8	-	8	Experiment readout and assays

Table 2. The controls used in experiments

Untreated control	Medium (DMEM + FBS) + vehicle
Differentiated control	Inductors of differentiation (IBMX + DEX + INS) in medium
Negative control	Isoproterenol 50 µM (as lipolytic agent) added at the same time as the tested compounds (table 1)
Positive control	Rosiglitazone 50 µM (as stimulator differentiation and adipogenesis) added at the same time as the tested compounds (table 1)
Vanadium reference compounds	Vanadyl sulfate 50 µM (VOSO ₄) (as inorganic vanadium compound), Bis(maltolato)oxovanadium(IV) 50 µM (BMOV) (as complex vanadium compound)

calculated as differences between incubation medium without cells and medium with cells after incubation with tested compound.

Cell number

The estimation of the cell number was performed on the basis cell reaction with PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific), a cell-permeable resazurin-based reagent that functions as a cell viability indicator by using the reducing power of living cells (mitochondrial enzyme activity) to quantitatively measure the proliferation of cells (cell numbers). After incubation of the cells with test compounds, 10 μ l of PrestoBlue reagent was added to each microplate well, and the microplate was placed on an orbital shaker for content mixing. Plates were incubated for 20 minutes, and the fluorescence intensity at 560 nm excitation and 590 nm emission was determined using a multi-mode microplate reader POLARstar Omega (BMG Labtech, Germany). The results were normalized to the untreated control (cells with solvent only), wherein the intensity of fluorescence was taken as 100%.

Statistical methods

For all results statistical analysis was performed by analysis of variance followed by the Tukey test for post hoc comparisons with $p < 0.05$. All tests were performed using GraphPad Prism version 6.0 for Windows, GraphPad Software, San Diego, California USA.

Results and Discussion

Vanadium complex (1) exerted potent inhibition of all tested tyrosine phosphatases, namely PTP1B, LAR, SHP1, and SHP2 (Table 3). Importantly, the percentage of inhibition of activity of these enzymes by compound (1) at a concentration of 10 μ M was similar to that by inorganic vanadium compound VOSO_4 and BMOV. This methyl analog of ethyl maltolate (BEOV) has been tested as an antidiabetic agent in phase II clinical trial and was used as a comparator of the activity for our new complex (1) [27]. Incubation of phosphatases with complex (1) at the concen-

tration of 10 μ M led to the almost total inhibition of the activity of the investigated enzymes; therefore, there are limited methods to distinguish the inhibitory action of the tested compounds. Control compounds VOSO_4 and BMOV at a concentration of 1 μ M showed similar inhibitory activity for all tested phosphatases, and only LAR phosphatase activity was more strongly inhibited by both control vanadium compounds (Table 3).

With regard to the results obtained for the comparative compounds, the results for complex (1) showed significant differences in the inhibition of particular phosphatases. The degree of inhibition of SHP1 and SHP2 phosphatases (61% and 66%, respectively) was approximately two-fold higher than that of PTP1B and LAR (36% and 31%, respectively). While the inhibition of SHP1 and SHP2 phosphatases by complex (1) was slightly less than that caused by BMOV, the differences between the two compounds were distinctly greater for inhibition of PTP1B and LAR phosphatases (Table 3).

The observed differences in the degree of inhibition of tyrosine phosphatases (SHP1 and SHP2 vs. PTP1B and LAR) obtained in the screening tests seemed to be too small to indicate the selectivity of this complex. However, this does not indicate biological activity on 3T3-L1 cells (results given below), and further studies of the relationship between the adipogenesis process and the differences in the degree of inhibition of tyrosine phosphatases by various vanadium complexes seem to be reasonable and needed.

To assess the influence of tested complex (1) on the adipogenesis process, 3T3-L1 preadipocytes were used. Incubation of the cells was carried out in two stages of differentiation of preadipocytes to adipocytes (adipogenesis). After short-term exposure of the cells to inducers of adipogenesis for 24 hours (IBMX, dexamethasone, and insulin as described in Materials and Methods section), the inducers were withdrawn from the medium and replaced with tested or reference compounds at the concentration of 50 μ M. After a further 24 hours of incubation, intracellular lipid content in 3T3-L1 cells was determined as a marker of adipogenesis (Figure 1). The lipid content in cells incubated with complex (1) ($493\% \pm 22\%$) was significantly higher than that in the control cells subjected to differentiation ($328\% \pm 23\%$) and

Table 3. The results an *in vitro* human phosphatases (PTP) inhibition activity assay

Compound	Phosphatase (% of inhibition \pm SD)							
	PTP1B	LAR	SHP2	SHP1	PTP1B	LAR	SHP2	SHP1
	10 μ M				1 μ M			
VOSO_4	79 \pm 1	92 \pm 1	95 \pm 3	92 \pm 1	62 \pm 3	44 \pm 3	77 \pm 1	70 \pm 2
BMOV	90 \pm 2	96 \pm 2	95 \pm 1	91 \pm 2	77 \pm 2	58 \pm 2	82 \pm 2	76 \pm 1
Complex (1)	72 \pm 1	96 \pm 1	91 \pm 1	85 \pm 1	36 \pm 3	31 \pm 3	66 \pm 3	61 \pm 2

over two times higher than that in cells incubated with rosiglitazone and BMOV ($200\% \pm 21\%$ and $219 \pm 21\%$, respectively). All comparisons in the presented results were expressed in relation to untreated control without any differentiation inducers, and statistical differences between the results mentioned above are considered at $p < 0.001$.

