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Journal of Cancer Biology & Research

Research Article

Monitoring the Effectiveness and Time Dependency of Vanadium Pentoxide Cytotoxicity on A549 and Beas-2bcell Lines

Ayşe Kübra Karaboğa Arslan*, Ebru Öztürk, and Mükerrem Betül Yerer

Department of Pharmacology, Erciyes University, Turkey

*Corresponding author

Ayşe Kübra Karaboğa Arslan, Department of Pharmacology, Erciyes University, 38030, Kayseri, Turkey, Tel: 90-352-207-6666/28276; Fax: 90-352-437-9169; Email: aysekubrakaraboga@gmail.com

Submitted: 11 January 2017

Accepted: 14 February 2017

Published: 16 February 2017

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OPEN ACCESS

Keywords

- A549
- Beas-2b
- Cytotoxicity
- Xcelligence
- Vanadium pentoxide

Abstract

Lung adenocarcinoma is one of the most commonly occurring cancer types and it is the leading cause of cancer-related deaths worldwide. Vanadium compounds have different pharmacological effects and have cytotoxic properties. Aim of this study was by continuous monitoring to assay the cytotoxicity of vanadium pentoxide (V2O5) on human lung carcinoma cell line (A549) and human bronchial epithelial cell line (Beas-2b). Eight different concentrations of V2O5 between 2.5-40µM were applied on the cells and xCELLigence Real Time Cell Analysis (RTCA) was conducted to evaluate the impedance alterations over the Cell index values. Our results suggest the idea that V2O5 causes toxicity both on A549 and Beas-2b. We observed a dose-dependent cytotoxicity at 15 µM and higher doses in the A549 cells which might reveal its anticancer metallodrug potential. However, for Beas-2b cells although the cytotoxicity of V2O5 started after 5 µM, after 10 µM, the CI alterations reached a stable value at all doses applied, resulting in a maximum reduction of 50% in this healthy cells. Therefore these results revealed us that, 20 µM V2O5 at which cytotoxicity is initiated or up to 40 µM at which the IC50 level have been reached in A549 can also be used in Beas-2b since the maximum toxicity have been already reached at/over 10µM. We demonstrated that in cytotoxicity assays, the xCELLigence system can be used to optimize parameters such as the exposure time and compound concentrations. This study is the known first study to show V2O5's effects at these concentrations on A549 and Beas-2b in a real-time manner.

ABBREVIATIONS

A549: Human Lung Carcinoma Cell Line; Beas-2b: Human Bronchial Epithelial Cell Line; RTCA: Real Time Cell Analysis; V_2O_5: Vanadium Pentoxide

INTRODUCTION

Lung adenocarcinoma is one of the most commonly occurring cancer types among men and women and it is the leading cause of cancer-related deaths worldwide [1]. The presence of vanadium as an essential trace element at 20nM doses in body fluids is necessary for several physiological functions [2]. Although there are toxicity reports on several vanadium compounds including vanadium pentoxide (V_2O_5) on especially occupational exposure conditions [3], recent studies haveshown that certain vanadium compounds exhibit cytostatic/cytotoxic activity and suppress tumor cell growth *in vitro* and *in vivo* [4-6]. Exhibiting antitumor actions in several cancer cell lines such as lymphomas, T-cell leukemia, basophilic leukemia, and cancer of the liver, ovaries, testes, nasopharynx, bone and neuroblastoma, made vanadium compounds more popular for cytotoxicity tests [5]. After the invention of efficiency in treatment of cancer with various metallodrugs such as cis-platine, some other metallodrug candidates have been also investigated for their anticancer activities including vanadium, rutenyum and copper [7]. Vanadium oxides could be more toxic than vanadium salts therefore, vanadium oxide nanoparticles have been formulated by the scientists to reduce the side effects and increase the efficiency of vanadium oxides via nanoparticule formulations. Additionally, the same vanadium compounds could possess selective cytotoxicity to various cell lines such as L929 fibroblasts, FsaR fibrosarcoma cells and epithelial kidney cell line; Ma-104 cells [6].

