# Cell-based Screening for Identification of Novel Vanadium Complexes with Multidirectional Activity Relative to Cells Associated with Metabolic Disorders

Grzegorz Kazek[a, *], Monika Głuch-Lutwin[b], Barbara Mordyl[b], Elżbieta Menaszek[c], Janusz Szklarzewicz[d], Ryszard Gryboś[d], Monika Papież[c]

[a] *Department of Pharmacological Screening, Chair of Pharmacodynamics, Faculty of Pharmacy, Jagiellonian University Collegium Medicum, Medyczna 9, 30-688 Krakow, Poland*

[b] *Department of Radioligands, Chair of Pharmacobiology, Faculty of Pharmacy, Jagiellonian University Collegium Medicum, Medyczna 9, 30-688 Krakow, Poland*

[c] *Department of Cytobiology, Chair of Pharmacobiology, Faculty of Pharmacy, Jagiellonian University Collegium Medicum, Medyczna 9, 30-688 Krakow, Poland*

[d] *Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, 30-387, Krakow, Poland*

**Abstract**

In this study, 110 newly synthesized vanadium complexes from different structural groups were screened in three cell-based models representing the main target tissues for anti-diabetic drugs. In glucose utilization in C2C12 myocyte experiments, 93% of vanadium complexes were shown to have equal or greater activity than bis(maltolato)oxovanadium(IV) (BMOV), the methyl analog of bis(ethylmaltolato)oxovanadium(IV) (BEOV) which has been tested in clinical trials. Moreover, 49% and 50% of these complexes were shown to have equal or greater activity than BMOV in lipid accumulation in 3T3-L1 adipocytes and insulin secretion in RINm5F beta cell experiments, respectively. These results were the basis for the selection of compounds for the subsequent steps in the characterization of anti-diabetic properties. This study provides strong support for the application of screening cell-based assays with a phenotypic approach for the discovery of novel anti-diabetic drugs from the vanadium complex class. This is especially desirable due to the multiple and not fully defined mechanisms of action vanadium compounds.

**Keywords:** vanadium complexes, cell-based screening, anti-diabetic activity

## Introduction

Diabetes is the 7th the most common of death worldwide [1]. Currently, 14 classes of drugs are available to treat type 2 diabetes mellitus, but only 36% of patients with type 2 diabetes achieve glycemic control with currently available therapies. Therefore, new treatment options are desperately needed, especially against various metabolic disturbances and diseases such as insulin resistance, glucose intolerance, pre-diabetes and non-alcoholic fatty liver disease [2]. Unfortunately, there are no approved medications and therapeutic interventions in these states are not well established [3].

The clinical use of vanadium in diabetes has occurred for over one hundred years and the current state of knowledge about the mechanisms of action of vanadium compounds and complexes and evidence of their pharmacological effectiveness means that they are an interesting and promising object for research [4].

The best known and most widely described mechanism of action of vanadium and its complexes is inhibition of protein tyrosine phosphatases, especially PTP1B. This leads to the maintenance of the phosphorylated state of the insulin receptor and thus to upstreaming signaling analogous to the action of the insulin hormone. In a subsequent multi-step process, the glucose transporters are activated and cellular glucose uptake is then activated [5, 6, 7]. However, the importance of PTP1B inhibition as a key mechanism for vanadium does not limit the long list of identified molecular targets for vanadium action. Other enzymes (mostly phosphatases and kinases) in the glucose uptake pathway have been considered as targets; however, the insulin-like effects of vanadium remain still not fully answered [8, 9].

Among the main therapeutic strategies for treating type 2 diabetes are increasing insulin sensitivity (insulin sensitizers) and increasing insulin secretion (insulin secretagogues). Treating insulin resistance includes two mechanisms: activation of peroxisome proliferator-activated receptors (PPARs) such as thiazolidinediones (e.g., rosiglitazone and pioglitazone); and AMPK-activated protein kinase (AMPK) such as metformin. These two classes of drugs are the most commonly used medications for the treatment of type 2 diabetes. Anti-hyperglycemic drugs such as sulfonylureas and meglitinides increase insulin secretion from pancreatic beta-cells [10, 11, 12, 13].