A well-studied and described effect of adipogenic compounds is the stimulation of proliferation of preadipocytes, and this effect was demonstrated in this experiment for rosiglitazone ($249\% \pm 13\%$) (Figure 2). The same effect was also observed for BMOV and complex (1) ($283\% \pm 15\%$ and $200\% \pm 17\%$ of untreated control, respectively, $p < 0.001$).

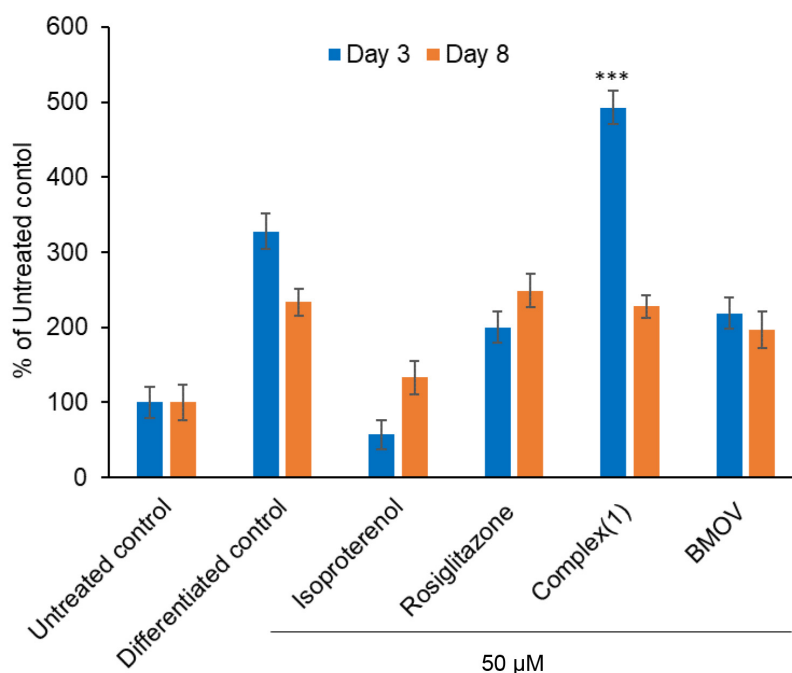


Figure 1. Intracellular lipid content in 3T3-L1 cells subjected to differentiation to adipocytes after incubation with control compounds (isoproterenol, rosiglitazone and BMOV) and vanadium complex (1). The selected statistical significance is marked on the figure for day 3. Tukey's post-hoc test was used. *** $p < 0.001$ vs BMOV, rosiglitazone and undifferentiated control

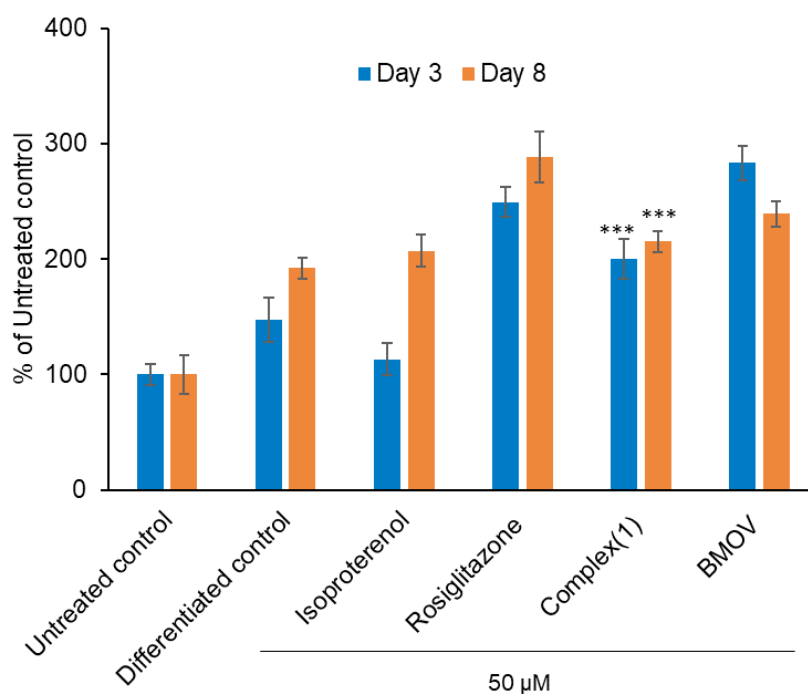


Figure 2. Number of 3T3-L1 cells subjected to differentiation after incubation with control compounds (isoproterenol, rosiglitazone and BMOV) and vanadium complex (1). The selected statistical significance is marked on the figure. Tukey's post-hoc test was used. *** $p < 0.001$ vs undifferentiated control and BMOV (day 3), *** $p < 0.001$ vs undifferentiated control and rosiglitazone (for day 8)

This proliferative effect together with the increase in intracellular lipid accumulation confirms the effect of complex (1) on the intensification of adipogenesis.

