Vanadium oxide 3-methoxy salen, a synthetic biologically active complex against HeLa and McCoy cell lines
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ABSTRACT

Background: Vanadium is an essential dietary microelement that plays a key role in metabolic pathways and has anti-neoplastic effects. In this regard, vanadium oxide 3-methoxy salen complex was produced and its anticancer effects were evaluated against HeLa and McCoy cell lines.

Methods: Schiff bases produced from equivalents of Vanadyl acetylacetonate [VO(acac)2] in methanol were used to make a vanadium oxide 3-methoxy salen complex. Then, The antioxidant property of compound, cell viability and cytotoxicity assay, DNA fragmentation analysis and determination of the apoptosis pathway genes were evaluated.

Results: The result showed that the compound with an RC50 value of 126.3 µM demonstrated considerable free radical scavenging activity. The combination strongly suppressed the viability and proliferation of HeLa and McCoy cell lines in a dose-dependent manner, with IC50 values of 213 µM and 175 µM, respectively. When the viability and cytotoxicity values of the treated cells were compared, it was discovered that the cells had died of apoptosis, which was validated by DNA fragmentation analysis. Caspase 3, Bcl2 antagonist/killer, and Bcl2 associated X protein (Bax) gene expression levels all increased significantly in a quantitative investigation of apoptotic pathway genes, with 2-CT values of 2.36, 2.63, and 3.18, respectively.

Conclusion: In HeLa and McCoy malignant cell lines, lower quantities of the complex caused programmed cell death. This potential of complex can be used in cancer chemoprevention and cancer therapy.

Keywords: Apoptosis, Bax, Caspase, Salen Complex, Vanadium
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INTRODUCTION:

Vanadium is an essential dietary microelement that, along with its chemical compounds is known to have wide physiological effects [1]. Vanadium not only plays a key role in the metabolic pathways of thyroid glands, bone growth, lipids, and carbohydrates but also shows in vitro and in vivo anti-neoplastic effects [2, 3]. Recent studies have demonstrated that vanadium and its derivatives could not only induce apoptosis and DNA cleavage, but also retard cell proliferation, reduce the incidence of mammary tumors, kill yeast cells, inhibit hepatoma cells, and improve glucose homeostasis by insulin-like effects [4-7]. Depending on the type of the complexes and/or valence of the salts, vanadium and its derivatives have been reported as both preventive and anti-tumor agents, but some of their complexes or ions have shown carcinogenic effects through induction of retrotransposition [8-10]. However, there is little difference between the effective and toxic doses of the element. The surrounding ligands of the vanadium compounds may improve the adsorption of the compound into cells, and the anticancer effects of the compounds may ensue from the inhibition of protein tyrosine phosphatase or the activation of tyrosine phosphorylation [10]. However, some chemical conditions like lower pH of cytoplasm and/or the tissues immediately surrounding cancer cells can improve the cytotoxic effects of some vanadium complexes [5]. Recent studies have shown that the vanadium oxide 3-methoxy salen complex could serve as an anti-oxidative, anti-diabetic and anti-proliferative agent [11, 12]. Diabetic rats were given the methoxy VO-salen complex, which lowered their blood glucose levels [11]. According to recent research, vanadium may cause chromatin condensation and cell cycle arrest in treated cells, suggesting that it has the potential to become an anti-cancer treatment in the future [13].

Materials and Methods:
The vanadium oxide 3-methoxy salen complex was produced and tested as a physiologically active molecule against In HeLa and McCoy malignant cell lines in the current investigation.

Synthesis of Schiff base ligands
The Schiff base ligands were synthesized quantitatively by reacting meso-1,2-diphenyl-1,2-ethylenediamine and 1,2-ethylenediamine in ethanol with 2 equivalents of salicylaldehyde, 3-methoxysalicylaldehyde, and 5-bromo-salicylaldehyde. A dropwise addition of diamine (1 mmol) in 40-50 mL ethanol was made to a vigorously agitated ethanolic solution of aldehyde (40 mL) (2 mmol). After that, the mixture was mixed and refluxed for 1 hour. The resulting yellow precipitate was collected by filtering, washed with ethanol, and dried in a desiccator after the mixture was cooled. The interaction of Schiff base ligands with equivalents of vanadyl acetylacetonate [VO(acac)2] in methanol produced the whole vanadyl Schiff base complex [14].

