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Research Article

Anti-Cancer Activity of Biotin-Polyoxomolybdate Bioconjugate

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Abstract

Objectives: Polyoxometalates (POMs), polyanionic metal clusters, have been well recognized for their anticancer activity in recent decades. Despite their potential anticancer activity, normal cell toxicity is one of the pressing issues that prevent their further clinical applications. In this work, we synthesized a new bio-conjugate based on POM and biotin. We used biotin as a bio-molecule to control the cytotoxicity of POM on healthy cells and simultaneously increase the toxicity on cancerous cells.

Methods: We synthesized the biotin derivative of POM via an amidation between the two molecules of biotin and amine groups on polyoxomolybdate. We approved the final structure using different spectroscopic data. We studied the cytotoxicity activity in-vitro using MTT protocol. We chose breast carcinoma cells (MCF-7) and hepatocellular carcinoma cells (HepG2) in comparison to the human umbilical vein endothelial cell (HUVEC).

Results: Results showed that biotin could improve the anticancer activity of polyoxomoybdate (IC50; 0.082 mM) on MCF-7 and (IC50; 0.091 mM) on HepG2 cells. Furthermore, the biotin-polyoxomolybdate conjugate showed lower toxicity on healthy cells versus the parent polyoxomolybdate and the Cis-platin as an approved drug.

Conclusion: Thus, we introduce promising novel POM bioconjugate, which can be further assessed in pre-clinical studies.

Keywords: Anti-cancer activity, biotin hybrid POM, polyoxometalats (POMs), MTT assay

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Cancer is a disease in which the cells of the body divide and reproduce abnormally. These cells are capable of penetrating and destroying healthy body tissues. Cancer is the second leading cause of death in the world and is responsible for an assessed 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to cancer. Chemotherapy is one of the most important methods of treatment and control of cancer that has not always been well received by patients due to its many side effects. In the field of pharmaceutical chemistry, there has always been a focus on finding new cytotoxic agents that have fewer side effects besides their potency.[1, 2] Polyoxometalates (POMs) are anionic clusters of oxygen-metal with remarkable chemical and physical properties. During the long history of POMs, we can see the different fields of application they involved comprising catalysis [3] magnetism,[4] material sciences,[5] and medicine.

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[6, 7] There have been relatively large studies since the last decades, which have introduced POMs as promising potential and cheap inorganic agents with remarkable anti-tumor, anti-viral, and anti-bacterial activities.[6]

POMs can inhibit tumor cell growth, for example, $[NH_3Pri]_6[Mo_7O_{24}]$. $3H_2O$ reveals good anticancer activity against breast, pancreatic, adenocarcinoma, and cancerous sarcoma cells.[8] Different research groups have reported the in-vitro anticancer activity of different polyoxotungstates. Lately, the cobalt-antimony derivative $\{CoSb_6O_4(H_2O)_3[Co(hmta)SbW_8O_{31}]_3\}_{15}$, (hmta; hexamethylenetetramine) was also displayed anticancer activity. POMs, specifically, Dawson-type $[P_2Mo_{18}O_{62}]^{6-}$ anions, were also recognized as protein kinase CK2 inhibitors, this multifunctional kinase is down-regulated in most of the cancers.[9]

The induction of cell apoptosis and the inhibition of ATP generation are the most reported mechanisms for the anticancer activity of POMs. Some other studies have reported the interaction of POMs with fibroblast growth factors, which has introduced them as new types of tumor angiogenesis inhibitors.[10-12]

All in all, POMs have unusual biological activities, with high efficacy. However, despite these achievements, after initial developments, so far, no POMs have been exposed to the marketplace; the main issue we should concern about is their side effects on healthy cells, which can be overcome either by increasing cell specificity or targeting.[6, 13]

The functionalization of POMs with organic groups via covalent bonding [14] can induce more stability under physiological conditions and offers to improve bio-distribution. [15] It seems that the hybrid organic-inorganic POMs have the potential ability to trigger the cellular receptors if the organic parts of the molecule can handle this process.[16] So, we can modify POMs through the conjugation to bioactive ligands, to get success, we can use more biologically reasonable organic ligands with well-defined structure and specific bioactivity to decorate the POMs. In this study, we have described a valuable research on tuning the anticancer activity and selectivity of a POM using targeting approach; this approach offers significant forthcoming advantages. The best of them is increasing drug delivery to the tumor site rather than the rest of the body. To get such a successful targeted delivery, we first need cellular targets that have a higher expression on the tumor cells than the healthy tissue.[17]