Further incubation of complex (1) and comparative compounds was performed at a later stage of the differentiation process of preadipocytes to adipocytes. After approximately 7–10 days of 3T3-L1 preadipocyte differentiation, this process was

control ($p < 0.001$) (Figure 3). The same differences were also seen on day 8 of the experiment. This indicates that complex (1) exerts effect on both preadipocytes and differentiated adipocytes, showing both pro-adipogenic and insulinomimetic effects. Rosiglitazone and BMOV also showed similar differences, with the effect of BMOV on glucose utilization significantly higher than that for complex (1).

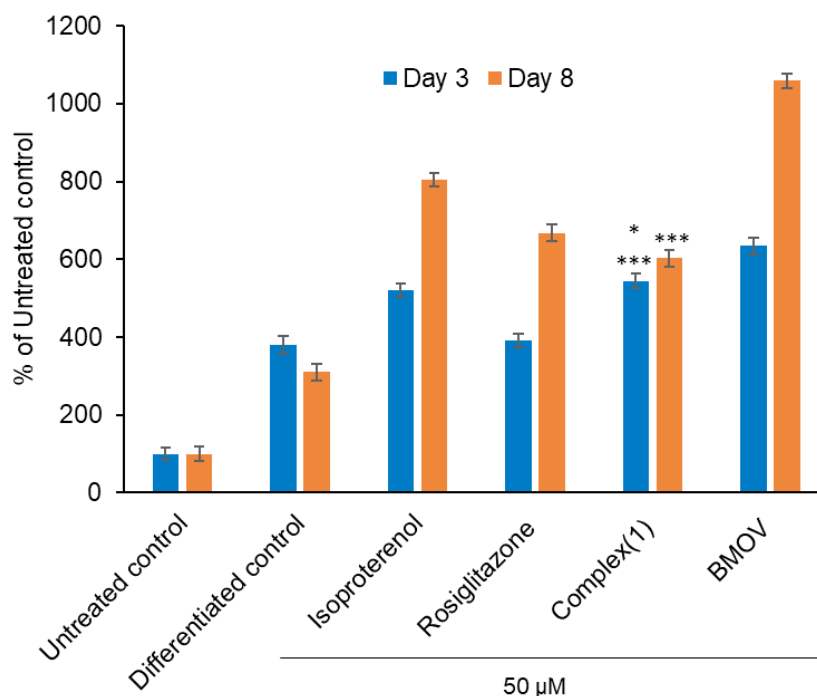


Figure 3. Glucose utilisation in 3T3-L1 cells subjected to differentiation to adipocytes after incubation with control compounds (isoproterenol, rosiglitazone and BMOV) and vanadium complex (1). The selected statistical significance is marked on the figure. Tukey's post-hoc test was used. *** $p < 0.001$ vs undifferentiated control and rosiglitazone (day 3) and * $p < 0.05$ vs BMOV (day 3), *** $p < 0.001$ vs undifferentiated control and BMOV (day 8)

completed, and most cells exhibited mature adipocyte phenotype with numerous intracellular lipid droplets. A 24-hour incubation with the examined compounds on day 7 after the initiation of the differentiation process showed that the intracellular lipid content influenced by rosiglitazone, BMOV, and complex (1) was approximately two-fold higher than that in 3T3-L1 cells not treated with any adipogenesis inducers (untreated control). For isoproterenol used as a negative control (lipolytic activity), the intracellular lipid content was approximately half of that in differentiated control cells. Differences in the effect on lipid accumulation between rosiglitazone, BMOV, differentiated control, and complex (1) on day 8 of the experiment were aligned, which indicate that the adipogenesis process was completed at this stage, and these compounds did not cause further increase in lipid accumulation. Further, the lack of significant differences in the cell number on days 3 and 8 of differentiation confirmed this conclusion.

Glucose utilization in 3T3-L1 preadipocytes under the influence of complex (1) on day 3 of differentiation was significantly higher than that in differentiated control as well as untreated

The obtained results indicate that complex (1) is an effective factor strengthening the previously initiated process of differentiation of preadipocytes into adipocytes, which is accompanied by an increase in cell proliferation and intracellular lipid accumulation. These results are consistent with those of other studies in which vanadium compounds showed similar properties [19, 21].

With regard to the published results [29], our study is the first with the 3T3-L1 cell model in which vanadium complex was used not as a stand-alone adipogenic agent, but was used after a short period of induction by “classical” agents initiating the differentiation of preadipocytes. This approach seems to be important because of the fact that pharmacological interventions in metabolic disorders usually occur when the process of differentiation has already been initiated or progressed.

Conclusion

The present preliminary results support the hypothesis that vanadium complexes offer new opportunities for the development of therapeutic strategies for the treatment of type 2 diabetes

and other metabolic diseases that involve adipocytes in pathophysiological mechanisms. Because there are a limited number of studies on the influence of vanadium complexes on adipose physiology, more extensive and comprehensive studies are required.

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