The common cytotoxicity tests such as MTT, XTT, WST are still being widely used on determination of cell viability of cancer cell lines [8]. However these tests are end point tests which only reflect the effect of the tested compound on that precised time point chosen by the researcher. Sometimes the drug candidate might cause an acute effect on earlier time-points than the timepoint/s tested by the scientist by these common cytotoxicity tests and then the cells might start proliferating again. Therefore, the scientist might miss these acute effects by such end point tests which make real-time cytotoxicity tests inevitable for a real-time evaluation. The Roche xCELLigence System allows assessment of cell proliferation and cytotoxicity with quantitative and real-time monitoring of cells and based on impedance measurements for analyzing the status of adherent cells *in vitro*. Measurement of the

electrical impedance gives an idea about adhesion, proliferation and viability of cells. When the changes observed in impedance due to the cell attachment and spreading, it is expressed as the parameter termed Cell Index (CI) is formed. The CI reflects the cell viability thus cell number, attachment quality and cell type [9,10]. Monitoring of cell viability is critical and the xCELLigence system enables continuous measurement and quantification of cells [11,12]. Furthermore, the xCELLigence system allows for the calculation of time-dependent IC_{50} values in real-time [10].

In our present study, we conducted experiments with thexCELLigence system that investigated the cytotoxicity of the V_2O_5 on A549 and Beas-2b cell lines by real-time and continuous monitoring of the cell growth, proliferation and viability.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO, CAS: 67-68-5), Trypsin-EDTA (T3924), Fetal Bovine Serum (FBS, F2442), Penicillin-Streptomycin (P4333), Vanadium Pentoxide (V_2O_5) (CAS Number:1314-62-1), Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (DMEM-F12, D5546), RPMI-1640 Medium (R8758) purchased from Sigma Aldrich.

Cell culture

A549 (ATCC, CCL-185) human lung carcinoma cancer cell line, Beas-2b(ATCC, CRL-9609) human bronchial epithelial cell line purchased from American Type Culture Collection (ATCC). A-549 cells were considered to be alveolar epithelial cells with properties of type II cells, as they were isolated from an alveolar cell carcinoma [13]. Beas-2b cells were from healthy human bronchial epithelium and considered to be airway epithelial cells which transformed by an adenovirus 12–SV40 hybrid virus [14]. They have been shown to sustain typical epithelial morphology and many epithelial functional features [14,15]. A549 and Beas-2bcells cultured with DMEM-F12 and RPMI respectively which are contain with FBS 10%, L-glutamine 1%, 100 U/ml penicillin and 100 µg/ml streptomycin. A549 and Beas-2b were grown on 75 cm^2 cell culture flasks to approximately 80 and 70% confluence at 37^0C and humidified atmosphere with 5% carbon dioxide (CO_2) respectively. When cells reached the confluence, we detached cells with 0.25% trypsin-EDTA. The cells were centrifuged with the Universal 320R (Hettich, Zentrifugen, 1406 Germany) at 1000 rpm for 5 mins at 25^oC and seeded on 96 wells E-plate xCELLigence analysis. When cells reached log growth phase approximately 24 h later from seeding to E- plate, we treated cells with determined V_2O_5 concentrations.

Principle of xCELLigence system

The xCELLigenceReal-Time Cell Analyser (RTCA) systemallows monitoring of living cells through an integrated microelectronic sensor that found in each 96-microwell plate. The xCELLigence RTCA single plate (SP) system is composed of four components: a computer, electronic sensor analyzer, workstation and 96-microwell plate device. The E-plate is mounted on a device station located inside a CO_2 incubator. The device station is related to an electronic analyzer which is found at outside that automatically evaluates and transfers to a computer the electronic impedance values. The impedance data from the selected wells is exported to the computer and analyzed using RTCA software. CI is used to evaluate cell status based on detected cell-electrode impedance [16]. Cytotoxic effect of V_2O_5 was monitored with xCELLigence RTCA as described by manufacturer's instructions (Roche Applied Science and ACEA Biosciences), with slight modifications. Firstly, optimal seeding amounts of A549 and Beas-2b cell numbers were determined and then the cells were seeded in E-plate 96 wells. Cell proliferation, attachment and spreading for selected wells were monitored and recorded every 15 mins via the impedance of E-plate wells. Approximately 24h post-seeding when the cells were in the log growth phase, the cells are treated with V_2O_5 and control groups received only medium and replicated 4-times and the experiments were run for about 72 h.