Biotin (B7) is an essential coenzyme for five mitochondrial carboxylases; it needs for normal mitochondrial and cellular functions. Biotin has a critical role in the biotinylation of histones, which can affect cell proliferation, gene silencing, DNA repair pathways, and cell apoptosis.[18]

There are so many different scientific reports emphasizing the better efficacy of biotin- drug conjugated hybrids. Hu et al. reported that Biotin-Pt (IV)-indomethacin hybrid could provide enhanced cancer cellular uptake and reverse cis-platin resistance.[19] So, biotin-targeting could improve the bioavailability of the drug conjugate along with better selectivity and markedly could enhance the water solubility of the drug.[20-23]

Cancer cells such as MCF-7 and HepG2 for their rapid cell growth have high levels of biotin receptors (sodium-dependent multivitamin transporter, SMVT) on their surface. [24, 25] Chen et al., prepared a biotin conjugates with one of the new-generation toxoids, SBT-1214, the approach of this work is evident, use of the biotin- SBT-1214 conjugate [26] which is characterized by an intracellular labile disulfide linkage, to target the cancer cell by exploiting the overexpression of biotin receptor on the tumor cell surface.

So, biotin seems to be the promising targeting agent that can selectively deliver POM as a cytotoxic agent to the cancer cells. Therefore, we predicted that the Biotin–POM Conjugate had improved cellular uptake via cell surface biotin receptors. In this study, we examined the biotinylation effect on the cytotoxicity profile in a molybdenum based POM.

Methods

All reagents and solvents were purchased commercially and used without further purification unless specially noted. We used Ultrapure Milli-Q water in all experiments. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), tetrabutylammonium bromide (TBAB), tris-(hydroxymethyl) aminomethane (TRIS), N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) were purchased from Sigma Aldrich (USA). Fetal bovine serum (FBS) was purchased from Gibco (Life Technologies AG, Switzerland). Dulbecco's modified Eagle's medium (DMEM) and RPMI- 1640 without folic acid were from Invitrogen Corporation. The breast cancer cell (MCF-7) and liver cancer cell (HepG2) were provided from the Pasteur Institute of Iran and cultured in DMEM, supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cord veins and maintained in RPMI-1640, supplemented with 10% human serum, 10% FBS, 1% glutamine (Invitrogen), 100 IU mL^{-1} penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 $mgmL^{-1}$ streptomycin (Sigma-Aldrich). We purchased the other remaining chemicals with the reagent grade purity from Sigma-Aldrich Co.

Synthesis of Compounds

Synthesis of Tetrabutylammonium Octamolybdate $[TBAB]_4[\alpha\text{-}Mo_8O_{26}]$ (1) (POM-1)

We acidified a solution of sodium molybdate dihydrate ($NaMoO_4.2H_2O$) (5.00 g, 20.70 mmol) in 12 mL of water with 5.1 7 mL of 6.0 N aqueous HCl (31.00 mmol) in a 50-mL flask with vigorous stirring for 1 to 2 min at room temperature. A solution of TBAB (99% pure, 3.34 g, 10.40 mmol) in water (10 mL) is then added as the stirring continued. We saw a white precipitate immediately, after stirring the mixture for l0 min, the precipitate is collected with suction and washed continuously with 20 mL of water, 20 mL of ethanol, 20 mL of acetone, and20 mL of diethyl ether respectively. This crude product (4.78 g) is dissolved in 35 mL of acetonitrile and stored for 24 hr at - 10 °C. The bright, colorless, block-shaped crystals were collected and washed continuously with 20 ml of distilled water, ethanol, acetone, and diethyl ether, again. The precipitations were dried for 12 h under vacuum (0.1 Torr). The obtained crystals became cloudy upon drying. Yield: 3.58 g (1.66 mmol), 64% of yield based on molybdenum.[27]

Synthesis of $[TBAB]_3[MnMo_6O_{18}(TRIS)_2]$ (2) (POM-2)

In 150 mL of acetonitrile, a mixture of 1 (8.00 g, 3.70 mmol), $Mn(OAc)_3.2H_2O$ (1.49 g, 5.60 mmol), and TRIS (1.56 g, 12.80 mmol) was refluxed for 16 h., the orange solution was cooled to room temperature and filtered to remove precipitates. The filtrate was exposed to ether vapor. A white precipitate has been filtered off after two hours. For several days, the orange filtrate was exposed to ether vapor. Large orange crystals were finally obtained in a yield of 80 percent. They were filtered and washed with a small amount of cold acetonitrile and ether and then dried in the vacuum.[28]

Synthesis of Biotin-POM-Biotin Conjugate (BP57)