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*Corresponding author: grzegorz.kazek@uj.edu.pl

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Sci, Tech. Innov 2019; 4 (1), 47-54

The molecular mechanisms of the pharmacological effects of vanadium complexes involve multiple mechanisms which are identical with some therapeutic targets in the current classification of diabetes according to their molecular group and signaling molecules [14]. The mechanisms of the anti-diabetic actions of vanadium compounds include, in addition to the mentioned inhibition of tyrosine phosphatases, activation of numerous signaling pathways and transcription factors – AMPK, PI3K-PKB-GSK-3β/Akt-mTOR, NF-κB, MAPK (MEK1/2-ERK) – and elevation of PPARγ levels [15, 16; reviewed in 4, 9, 17]. In addition to the effects on the metabolism of carbohydrates and lipids, vanadium complexes mediate regeneration of pancreatic β-cells which increase insulin production [18, 19].

Since vanadium complexes exert their wide range of metabolic effects via multiple mechanisms, in testing the anti-diabetic activity of vanadium compounds, it may not be sufficient to focus on a single mechanism, i.e. PTP1B inhibition [17]. The most appropriate research approach for the identification of novel compounds for therapeutic areas with complex disease pathomechanisms or where the compounds show activity against multiple molecular targets is an approach based on a phenotypic screening paradigm. These cell-based screens provide a substantial amount of biological information, such as the ability of a compound to ameliorate the phenotype of a intact cellular disease model, such as inhibition of a biological process, morphological or structural changes. They might provide direct evidence of efficacy, regardless of the intracellular molecular target [20, 21]. Cell-based assays are able to examine the interaction between target and compounds in a cellular context in an extracellular environment similar to body fluids, and phenotypic screens can give important information about tested molecule penetration into intact cells, which seems to be particularly important in the context of interchange of chelators in extracellular and intracellular body media ("speciation") [9].

In this study, screening assays were conducted with the use of cellular models corresponding to the main cellular pharmacological targets associated with metabolic disturbances in diabetes and other metabolic disorders. Our laboratory has adapted and validated models described and used in vanadium activity assessment including: glucose utilization (consumption) in C2C12 myocytes [22, 23], lipid accumulation in 3T3-L1 adipocytes [24, 25, 26, 27] and insulin secretion in RINm5F beta-cells [28, 29]. These experiments were used in the assessment of the anti-diabetic activity of vanadium complexes.

The level of glucose consumption by cells is the result of the transport of glucose to the cells and the degree of activation of intracellular metabolic processes; therefore, these assays best assess the influence of compounds acting on various mechanisms. Insulin plays an essential role in glucose homeostasis by increasing its storage or utilization by regulating the transport of glucose into the cell. Glucose transporter type 4 (Glut4) plays a fundamental role in glucose utilization in muscle and, upon stimulation by insulin, increases glucose transport into the cells. This is critical for the maintenance of glucose homeostasis and normal blood glucose level [30]. Adipose tissue and cells represent the targets of first choice for the development of cell-based assay systems for the insulin-like activity of compounds. This assay measures the cellular triglyceride level as an end-point readout and has the advantage of capturing the lipid lowering efficacy of potential candidates at different sites in lipid metabolic pathways [31]. Functional insulin-secreting pancreatic β cells are used in the study of targets and drugs to promote insulin secretion [32]. Insulin resistance causes a rise in insulin secretion in a compensation mechanism leading to pancreatic beta cell stress and damage with a progressive decrease in insulin secretion and subsequent hyperglycemia [33].

Following validation by a series of biochemical and pharmacological tests, these three models were applied to screen over one hundred vanadium complexes from different structural groups. The purpose of this study stage was the selection of the most active new vanadium complexes as potential compounds for metabolic disorders. Reference compounds and drugs approved in diabetes treatment, the inorganic vanadium compound vanadyl sulfate (VOSO$_4$) and vanadium complex bis(maltolato)oxovanadium(IV) (BMOV), a methyl analog of ethylmaltolate (BEOV) which has advanced to phase II clinical trials [34] were used for comparisons of biological activity. We used an approach based on high-throughput screening (HTS) methods and we adapted this strategy for academia-based projects in drug discovery with a small number of test compounds. In the current HTS data analysis, an activity cutoff value is set to select a certain number of compounds whose tested activities are greater than this threshold. In our study, the initial threshold values were the results for BMOV, because our goal was to discover complexes better than this well-studied and clinically tested vanadium complex.