Derivation of cell index (CI)

At the end of experiment all calculations were done with the RTCA-integrated software of the xCELLigence system. For each well, the baseline CI was calculated by subtracting the baseline CI from the original CI. The CI is derived to represent cell status based on the measured relative change in electrical impedance that occurs in the presence and absence of cells in the wells, which is calculated based on the following formula: $CI = (Zi–Z_0)/15$, where Zi is the impedance at an individual point of time during the experiment and Z_0 is the impedance at the start of the experiment. Impedance changes can occur depending on mainly two factors: The number of cells attached to the electrodes and the dimensional change of the attached cells on the electrodes [10,16,17].

Cell growth and proliferation assay using xCELLigence system

A549 and Beas-2b cells were grown and expanded in the culture flasks. When cells reached the determined confluence, we detached cells with 0.25% trypsin-EDTA. The cells were centrifuged with the Universal 320R (Hettich, Zentrifugen, 1406 Germany) at 1000 rpm for 5 mins at 25^oC. A549 (12500 cells/well) and Beas-2b (10000 cells/well) cells seeded on E-plate at 100 µL of cell culture media into the each well. After this the E-plate 96 was connected to the system and checked in the cell culture incubator for proper electrical-contacts and the background impedance was measured during 24 h. The adhesion, growth and proliferation of the cells were monitored every 15 min for a period of up to 24 h. When cells reached log growth phase approximately 24 h later from seeding to E- plate, we treated cells with V_2O_5 for 2.5; 5; 7.5; 10; 15; 20; 30 and 40 µM concentrations. The electrical impedance was measured by the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed CI.

Cytotoxicity assay using xCELLigence system

Approximately 24 h after seeding, when the cells were in the log growth phase, the cells were exposed to 25 µL of medium containing V_2O_5 for 2.5; 5; 7.5; 10; 15; 20; 30 and 40 µM concentrations. Controls received medium only. All experiments were run for 72 h.

Statistical Analysis

All calculations were obtained using the RTCA-integrated software of the xCELLigence system. The RTCA software performs a curve-fitting of selected "sigmoidal dose–response equation" to the experimental data points and calculates logarithmic half maximum effect of concentration (log $[IC_{50}]$) values at a given time point based on log of concentration producing 50% reduction of CI value relative to solvent control CI value (100%), expresses as log IC_{50} [18]. The CI was calculated from repeated experiments (n = 4) with the xCELLigence system. Furthermore, the integrated software of the xCELLigence system allows users to obtain parameters such as: average value, standart deviation (SD) and IC_{50}. Since these values are recorded and expressed in CI units, it can be both analyzed on the xCELLigence system and exported to Excel for any type of mathematical analysis, or graphics, facilitating the scientific analysis of the obtained data. Statistical analysis was performed GraphPad Prism Software Version 7.01 using to compare differences in values between the control and experimental group. Data were expressed as mean±standard deviation. Values of all significant correlations (p<0.05) are given with degree of significance indicated (*p<0.01, **p<0.001, *** p<0.0001, **** p<0.00001).

RESULTS

Monitoring of cytotoxicity in real-time using xCELLigence system

V_2O_5 exhibited cytotoxic effect on A549 at 20 µM, 30 µM and 40µM concentrations (Table 1, Figure 1,2). In Beas-2bcytotoxic effectis observed and the CI alterations decreased at all the concentrations compared with the untreated control (Table 1, Figure 3,4). By repeated xCELLigence measurements of V_2O_5 concentrations dose–response CI curves were obtained.

While the cells CI were increasing proportionally to cell number, we treated cells with the V_2O_5 concentrations. Alterations of CI for both cell types at 12 h, 24 h and 48 h after the treatment is given below (Table 1).