Biotin (0.25 gr, 1 mmol), was first activated in the presence of NHS (0.12 gr, 1.04mmol) and EDC (1.20 mmol, 0.19 gr) in anhydrous DMF/CH3CN, the conjugation between biotin and the amine moieties of POM-2 (0.5 mmol, 0.94 gr) was carried out in the presence of TEA for 48 hours at room temperature. The BP57 bio-conjugate was precipitated by adding diethyl ether, filtered, and further purified by acetone and water wash to remove residual impurity of reagents and recrystallized from acetonitrile then dried under vacuum.[29]

Cell Viability Assay

With 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2, the cells were cultured and retained in RPMI-1640 (GIBCO) medium. The antitumor activity of compounds has been tested using standardized MTT protocol. Typically, stock solutions were prepared at a concentration of 0.01 $molL^{-1}$ in phosphate-buffered saline (PBS) (pH 7.4). Cells were densely seeded at a density of 5×10^3 cells per well, then incubated under the same as the culturing conditions for 24 h. Various concentrations of the tested compounds ranging from 50, 100, 200, 300, 400, and 500 µg/mL were added to the plates (MCF-7, HepG2, and HUVEC Cells). After 24 h, 20 µL of MTT solution at a concentration of 5 mg/mL in PBS buffer (pH 7.4) was added to each well. The medium was removed after 4 hr. of incubation, and 150 µL DMSO was added to each well. The purple formazan precipitate was then solubilized thoroughly, and we measured the absorbance using a micro-plate reader at 570 nm. We assigned three replicates per plate for each concentration, and all tests were repeated for all cell lines on three consecutive days. The cell viability was calculated using the following equation;

$$\text{Cell Survival\%} = \frac{(Tm\text{-}Bm)}{(Cm\text{-}Bm)} \times 100$$

in which Tm, Bm, and Cm represent mean absorbance of the treatment, blank and negative control, respectively.[30]

The cytotoxicity assay was run triplicate, and final average cell survivals used to calculate IC50s using excel software. To measure statistical relations between each treatment, one way ANOVA test was run in SPSS software, with Tukey alfa as post hoc test and significance level set at 0.05.

Results

Synthesis of Compounds

The schematic presentation of our synthesis has presented in figure 1. Furthermore, all of spectral evidences for correct synthesis of POM-1, POM-2, and BP57 have been provided as follow:

$[TBAB]_4[\alpha\text{-}Mo_8O_{26}]$ (POM-1); FTIR (KBr): v (cm^{-1}) 3445 (w, br), 2968(s), 2938 (s), 2875 (s), 1615 (w), 1473 (s), 1371(m), 1339 (w), 1149 (w), 957 (s), 928 (s), 910 (s), 862 (s), 810 (s), 663 (s), 562 (w), 505 (w), 413 (w).

$[TBAB]_3[MnMo_6O_{18}(TRIS)_2]$ (POM-2); FTIR (KBr): v (cm^{-1}) 3447 (w, br), 3291 (w), 2960 (s), 2934 (s), 2873 (s), 1619 (w), 1479 (s), 1384 (m), 1341 (w), 1172 (w), 1138 (w), 1040 (s), 939 (s), 918 (s), 900 (s), 797 (w), 739(w), 664 (s), 566 (m), 520 (w), 455 (m), 413 (m). 1H-NMR (400 MHz, DMSO-d6): 0.94 (t, 36 H), 1.32 (m, 24 H), 1.57 (m, 24 H), 3.12 (m, 24 H), 61.8 (s, 12 H); Elemental analysis: calcd. for C56H124MnMo6N5O24: C 35.73%; H 6.64%; N 3.72%; found experimental C 35.63%;