Synthesis of the [VO(3-methoxy-salen)]
In a hot methanolic solution (70 mL) of VO(acac)2 (1 mmol), 3-methoxy-salen (1 mmol) and pyridine (1.5 mL) were added, and the mixture was rapidly agitated for 90 minutes under reflux. Filtration was used to collect the green precipitate, which was then washed with ethanol and ether before being dried in the air. [VO(3-methoxy-salen)] yield: (%82). C18H18N2O5V anal. calcd: 54.96; H, 4.58; N, 7.12. C was 54.79, H was 4.31, and N was 7.43. 981 [(V=O)], 1621 [(C=N)] IR (KBr, cm-1) (Fig. 1).

DPPH radical scavenging activity
According to a previously reported approach, the complex’s antioxidant property was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging test [15]. The RC50 value was established as the minimum concentration of an agent (µM) necessary to reduce DPPH radicals by 50% in the experiment condition [16].

Cell line and culture conditions
The National Cell Bank of Iran provided the HeLa and McCoy cell lines. The cells were grown in 25 cm2 culture flasks at 37 ºC in a humidified environment with 5% CO2 in RPMI 1640 (Gibco) media supplemented with fetal bovine serum (10%), penicillin (100U/ml),
and streptomycin (100g/ml). In the next experiments, all cells had a passage number of 3 - 5.

**Cell viability and cytotoxicity assay**

The MTT colorimetric test and the trypan blue exclusion experiment were used to assess the VOMS complex’s cell viability and antiproliferative properties [17, 18]. The cells were treated with concentrations of the complex of 75, 150, 300, 600, and 1200 M while in exponential development, and the results were recorded after 8 and 16 hours of incubation. The IC50 and CC50 values were established as the concentrations of a substance necessary to impede 50% of the cell growth and disrupt 50% of cell membrane, respectively [16].

**DNA fragmentation analysis**

A previously reported approach was used to assess DNA internucleosomal breakage as a crucial aspect of programmed cell death. Actinomycin D-treated cells were employed as an apoptosis-positive control [18].

**Determination of the apoptosis pathway genes by SYBER Green quantitative real-time PCR**

At first, 1 ml of TRIzol reagent (RNX, Cinnagen Co., Iran) per cell culture flask was used to homogenize the cells. Total RNAs were extracted from the cells using the manufacturer’s recommended methodology, followed by an additional chloroform extraction step to achieve phase separation. The total RNAs were resuspended in DEPC-treated double-distilled water, and the final concentration was measured using a 260 nm absorbance measurement (A260). The quality of the RNA was determined using electrophoresis on a 1% agarose gel. In the presence of 0.01 M DTT, 0.5 mM each dNTP, 0.5 µg oligo-dT, Primer, 40 U RNase free ribonuclease inhibitor, and 200 U M MuLV reverse transcriptase, first-strand cDNA was synthesized from 2 µg of each RNA sample (Fermentas, Germany) [19]. The reverse transcriptase enzyme was omitted from one of the RT PCR processes, which was carried out in triplicate. This extra reaction was carried out to determine the amount of genomic DNA contamination in each sample. The quantitative RT-PCR (qPCR) was performed using the Real-time PCR SYBER GREEN I kit (Qiagen, USA) to amplify the caspase 3, Bcl2 antagonist/killer gene [20], in triplicate, the Bcl2 associated X protein (Bax) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes [21, 22]. The GAPDH primer was chosen with the help of the beacon designer program (Version 7.5) (Table 1).

The qPCR was performed on the Rotor-Gene 3000 in two phases, according to the supplier’s instructions, and using first strand cDNA as a template (Corbett Research, Australia). The data were processed using the previously published $2^{-\Delta\Delta Ct}$ approach [23]. [(Ct target - Ct GAPDH)time x – (Ct target - Ct GAPDH) time 0] was used to determine the $2^{-\Delta\Delta Ct}$ value, and are expressed as gene expression levels that are related to one another.

