Abstract – Objective: The anti-cancer effects of 4-bromo-2-(((5-chloro-2-hydroxyphenyl) imino) methyl) phenol ([IV(L)] complex) have been verified. The main mechanisms used by the [IV(L)] complex to induce apoptosis in cancer cells have yet to be clarified. This study has been designed to explore the effects of the [IV(L)] complex on the expression of apoptosis-related genes, including P53, caspase-8, bax, bcl-2, bim, P21, and bid, in human liver hepatocellular carcinoma (HepG2) cell lines and mouse fibroblast cells (L929), as normal cells.

Materials and Methods: The RPMI medium was used to culture L929 and HepG2 cells at the IC50 concentration of the [IV(L)] complex. The expression of some apoptosis-related genes (p53, caspase-8, bax, bcl-2, bim, P21, and bid) was evaluated before and after 48 h from treating the cells by the [IV(L)] complex at the IC50 concentration using the real-time PCR. Data analyzed via SPSS 18 (SPSS Inc., Chicago, IL, USA) software and p < 0.05 was designated as the significant level.

Results: The [IV(L)] complex increased the mRNA expression levels of P53 and P21 genes in HepG2 (p < 0.05) cells. In addition, the expression levels of caspase-8 and bid decreased after treatment with the [IV(L)] complex (p = 0.05), and the expression levels of bax and bim genes remained fixed in the HepG2-treated cells. In L929 cells, the mRNA expression levels of P53, caspase-8, bim, P21, and bid increased, with the expression level of the bax gene significantly decreased after 48 h from treatment with the [IV(L)] complex (p = 0.001).

Conclusions: The Vanadium [IV(L)] complex induced apoptosis in HepG2 cells using the P53-P21 pathway-dependent method. In L929 cells, the mRNA expression levels of caspase-8 and bid were up-regulated in the treated cells. Therefore, it seems that apoptosis is triggered by both the P53-P21 pathway and the extrinsic apoptotic pathway in L929 cells.

Keywords: Vanadium complex, HepG2 cells, L929 cells, Hepatocellular carcinoma, Apoptosis.

List of Abbreviations: HCC: Hepatocellular carcinoma, Vanadium [IV(L)] complex: IV complex with 4-bromo-2-(((5-chloro-2-hydroxyphenyl) imino) methyl) phenol (L), FBS: Fetal Bovine Serum, EDTA: Ethylene Diamine Tetra Acetic Acid, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide, L929: Mouse fibroblast cells, HepG2: Liver hepatocellular carcinoma.
INTRODUCTION

Cancers are nowadays the main causes of morbidities and mortalities in both developed and developing countries. Liver cancer is most common in less developed countries, as demonstrated by the fact that in 2012 as many as 83% of the estimated 782,000 new cases worldwide occurred in such areas. Liver cancer is the second leading cause of cancer-associated mortality worldwide. Hepatocellular carcinoma (HCC) is the most prevalent type of cancer, ranked fifth among human cancers, with approximately 750,000 new cases arising each year. HCC could be induced by several factors: well-known risk factors for liver cancer including hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxins, alcohol consumption, oral contraceptives, tobacco smoking, androgenic steroids, and diabetes mellitus are suspected risk factors. Current knowledge regarding non-infectious occupational risk factors and liver cancer is inconclusive except for the well-established association between vinyl chloride monomer (VCM) and angiosarcoma of the liver (ASL). High liver cancer mortality has been described among heavy construction equipment operators, chimney sweepers, chemical workers, seamen, painters and exposure to solvent methylen chloride and textile machinery. Even though some therapeutic approaches such as chemotherapy, radiotherapy, and immunotherapy are used to treat cancers, they are associated with various complications due to their effects on noncancerous normal cells. Thus, finding new cancer treatment strategies is one of the major concerns of researchers to reduce complications. HepG2 is a famous HCC cell line that is used by researchers to examine new treatment strategies in vivo. Vanadium (IV) is a metal ion with its complex introduced as a candidate to treat cancers. Due to the low side effects of Vanadium IV, as compare to other metal ions such as platinum, current studies are mainly focused on Vanadium complexes to probably develop a safe and effective drug. Previous studies revealed that the IV complex with 4-bromo-2-((5-chloro-2-hydroxyphenyl)imino)methyl) phenol (L), abbreviated as the [IV(L)] complex, induces apoptosis more in HepG2 cell lines than in L929 cells, as normal cell lines. It has been suggested that the IV complexes could overcome cancer cells by several mechanisms, including the upregulation of free radical reactions, the alteration of expression in molecules involved in apoptosis, such as phospho-p21-activated protein kinases (PAK), oestosterone-3-kinase-protein kinase B/Akt (PI3K-PKB/Akt), cyclin-dependent kinase (CDK) 4, 6, and 7, death-associated protein kinase (DAPK), protein 2-alpha (AP2), Fas-associated protein with death domain (FADD), c-Jun N-terminal kinase (JNK), Caspases 3, 6, 7, 10, and 11, as well as B-cell lymphoma-extra (Bcl-x) and (Bcl-2). However, the effects of the [IV(L)] complex on the expression levels of p53, caspase-8, bax, bcl-2, bim, p21, and bid genes in HepG2 and L929 cells are yet to be clarified.