The IC_{50}values were calculated as 22.6 µM and 3.0 µM for A549 cells and Beas-2b cells after exposure V_2O_5 concentrations, respectively.

DISCUSSION AND CONCLUSIONS

Metallodrugs are novel and powerful tools for various therapeutic applications and display interesting biological activities for chemotherapy. In this field, cisplatin was the first inorganic compound with high relevance in cancer treatment and was major drug in clinical use. Toxicity and resistance problems trigger the development of other related drugs. Leon et al. show that complexes of metals are the new metal-based drugs used in the treatment of several cancers, such as, lung, colon, breast etc.[7].Vanadium compounds exhibit antitumor actions in several cancer cell lines such as lymphomas, T-cell leukemia, basophilic leukemia, and cancer of the liver, ovaries, testes, nasopharynx, bone and neuroblastoma was reported [5]. There are i*n vitro* and *in vivo* studies on lung cells [4,5,19,20]. According to that studies that can be helpful in determining the underlying mechanism(s) of carcinogenicity due to V_2O_5 in the lungs of mice. It is important to carry out studies on a range of pulmonary cell types, since carcinogenic effects were observed in the lung and vanadate (V^{+5}) affect different cell types disproportionately [21]. Waters M. D. et al. revealed that exposure to vanadium oxides could alter alveolar macrophage integrity and function to the detriment of pulmonary defense [19]. Rajendran N. et al., suggested that an interaction of characteristics, i.e., bioavailability, solubility and oxidation state, also species sensitivity, likely affect the toxicity potential of vanadium compounds. The lung is the target organ for all vanadium compounds. Vanadium compounds of different oxidation states (i.e., +3, +4 and +5) and solubility was associated with concentration-dependent toxicity in rats [20].

Vanadate oxidation states of biologic interest can lead to a lot of adverse effects in mammals etc. loss of body weight, reproductive and developmental toxicity, morbidity, and even death were found following vanadium exposure [3].

The aim of this study was to investigate the RTCA SP device of the xCELLigence system as a monitoring of cell proliferation the cancer and the investigation of the cytotoxicity of the V_2O_5 for

Table 1: The alterations of CI for both cell types at 12 h, 24 h and 48 h after treatment withV_2O_5 concentrations using the xCELLigence system.

Cell type/ number seeded	**A549/ 12.500 cells/well**			**Beas-2b/ 10.000 cells/well**		
Time	***12 h***	***24 h***	***48 h***	***12 h***	***24 h***	***48 h***
CI(control)	7.66±0.32	7.59±0.25	7.27±0.33	1.67±0.06	2.80±0.15	4.97±0.22
CI (2.5 µM)	7.51±0.29	7.72±0.50	7.33±0.38	1.35±0.09	2.01±0.32[d]	4.52±0.50[a]
CI (5 µM)	7.57±0.20	7.72±0.39	7.57±0.43	0.97±0.06[d]	1.03±0.17[d]	1.48±0.67[d]
CI (7.5 µM)	7.49±0.24	7.75±0.41	7.65±0.59	0.84±0.04[d]	0.76±0.07[d]	0.87±0.24[d]
CI (10 µM)	7.31±0.07	7.50±0.02	7.54±0.51	0.73±0.02[d]	0.64±0.05[d]	0.55±0.21[d]
CI (15 µM)	7.14±0.09	7.04±0.14	7.24±0.20	0.69±0.06[d]	0.58±0.09[d]	0.37±0.13[d]
CI (20 µM)	6.66±0.19[c]	6.07±0.36[d]	5.12±0.49[d]	0.65±0.01[d]	0.59±0.02[d]	0.33±0.02[d]
CI (30 µM)	5.70±0.20[d]	4.65±0.32[d]	2.50±0.30[d]	0.62±0.02[d]	0.64±0.02[d]	0.37±0.04[d]
CI (40 µM)	5.13±0.39[d]	4.31±0.66[d]	0.79±0.47[d]	0.60±0.06[d]	0.67±0.05[d]	0.34±0.04[d]

CI of during 12, 24 and 48 h exposure on the viability for A549 and Beas-2b cell lines were analyzed by GraphPad Prism Software Version 7.01 using to compare differences in values between the control and experimental group. Data were expressed as mean±SD (n = 4). Values of all significant correlations (p<0.05) are given with degree of significance indicated (a: p<0.01, b: p<0.001, c: p<0.0001, d: p<0.00001).