### Table 1. The oligonucleotides which were used for the quantitative analysis of the apoptosis pathway genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplification Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>caspase3</td>
<td>AGAACGGGACCTGGGATGGGAGGCTGGTGCTGTTCAG</td>
<td>191</td>
<td>(21)</td>
</tr>
<tr>
<td>bak</td>
<td>GAACGGGAGGCTGGAAGGGTGTCAGCCATCTGTTACG</td>
<td>307</td>
<td>(21)</td>
</tr>
<tr>
<td>bax</td>
<td>TGCTTCAGGGTTTCATCCAGGCGGCGCCATCTCACGG</td>
<td>170</td>
<td>(22)</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AGAACATCATCCTGGCTACTGCTCCGAGCGCTCTCAC</td>
<td>192</td>
<td>This work</td>
</tr>
</tbody>
</table>
Results:

Synthesis of the complex

The interaction between tetradeutate ligands and VO produced the required oxovanadium(IV) complex (acac) [24]. The complex had a decent yield. The IR spectra of VO(3-methoxy-Salen) displays a V=O stretching vibration at 981 cm⁻¹, indicating that the compound is monomeric. The complex's C=N stretching vibrations were centered at 1612 cm⁻¹. The production of the VO-schiff base complex was also validated by elemental analysis (Figure 1).

Antioxidant Activity:

The antioxidant properties of the vanadium oxide 3-methoxy salen complex were assessed using the DPPH test after it was produced and purified. With an RC50 value of 126.3 µM, substantial DPPH radical scavenging activity was observed. The complex’s antioxidant activity increased in a dose-dependent way.

Cytotoxic and viability assay

For 8 and 16 hours, log-phase monolayer cells were treated with the complex at concentrations of 40, 80, 160, 320, and 640 µM. After 8 hours of treatment, the vitality of McCoy and HeLa cells was suppressed with IC50 values of 175 µM and 213 µM, respectively. With increasing treatment time, the IC50 value decreased significantly. The inhibitory potential of the complex is dependent on the kind of treated cells, according to a comparison of the viabilities of the treated cells. The McCoy cells were more susceptible to the complex’s cytotoxic action than the HeLa cells (Table 2). Until ~200 µM of the complex, the percentage viability of the cells vs. the concentrations of the complex showed a significant linear relationship. The linear curve reached a plateau at concentrations larger than ~200 µM, and the viability was unaffected by increasing the complex concentration (Figure 2).

When the IC50 and CC50 values were compared, it was

Figure 1. The structure VOS complex

Figure 2. The effects of vanadium oxide 3-methoxy salen complex on the McCoy and HeLa cells. Figures A and B show the linear dependency of the viability vs. the concentrations in the McCoy and HeLa Cells after treatment.
discovered that cell death may be classified as necrosis as well as apoptosis. More than 35% and 20% of the HeLa and McCoy cells, respectively, were presumably experiencing apoptotic cell death at a concentration of 160 µM (Figure 3).

Analysis of Apoptosis
The treated cells were tested for DNA fragmentation, which is a sign of apoptosis. The degree of fragmentation rose in a dose-dependent manner, and fragmentation in the treated cells was more efficient than in the positive controls (figure 4).
In HeLa cells treated with 160 M, qPCR of genes implicated in the apoptotic pathway was done. The \(2^{\Delta \Delta CT}\) values for the cp3, bac, and bax genes were 2.36, 2.63, and 3.18, respectively, when compared to GAPDH (Table 3).

Discussion:
Vanadium is not carcinogenic, but its presence in cancer cells causes changes in the expression of p53 and Bax, as well as the downregulation of Bcl2 proteins and antiproliferative activity, due to interactions with several critical enzymatic processes. In vitro and in vivo research have shown that vanadium has anti-carcinogenic and anti-cancer properties in various forms [25].
Results of antioxidant evaluation demonstrated that the overproduction of reactive oxygen species, which causes oxidative stress, adds to an overburdening of the cellular defense system, which may end in cell death [26, 27]. According to the previous reports, the significant radical scavenging activity of the methoxy VO-salen is attributed to its vanadium elements which can reduce the cytotoxic effect of H2O2 [12].