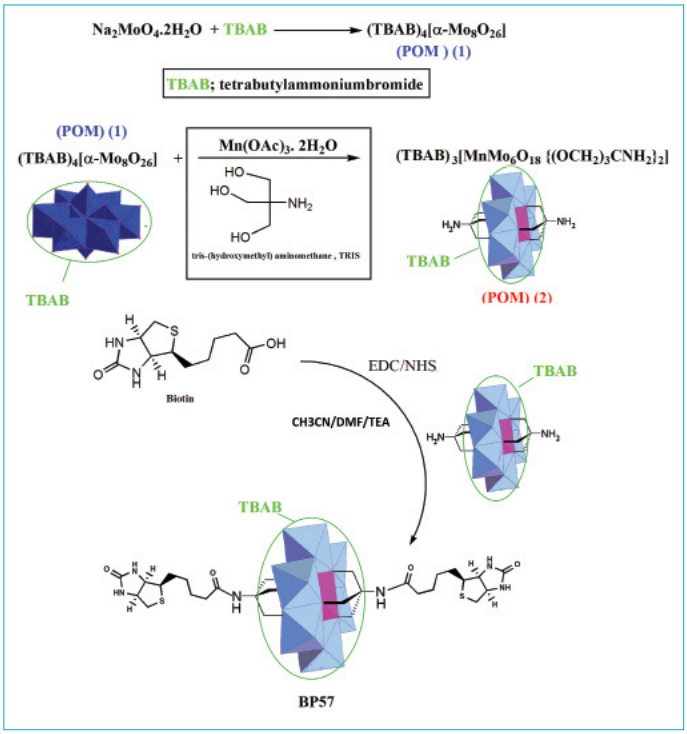

Figure 1. The schematic view of Biotin-POM conjugate (BP57) synthesis.

H 6.89%; N 3.88%; MW: 1882.18 gr/mol UV-Vis. (CH_3CN): λmax=220 nm, ε=47824 cm^{-1} M^{-1}.

Biotin-POM-Biotin conjugate (BP57); FTIR (KBr): ν (cm^{-1}) 3375 (w, br), 3288 (w), 2960 (s), 2870 (m), 1690 (bs, amide C=O), 1641 (m, NH amide def.), 1464 (m), 1378 (w), 1341 (w), 1157 (w), 1106 (w), 1031 (s), 942 (s), 918 (s), 900 (s), 797 (w), 738 (w), 662 (s), 564 (m), 518 (w), 455 (m), 413 (m). 1H-NMR (400 MHz, DMSO-d6): 0.96 (t, 36 H, POM), 1.32 (m, 24 H, POM), 1.56 (m, 8 H, Biotin), 1.69 (m, 4H, Biotin), 1.72 (m, 24 H), 3.16 (m, 24 H), 3.34-342 (m, 6 H, Biotin), 4.15 (s, 2H, Biotin), 4.30 (s, 2H, Biotin), 6.38 (m, 4H, Biotin), 9.38 (bs, 2H, newly formed amide NH), 62.30 (s, 12 H, POM); Elemental analysis: calcd. for C78H158MnMo6S-2N9O28: C, 39.62%; H, 6.73%; N, 5.33%; S, 2.71%; found experimental C, 39.49%; H, 6.80%; N, 5.42%; S, 2.68%; MW: 2364.84 gr/mol.

Cytotoxicity Assessments

The results of *in-vitro* cytotoxicity assessments for the final conjugate (BP57) in comparison to the POM, on the MCF-7, HepG2, and HUVEC cells have been presented in figures (2,3 and 4). Figure 2 represents the comparative cytotoxicity of BP57 and POM on the MCF-7 cell line. Figure 3 represents the same evaluation on the HepG2 cell line, and Figure 4, represents the comparative cytotoxicity of POM and BP57 on the HUVEC cell line.

Discussion

Synthesis of Compounds

We synthesized the $[N(C_4H_9)_4]_4[\alpha\text{-}Mo_8O_{26}]$ (1) as the core of our POM, according to the previous reports without any difficulty. We isolated $[N(C_4H_9)_4]_3[MnMo_6O_{18}\{(OCH_2)_3CNH_2\}_2]$ (POM-2) after the incubation of $[N(C_4H_9)_4]_4[\alpha\text{-}Mo_8O_{26}]$ with manganese (III) acetate in the presence of TRIS as orange crystals without any significant difficulty in 80% yield from 1 according to the previously reports.[28] The reaction of biotin with both ends of POM led to the final conjugate BP57 as a pale orange powder (Fig. 1). Since the synthesis and the characterization of Anderson type polyoxomolybdate (POM-1) was previously reported,[27, 28] so in this study, we compare the NMR, FTIR, and elemental analysis results with them to ensure about the accurate synthesis of the core structure of POM.

The structure of (POM-1) was approved by FTIR spectroscopy. We characterized the structure of (POM-2) and biotin-conjugated POM (BP57) by ^{1}HNMR spectroscopy, FTIR spectroscopy, and CHNS elemental analysis.