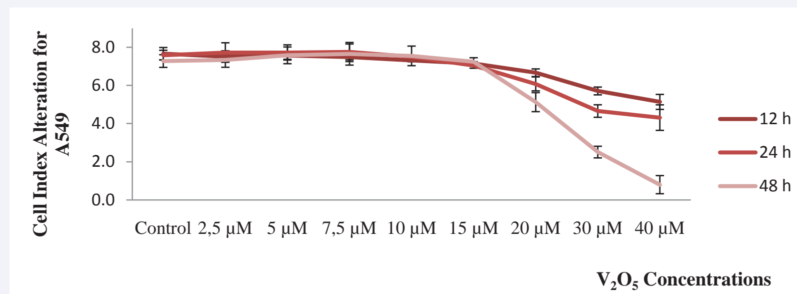

Figure 1 During 12, 24 and 48 h exposure on the viability of A549 cell line was measured based on the dose–response curves of the cell index by the xCELLigence system. Data were expressed as mean±SD (*n* = 4).

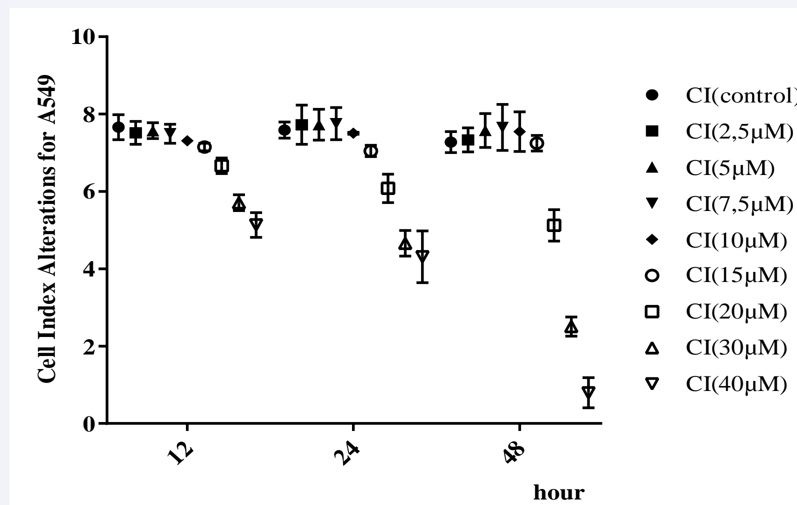

Figure 2 CI alterations in a time-dependent manner in A549 cell line.

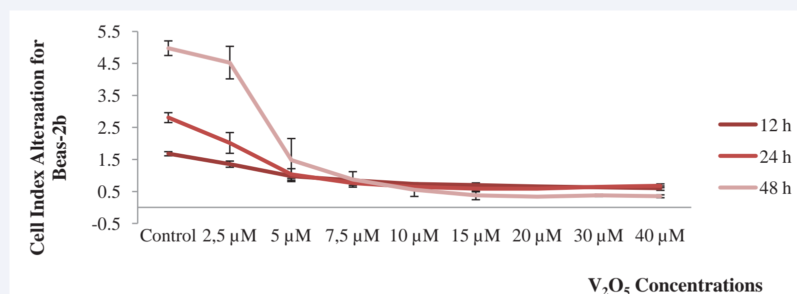

Figure 3 During 12, 24 and 48 h exposure on the viability of Beas-2b cell line was measured based on the dose–response curves of the cell index by the xCELLigence system. Data were expressed as mean±SD (*n* = 4).

2.5; 5; 7.5; 10; 15; 20; 30 and 40 µM concentrations during 72 h.