<table>
<thead>
<tr>
<th>Cell line</th>
<th>8-our treatment</th>
<th>16-hour treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (µM)</td>
<td>Viability at 40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>µM (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>McCoy</td>
<td>175</td>
<td>90</td>
</tr>
<tr>
<td>Hela</td>
<td>213</td>
<td>90</td>
</tr>
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<td></td>
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</tbody>
</table>

Table 2. The antiproliferative effect of vanadium oxide 3-methoxy salen against the cancerous cell lines.
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Table 3. The Relative quantitation results of the apoptosis pathway genes (t: treatment; c: control).

<table>
<thead>
<tr>
<th>gene</th>
<th>bax</th>
<th>bax</th>
<th>bak</th>
<th>bak</th>
<th>cp3</th>
<th>cp3</th>
<th>GAPDH</th>
<th>GAPDH</th>
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<tbody>
<tr>
<td></td>
<td>18.09</td>
<td>18.67</td>
<td>22.21</td>
<td>22.32</td>
<td>22.82</td>
<td>22.97</td>
<td>18.34</td>
<td>17.25</td>
</tr>
<tr>
<td></td>
<td>-1.67</td>
<td>-1.4</td>
<td>-1.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.18</td>
<td>2.63</td>
<td>2.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.7</td>
<td>0.24</td>
<td>cc</td>
<td>3.3</td>
<td>0.23</td>
<td>0.9</td>
<td>1.03</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Figure 4. The vanadium oxide 3-methoxy salen complex-induced DNA fragmentation in the McCOy (A) and HeLa (B) Cell lines. C- ) Negative control, t1 and t2) Treatment with 50 µM and 160 µM of the vanadium oxide 3-methoxy salen complex.

The IC50 values of vanadium salts have previously been reported to range from 27 to 47 µM [28]. It is obvious, however, that the kind of ligands used may impact the cytotoxicity of vanadium complexes, and these results demonstrated a low amount of cytotoxicity.

The predominant mechanism of cell death, according to DNA fragmentation experiments, was dose-dependent activation of apoptosis. The DNA fragmentation caused by vanadium compounds, as previously documented, might cause the cell-cycle arrest and/or cytotoxicity through nucleosomal fragmentation. Through DNA breakage and fragmentation, as well as plasma membrane lipoperoxidation, the chemicals may cause the cell-cycle arrest and/or cytotoxicity. After interacting with the nucleotide phosphate groups in DNA, vanadium compounds may have antiproliferative effects [29, 30]. These findings suggest that in the complex-treated cells, programmed cell death was effectively triggered.

Caspase 3 is a key enzyme in the apoptotic pathway’s execution stage, causing internucleosomal DNA cleavage, chromatin condensation, and the activation of other proteases [31]. The therapy with vanadium generates considerable chromatin condensation and cell cycle arrest, which results in apoptosis. Vanadium has the potential to be turned into an anti-cancer medication in the future, according to apoptosis-based studies. In the treated cells, the expression of the bax gene was much higher than that of the other genes, according to the findings. The translocation of Bax from the cytosol to the mitochondrial intermembrane contact sites causes mitochondrial permeability transition, loss of mitochondrial potential, the release of cytochrome C, activation of caspases, and DNA breakage, all of which lead to apoptosis [32, 33]. Vanadium’s anticancer effects were discovered to be mediated via the inhibition of cellular tyrosine phosphatases and/or the activation of tyrosine phosphorylases, which resulted in the induction of death in numerous cell lines [29]. Overexpression of the protein tyrosine phosphatase triggered the caspase pathway directly and caused p53-independent apoptosis, according to previous research [34]. The vanadium oxide 3-methoxy salen compound then activates the protein tyrosine phosphatase, causing apoptosis. Although SH2 domain-containing protein tyrosine phosphatase is predominantly a positive regulator of cell growth and development, it also plays a negative role in IFN-induced growth inhibition. IFN- also caused a greater degree of caspase expression [35].

In conclusion, our results showed that lower quantities of the complex in malignant cell lines by antiproliferative effects [29, 30]. These findings suggest that in the complex-treated cells, programmed cell death was effectively triggered.
Acknowledgments:
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