## Materials and Methods

### Chemical structure of tested vanadium complexes

The vanadium complexes that have been subjected to pharmacological activity assays were synthetized in the Faculty of Chemistry, Jagiellonian University. They represent vanadium complexes (III, IV and V) with Shiff bases with donor atoms ONO. In the general, simplified formula [VX(ONO)y(L)n], X is oxygen or is absent and L is L1 or L2, wherein: L1 represents a halogen anion or a neutral or a deprotonated solvent molecule selected from the group consisting of C1-C12 alcohols, e.g. ethyl alcohol or methyl alcohol and/or water; L2 is a neutral or anionic ligand NN, NO or OO-donor selected from the group consisting of: polypyridine, e.g. 2,2'-bipyridine (bpy), 1,10-phenanthroline (phen), pyrones, deprotonated 3-hydroxy-2-methyl-4-pyrone (mal-), quinoline, e.g. deprotonated 8-hydroxyquinoline (quin-) or pyridine carboxylic acids, e.g. deprotonated 2-picolinic acid.

G. Kazek et al. | Cell-based Screening for Identification of Novel Vanadium Complexes with Multidirectional Activity Relative to Cells Associated with Metabolic Disorders

49

The letters O and N represent atom, through which the ligand is bound to the vanadium, means a triple-bond ligand of the general formula 1 (Figure 1) in the keto or enol form, neutral or deprotonated. R1, R2, R3, R4 independently represent hydrogen atom, fluorine atom, chlorine atom, bromine atom, iodine atom or a SO3, hydroxo, nitro, alkoxy, aryloxy, dialkylamino, alkyl or aryl group, wherein at least one of the R1, R2, R3, R4 substituents is different from hydrogen atom, R5 independently stands for hydrogen atom, alkyl or aryl group, R6 is a group selected from those given in Figure 2. In addition to these generally described complexes, several vanadium complexes with thioanilide amino acid derivatives has been tested in this study.
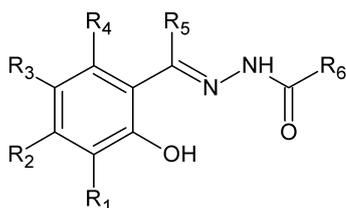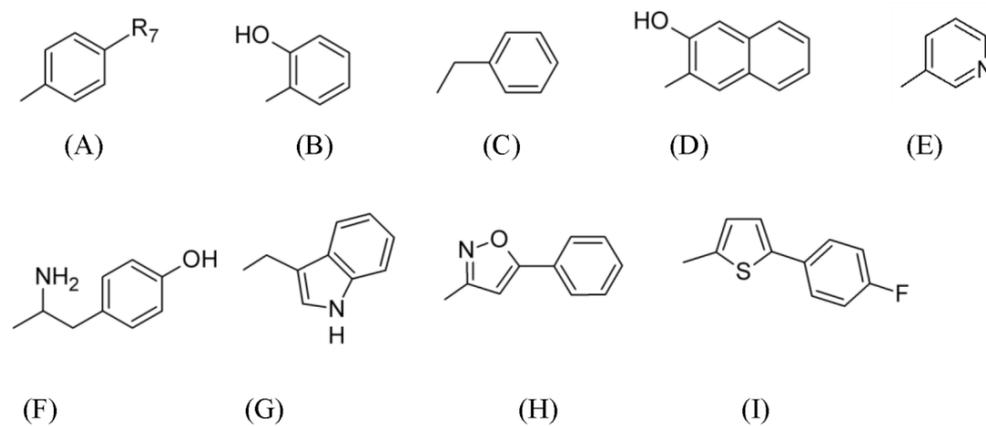


**Figure 1.** General structural formula **1**

ciently high signal from the experimental setup in the absence of significant cytotoxic activity. Based on the results for BMOV and VOSO$_4$, the concentration 100 µM for 60 min incubation with pancreatic beta-cells (RINm5F) and the concentration 50 µM in 24-hour incubations of myocytes (C2C12) and adipocytes (3T3-L1) were selected for the tests. All cytotoxicity tests were conducted at 50 µM and 24-hour incubations. In other *in vitro* studies using cell models, the concentrations of vanadium compounds were up to 1 mM [35, 36].