In our study we have investigated the effects of V_2O_5 on 2 different cell lines, including human lung carcinoma and human bronchial epithelial cell line; the CI alterations decreased strongly 20 µM, 30 µM and 40 µM for A549, at all the concentrations for Beas-2b. Our results suggest the idea that V_2O_5 causes toxicity on A549 and Beas-2b. The cytotoxic effect was observed in the A549 cell at 20 µM and there was a dose-dependent cytotoxicity at 15 µM and higher doses. For Beas-2b cells; after 10 µM doses, the cell index alterations were almost the same at all doses applied. The CI alterations after 10 µM reached a stable value, resulting in a maximum reduction of 50% in Beas-2b cells. Therefore, 20 µM V_2O_5 at which cytotoxicity is initiated in A549 can also be used in Beas-2b. It is for sure that, changes in cellular and molecular properties at 20 µM dose should also be investigated in Beas-2b non-cancer cell line.

We demonstrated that in cytotoxicity assays, the xCELLigence system can be used to optimize parameters such as the exposure time and compound concentrations. Finally, the IC_{50} values can be obtainedvia the xCELLigence system in real-time.

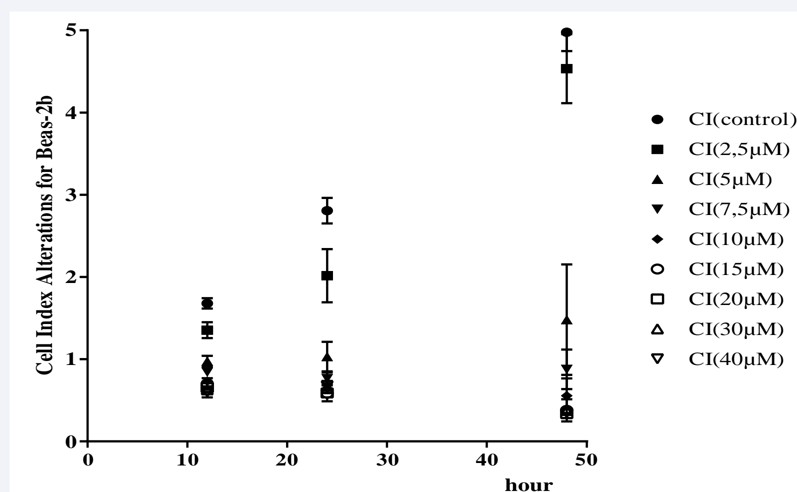

Figure 4 CI alterations in a time-dependent manner in Beas-2b cell line.

V_2O_5 shows different cytotoxicity profiles on the A549 and Beas-2b at the same concentrations. The findings of this study suggest that exposure to V_2O_5 can result in cytotoxicity 20 μM and the higher doses both A549 and Beas-2b cell lines. Beas-2b cell line exhibits similar results at 20 μM, 30 μM and 40 μM concentrations. Our results suggest that V_2O_5 has disparate effects on the cancer cells at different doses.Our data indicate that bronchial epithelial cells respond differently than lung epithelial cell lines to V_2O_5 with regard to CI alterations and real-time cytotoxicity profile. This different response made us to consider that cellular structure and type are important in terms of cytotoxic effect. Future studies may elucidate V_2O_5 effect differences between the cancer and normal cell lines and define the cellular toxicity potential as well as molecular mechanismsof V_2O_5 *in vitro* and *in vivo*. That will be important in understanding how the physicochemical and biological features of specific V_2O_5 interact to affect toxicity potential. V_2O_5 can be important for the metallodrugs search studies. Additionally, the RTCA System is a useful comparision tool for evaluating the cytotoxic effect of compounds in *in vitro* cell culture studies. This study is the known first study to show V_2O_5's effects on A549 and Beas-2b cell lines in a real-time manner.

ACKNOWLEDGEMENTS

We thank gratefully N. Nalan İmamoğlu Şirvanlı for supplying to us Beas-2b cell line.

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Cite this article

Karaboğa Arslan AK, Öztürk E, Yerer MB (2017) Monitoring the Effectiveness and Time Dependency of Vanadium Pentoxide Cytotoxicity on A549 and Beas-2bcell Lines. J Cancer Biol Res 5(2): 1097.