Therefore, based on our investigation that confirmed the cytotoxicity of the [IV(L)] complex more on HepG2 cells than L929 cells, the major goal of this study is to find the main genes targeted by the [IV(L)] complex to induce apoptosis in cancerous cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions

Human liver cancerous cells (HepG2), and mouse fibroblast (L929) cell lines were purchased from the Pasteur Institute, Tehran, Iran. The cell lines were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640) culture medium under standard conditions as described in the previous investigation. The determination of the inhibiting cell growth by 50% (IC50), the percentage of the survived cells using the MTT assay (Roche, Mannheim, Germany), the preparation of 4-bromo-2-((5-chloro-2-hydroxyphenyl)imino)methyl)phenol (H2L), 2,2'-bipyridine[4-bromo-2-((5-chloro-2-hydroxyphenylimino)methyl)phenol] oxido-vanadium(IV) [VOL(bipy)], and the FTIR spectral data were described in detail in the previous study. Our research received the Ethics Committee approval by Rafsanjan University of Medical Sciences (RUMS) with code No. IR.RUMS.REC.1394.178. All the laboratory tests were performed at the Molecular Medicine Research Center and the Research Institute of Basic Medical Sciences of RUMS.

RNA Extraction and cDNA Synthesis

The total RNA of HepG2 and L929 cell lines was purified before and after 48 h of treatment with the [IV(L)] complex, using the RNX solution from Cinnaclo Company (Tehran, Iran). The cDNA was synthesizized using commercial kits from Pars Tous Company (Mashhad, Iran). The total mRNA, oligo-dT, and RNase-free water were added to the tubes and incubated at 70°C and then 4°C for 10 and 4 min, respectively. The premix was then added and incubated at 40°C for 60 min. The next step was to incubate vials at 95°C for 10 min to inactivate the reverse transcriptase enzyme.

Real-Time PCR Analysis

The real-time PCR analysis was performed using a master mix from Pars Tous Company (Mashhad, Iran) by specific primers for P53, caspase-8, bax, bcl-2, bim, P21, and bid (Table 1). The real-time PCR protocol was
Besides, Bcl-2 mRNA levels decreased 3 times in HepG2 cell lines (p = 0.05), while the levels remained fixed in L929 cell lines. Although the expression levels of bim did not change in HepG2 cells after treatment with the \([IV(L)]\) complex (p > 0.05), the values increased significantly in L929 cells (p = 0.001).

The results also demonstrated that the mRNA expression levels of P21 increased significantly in both HepG2 (p = 0.05) and L929 cells (p = 0.001) after 48 h from treatment with the \([IV(L)]\) complex. The treatment of cells with the \([IV(L)]\) complex led to a significant increase in the mRNA expression levels of the bid gene in L929 cells (p = 0.001). However, the mRNA expression levels of the bid gene in treated HepG2 cells decreased significantly (p = 0.05) compared with the untreated cells.

Table 2 illustrates the data on the relative expression levels of all genes studied in both HepG2 and L929 cells before and after the treatment of the cells with the \([IV(L)]\) complex at the IC-50 concentration in 48 h. Figure 1 illustrates the effects of the \([IV(L)]\) complex on the expression levels of P53, caspase-8, bax, bcl-2, bim, P21, and bid in HepG2 and L929 cells.