## Glucose utilization in myocytes

C2C12 cell line (ATCC CRL-1772), subclone of myoblasts from mouse muscles were cultured according to a standard protocol in DMEM supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 µg/mL streptomycin at 37°C in 5% CO$_2$. Cells were plated on a 96-well microplate and after reach confluency were differentiated in medium with 2% horse serum. After differentiation, the medium was changed for medium with 0.2% bovine serum albumin, 100 IU/mL penicillin, 100 µg/mL streptomycin and after 2 hours incubation the medium was changed



**Figure 2.** Substituents R6 in the formula **1**

## Cell models and treatment

All cell lines were obtained directly from ATCC (American Type Culture Collection). The passage number of cells used in the experiments was between 4 and 10. The evaluation of the functional stability of the cell lines was conducted based on the results for control compounds, which were tested in each experimental series and compared with the results obtained in the process of validation and optimization of experimental models. Control compounds in all experiments were BMOV and VOSO$_4$. In the experiments of glucose consumption and lipid accumulation and in the experiments of insulin secretion rosiglitazone and glibenclamide, respectively, were additionally tested. The concentrations of the control compounds were the same as those of the tested complexes.

The concentrations used in the studies were selected in the process of the validation of research models to ensure a suffi-

for fresh medium containing vanadium complexes in a 50 µM final concentration. After 24 hours incubation, supernatants were collected. Incubations of vanadium complexes with cells were performed in triplicate and each compound was tested in three to four independent experiments.

Glucose concentration was determined based on an enzymatic reaction with glucose oxidase and a final fluorometric detection reaction end product using an Amplex Red Glucose/Glucose Oxidase Kit (Invitrogen) according to the manufacturer's protocol. A total of 10 µL supernatant was diluted in 50 mM PBS pH 7.4 and 10 µL reagents containing 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 a 384-well black microplate and incubated for 30 minutes at 37°C. All assays were conducted in triplicate. Fluorescence signal was measured at an excitation in 530 nm and emission in 580 nm us-

www.stijournal.pl

Sci, Tech. Innov 2019; 4 (1), 47-54

ing a multimodal microplate reader POLARStar Omega (BMG Labtech) and glucose concentration in samples was calculated in MARS Data Analysis Software based on glucose standards. Working range of calibration was 1.56 - 50 μM with mean sensitivity (slope) 7465 and mean linear fit R2=0.9962. Glucose utilization was calculated as the differences between incubation medium without cells and medium with cells after incubation with the tested compound. Final results were expressed as the per cent of controls containing cells and solvent only as well as the per cent of results for BMOV.

### Insulin secretion experiments

RINm5F cell line (ATCC CRL-11605), pancreatic beta-cells derived from rat islets of Largenhans were cultured according to a standard protocol in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% $CO_2$. For insulin secretion experiments, cells were seeded in 96-well plates and cultured for 5 days. The medium was then aspirated, the cells were washed three times with Krebs-Ringer-HEPES buffer with 0.5% bovine serum albumin. Cells were incubated at 37°C for 60 min in the above buffer including the tested vanadium complexes in a 100 μM final concentration and then supernatants were collected. Incubations of vanadium complexes with cells were performed in triplicate and each compound was tested in three to five independent experiments.

Insulin concentration was determined based on an amplified luminescent proximity homogeneous assay with an AlphaLISA immunoassay kit (PerkinElmer) according to the manufacturer's protocol. A total of 5 μl supernatant added to 384-well dedicated microplate was incubated for 60 min at 23°C with AlphaLISA Anti-Insulin Acceptor beads (10 μg/mL final) and Biotinylated Antibody Anti-Insulin (1 nM final). Then, Streptavidin (SA)-coated Donor beads were added and incubated for 30 min at 23°C in the dark. All assays were conducted in triplicate. Alpha signal readings were performed using multimodal microplate reader POLARStar Omega (BMG Labtech) and insulin concentration in samples was calculated in MARS Data Analysis Software based on insulin standards. Dynamic range of analytical method was 26.6–100 000 pg/ml (0.8–3 000 μIU/ml). Working range of calibration in linear range was 207.5 pg/ml – 1.95 ng/ml (7.8–125 μIU/ml) with mean sensitivity (slope) 1882 and mean linear fit R2=0.9780. Final results were expressed as the per cent of control containing cells and solvent only as well as the per cent of results for BMOV.

### Lipid accumulation in adipocytes

3T3-L1 cell line (ATCC CRL-11605) derived from fibroblasts from mouse embryo tissue were cultured according to a standard protocol in DMEM medium supplemented with 10% bovine calf serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in 5% $CO_2$. For lipid accumulation experiments, cells

were seeded in 96-well poly-D-lysine coated plates and cultured to reach confluency. The medium was then switched to differentiation medium. After 48 hours incubation, the medium was changed for standard medium with human recombinant insulin and the tested vanadium complexes in a 50 μM final concentration were added after 1 hour. Incubations of vanadium complexes with cells were performed in triplicate and each compound was tested in three to four independent experiments. After 24 hours of cell incubation with compounds, wells were washed with PBS with calcium and magnesium and 5 μL AdipoRed reagent (Lonza) in 200 μl PBS was added and the mixture was incubated for 10 minutes at 22°C. The fluorescence signal proportional to lipid content in cells was measured at an excitation in 530 nm and emission in 550 nm using a multimodal microplate reader POLARStar Omega (BMG Labtech). Final results were expressed as the per cent of control containing cells and solvent only as well as the per cent of results for BMOV.

### Cytotoxicity testing

A cytotoxicity assay was performed using PrestoBlue Cell Viability Reagent (ThermoFisher Scientific), a cell permeable resazurin-based reagent that functions as a cell viability indicator by using the reducing power of living cells (mitochondrial enzymes activity) to quantitatively measure the proliferation of cells. After 24-hours of incubation of compounds with C2C12, 3T3-L1 and RINm5F cells prepared as described above, 10 μL of PrestoBlue reagent was added to each well and the microplate was mixed on an orbital shaker. Plates were incubated for 20 min 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 control (cells with solvent only), wherein the intensity of fluorescence was taken as 100%.

## Results and Discussion

The presented results of cell-based screening of vanadium complexes showed that a large part of the tested compounds showed greater activity than BMOV (Figure 1-3), the methyl analog of ethylmaltolate (BEOV) which has been tested in clinical trials [34]. In glucose consumption assays in C2C12 myocytes, 103 out of 110 tested compounds (93%) showed greater activity than BMOV, and 28 of these complexes have activity greater than 150% of BMOV activity, and the most active complex showed activity at 290% of BMOV activity (Figure 3). Moreover, 54 out of 110 (49%) vanadium complexes showed equal or greater activity than BMOV in lipid accumulation in 3T3-L1 adipocytes, wherein the largest observed value is 164% of the activity BMOV (Figure 4). In the insulin secretion assay, 54 (50%) vanadium complexes showed higher activity in RINm5F beta-cells than BMOV and the most active complex show the level of 150% of BMOV activity (Figure 5).
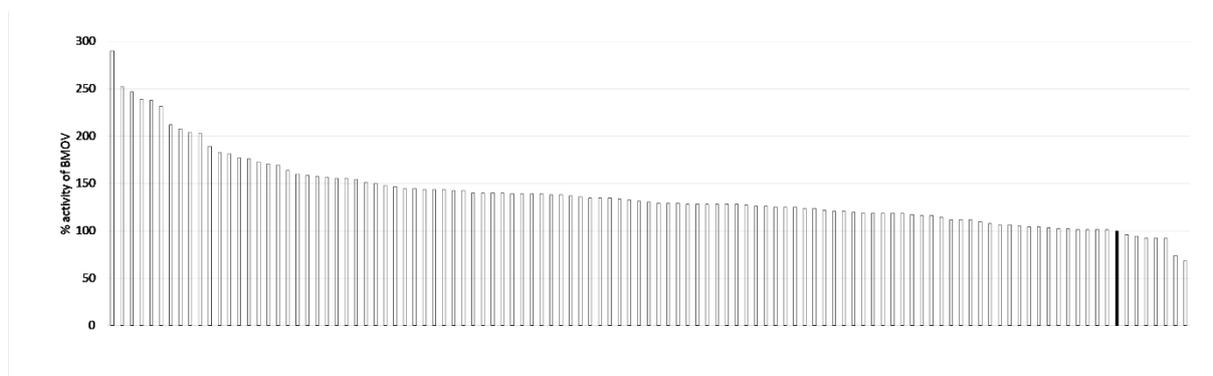
G. Kazek et al. | Cell-based Screening for Identification of Novel Vanadium Complexes with Multidirectional Activity Relative to Cells Associated with Metabolic Disorders