### DISCUSSION

It has been reported that HCC is a prevalent type of cancer in developed and developing countries\(^1,2\). Although HCC has drug-resistant attributes in some cases\(^20-22\), our previous study\(^16\) revealed that \([IV(L)]\) the complex treatment led to strong apoptosis in HepG2 cell lines, compared with L929 cells as nor-
of pro/anti-apoptotic genes in cancerous cells, i.e., HepG2, led to more apoptosis in HepG2 cells than in L929 cells. Besides, bax levels decreased in both cell lines. Due to the fact that the bax gene expression level is regulated by P53 and P53, it induces apoptosis via several mechanisms, including the up-regulation of the bax gene; thus, it may be implied that the [IV(L)] complex induces apoptosis in both cell lines independently of the bax gene. Besides, due to the significantly increased expression level of P53, this gene uses other mechanisms other than bax to induce apoptosis. The data revealed that bcl-2 was down-regulated in HepG2 cells, while it was unchanged in L929 cells. Hence, it can be concluded that bcl-2 was down-regulated in HepG2 cells, while it remained unchanged in L929 cells. Yet, it may be hypothesized that, although the mRNA levels of pro-apoptotic genes increased in normal cells, the positive feedback of anti-apoptotic genes might have regulated the effects of the [IV(L)] complex and inhibited apoptosis. Quite notably, although the pro-apoptotic gene, i.e., bax, did not increase in HepG2 and L929 cells, it appears that the asymmetrical expression levels of pro/anti-apoptotic genes in cancerous cells, i.e., HepG2, led to more apoptosis in HepG2 cells than in L929 cells. Besides, bax levels decreased in both cell lines. Due to the fact that the bax gene expression level is regulated by P53 and P53, it induces apoptosis via several mechanisms, including the up-regulation of the bax gene; thus, it may be implied that the [IV(L)] complex induces apoptosis in both cell lines independently of the bax gene. Besides, due to the significantly increased expression level of P53, this gene uses other mechanisms other than bax to induce apoptosis. The data revealed that bcl-2 was down-regulated in HepG2 cells, while it was unchanged in L929 cells. Hence, it can be concluded that the increased ratio of bax/bcl-2 in HepG2 cells, yet not in L929 cells, might have led to the activation of apoptosis via the intrinsic pathway. The up-regulation of caspase-8 and bid in L929 cells, yet not in HepG2 cells, indicates the activation of apoptosis via the extrinsic pathway. Furthermore, the fixedness of the expression levels of bim, as well as the down-regulation of bid in HepG2 cells, indicates the activation of apoptosis via the extrinsic pathway. The results demonstrated that P21 had the highest rate of the expression increase among the pro-apoptotic genes.

### Table 2. Relative expression of P53, caspase-8, Bax, Bcl-2, Bim, P21, and Bid in HepG2 and L929 cell lines before and after 48 h from treatment with the [IV(L)] complex.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>HepG2 1 ± 0.18</td>
<td>3.04 ± 1.39</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.05</td>
<td>771.62 ± 45.27</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>HepG2 1 ± 0.23</td>
<td>0.19 ± 0.04</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.08</td>
<td>328.81 ± 93.59</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Bax</td>
<td>HepG2 1 ± 0.20</td>
<td>0.68 ± 0.01</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.27</td>
<td>0.008 ± 0.01</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>HepG2 1 ± 0.37</td>
<td>0.35 ± 0.20</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.49</td>
<td>1.37 ± 0.9</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Bim</td>
<td>HepG2 1 ± 0.47</td>
<td>1.60 ± 0.4</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.07</td>
<td>1994.98 ± 582.31</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>P21</td>
<td>HepG2 1 ± 0.48</td>
<td>6.67 ± 0.65</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.58</td>
<td>2265.79 ± 167.53</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Bid</td>
<td>HepG2 1 ± 1.29</td>
<td>0.08 ± 0.10</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>L929 1 ± 0.87</td>
<td>1792.66 ± 490.32</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
that induces apoptosis in HepG2 cells by increasing the expression levels of two important pro-apoptotic genes, namely P53 and P21, in both HepG2 and L929 cells. Moreover, the complex induces apoptosis significantly in HepG2 and L929 cells via intrinsic and extrinsic pathways, respectively. Due to the fact that the intrinsic pathway is mainly the safe and plausible mechanism to fight cancers\textsuperscript{17}, it appears that the [IV(L)] complex can be considered as an important drug that needs to be evaluated through in vivo studies.

Besides, it has been documented that sodium orthovanadate, another component of vanadium, inhibits the proliferation of human HCC cells by increasing autophagy\textsuperscript{34}. Since autophagy is a mechanism that is different from apoptosis, it could be hypothesized that the [IV(L)] complex could suppress the proliferation of HepG2 cells by integrating apoptosis and autophagy. This is a condition that needs to be explored through further studies. Considering the strengths of this work in synthesizing and examining a new compound (Transitional Metal complex) in the prevention of liver cancer. Some restrictions were also encountered by the researcher, including the financial limitations of the study on the number of additional genes and different cell lines, as well as the time limit for testing on cancerous tissues and animals.
CONCLUSIONS

Finally the results of this study as primary work on a new complex of Vanadium ([IV(L)]) complex showed that this complex can induce apoptosis in HepG2 cells using the P53-P21 pathway-dependent method. In L929 cells, the mRNA expression levels of caspase-8 and bid were upregulated in the treated cells. Therefore, it seems that apoptosis was triggered by both the P53-P21 pathway and the extrinsic apoptotic pathway in L929 cells. Since this complex did not show many adverse effects on normal cells, it can be suggested as a potent compound. After carrying out the necessary clinical trials, it may also be used as an anticancer drug.

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ETHICAL APPROVAL:

This article includes no study on human participants or animals as performed by any of the authors.

INFORMED CONSENT:

This article involves no studies on human participants.

CONFLICT OF INTEREST:

The authors declare that they have no conflict of interests.

REFERENCES