51

**Figure 3.** Glucose utilisation in C2C12 myocytes screening activity results (24-hours incubation; final concentration of vanadium complexes 50 μM). Open bars graphs present ordered results for tested vanadium complexes as per cent of BMOV activity (filled bar)



**Figure 4.** Lipid accumulation in 3T3-L1 adipocytes screening activity results (24-hours incubation; final concentration of vanadium complexes 50 μM). Open bars graphs present ordered results for tested vanadium complexes as per cent of BMOV activity (filled bar)



**Figure 5.** Insulin secretion in RINm5F beta cells screening activity results (60-minutes incubation; final concentration of vanadium complexes 100 μM). Open bars graphs present ordered results for tested vanadium complexes as per cent of BMOV activity (filled bar)
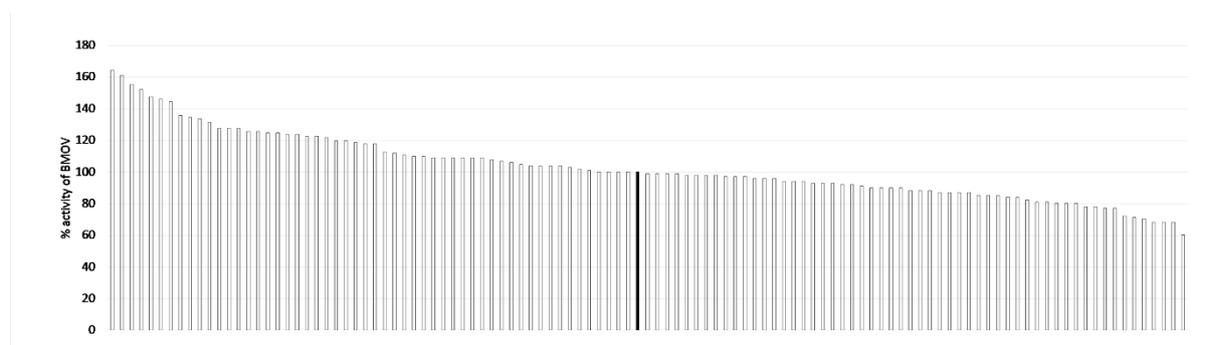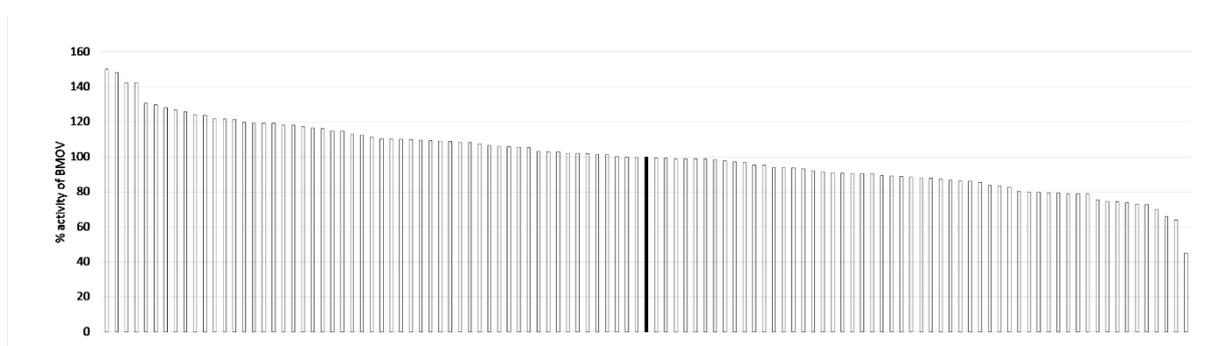
Based on the results presented in this study, those compounds were selected that showed high anti-diabetic activity and that simultaneously did not show significant toxicity in the same cellular models.

In the selection of complexes for further research, the profile of the anti-diabetic activity that complexes displayed for individual cell models was also identified. Table 1 shows examples of complexes illustrating high heterogeneity in cell activity pro-

files, from selective action in terms of insulin secretion against RINm5F pancreatic beta-cells (complex VC094) or selective action in terms of glucose consumption in C2C12 myocytes (complex VC024 and VC051) to complexes with high activity against all models tested (complex VC107). The results of cytotoxicity tests for the complexes given in Table 2 did not show any significant cytotoxic activity for any of the cell models used in the studies.

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Sci, Tech. Innov 2019; 4 (1), 47-54

**Table 1.** Activity profile of selected vanadium complexes in the used cell models.
Results were expressed as per cent of activity of BMOV

| Complex | Glucose utilization (C2C12 myocytes) | | Lipid accumulation (3T3-L1 adipocytes) | | Insulin secretion (RINm5F beta-cells) | |
|---|---|---|---|---|---|---|
| | Mean (%) | SD | Mean (%) | SD | Mean (%) | SD |
| **VC051** | 170 | 17 | 92 | 25 | 90 | 9 |
| **VC024** | 177 | 27 | 99 | 5 | 109 | 12 |
| **VC043** | 252 | 13 | 120 | 19 | 89 | 6 |
| **VC094** | 104 | 10 | 107 | 22 | 142 | 7 |
| **VC107** | 128 | 7 | 135 | 12 | 124 | 11 |

**Table 2.** The results of cytotoxicity testing for selected vanadium complexes in the used cell models.
Results were expressed as per cent of control containing solvent only; n.t. – not tested.

| Compound | C2C12 myocytes | | 3T3-L1 adipocytes | | RINm5F beta-cells | |
|---|---|---|---|---|---|---|
| | Mean (%) | SD | Mean (%) | SD | Mean (%) | SD |
| **BMOV** | 91 | 7 | 116 | 1 | 88 | 2 |
| **VOSO₄** | 84 | 7 | 123 | 7 | 89 | 2 |
| **ROSIGLITAZONE** | 120 | 3 | 120 | 6 | n.t. | n.t. |
| **GLIBENCLAMIDE** | n.t. | n.t. | n.t. | n.t. | 148 | 8 |
| **VC051** | 112 | 8 | 103 | 14 | 98 | 11 |
| **VC024** | 125 | 7 | 95 | 11 | 106 | 9 |
| **VC043** | 148 | 11 | 99 | 6 | 90 | 6 |
| **VC094** | 71 | 16 | 105 | 7 | 86 | 10 |
| **VC107** | 98 | 2 | 100 | 21 | 83 | 15 |

Previously published results concerning anti-diabetic activity of newly synthesized vanadium complexes include studies with a limited number of tested compounds. These studies are usually conducted in animal models, while studies in cell-based models are usually limited to one cellular model. For studies involving tens or hundreds of newly synthesized compounds, this approach is not possible; therefore, a multi-step process of early drug development from initial screening to final *in vivo* testing only for the most active compounds is the only rational approach. Due to the multidirectional action of vanadium compounds comprising various cells and molecular mechanisms, the most appropriate approach in the assessment of their anti-diabetic action and their impact on metabolism seems to involve several cell models representing different tissues. The approach based on cellular phenotypic screening and used in this study allowed for an effective assessment of the activity of vanadium complexes and confirmed this assumption.

In the next step these compounds were tested in a range of concentrations in the same experimental models for confirmation of the results observed in these preliminary tests. Subsequent studies of other mechanisms of anti-diabetic activity were carried out and profiling of the tested complexes against cells representing the main organs of the target action of compounds with anti-diabetic activity was performed. Based on this set of results, a few vanadium complexes have been selected for *in vivo* tests.

## Conclusions

The results of this study have allowed the selection of vanadium complexes with a multidirectional anti-diabetic activity, which has facilitated the choice of compounds for further tests in *in vitro* models. This study provides strong support for the application of screening cell-based assays involving a phenotypic approach for the discovery of novel anti-diabetic drugs from vanadium complexes class.

The tested 110 vanadium complexes are characterized by pronounced heterogeneity of activity profiles related to different cell models (myocytes, adipocytes, beta-cells) associated with pathomechanisms and treatment of metabolic disorders. This indicates interesting prospects for developing new compounds with targeted, desirable cellular activity, important not only in the treatment of metabolic diseases.

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G. Kazek et al. | Cell-based Screening for Identification of Novel Vanadium Complexes with Multidirectional Activity Relative to Cells Associated with Metabolic Disorders

53

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Sci, Tech. Innov 2019; 4 (1), 47-54

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