Based on the available reports, six edge-sharing MoO6 octahedral are arranged around a core of the MnO6 unit, making the Anderson structure. The TRIS are bound to the Mn(III) ion in the core via its alkoxy groups, so two amine groups of TRIS are oriented to outside of POM and are available for further modification The organic groups cover both sides of the planar hexagon through the chemical bonding to the amine groups of TRIS.[28]

As can see in Figure 1, we used both amine groups of POM for the functionalization with biotin. The amidation reaction between the carboxylic acid of biotin with Tris–POM–Tris was carried out through the carbodiimide strategy using EDC/NHS.[29] Purification through precipitation and recrystallization afforded the final product with a relatively high yield. The chemical structures of the biotin-POM-biotin conjugate (BP57) were confirmed by elemental analysis, FT-IR spectroscopy, and ^{1}HNMR spectroscopy. With an in-depth look at FTIR data of final conjugate compare to the POM, we find some changes after the conjugation, for example, N-H stretching frequency has somewhat increased from 3290 cm^{-1} to 3375 cm^{-1}, the carbonyl group stretching frequency moves from 1710 cm^{-1} in biotin to 1690 cm^{-1} in BP57 due to the conjugation, we also see the primarily amide band of biotin in the related place around 1641 cm^{-1}. We see some changes in the C–N stretching band from 1172, 1138 cm-1 to 1157, 1106 cm-1 after conjugation.

Furthermore, we can see the characteristic bands of Anderson- type POM at 943, 922, 903, 667, and 563 cm^{-1}, which remain unchanged after conjugation with biotin. These

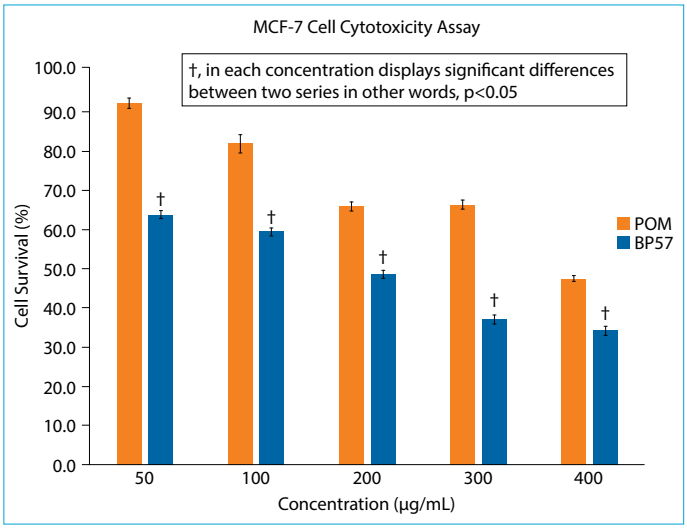

Figure 2. The comparative cytotoxicity of BP57 and POM on the MCF-7 cell line.

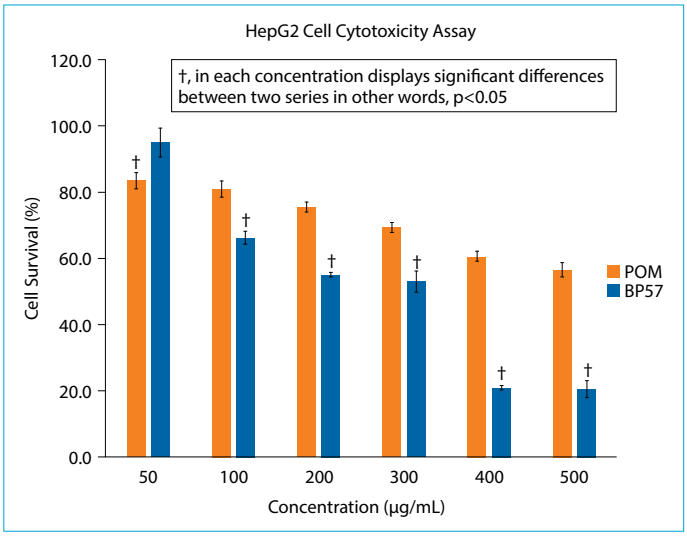

Figure 3. The comparative cytotoxicity of BP57 and POM on the HepG2 cell line.

IR proofs undoubtedly convinced us of the correct amide formation between biotin and POM. The data of ^{1}HNMR of BP57 are the best complementary data, the all fundamental signals of TBAB and TRIS in the POM scaffold are retain intact in BP57, beside them we can see the related signals of biotin in the correct spectral region, especially the signal of newly formed N-H amide proton around 9.3 ppm which is the best evidence for correct conjugation. As shown by Marcoux et al.,[28] because of the strong electron-withdrawing identity of POM, its methylene protons (belong to TRIS) appear around 62 ppm in HNMR with the right signal ratio for all related areas. Particularly the proper ratio of biotin signals to the methylene moieties of TRIS around 62 ppm confirmed the conjugation of 2 molecules of biotin to the POM.

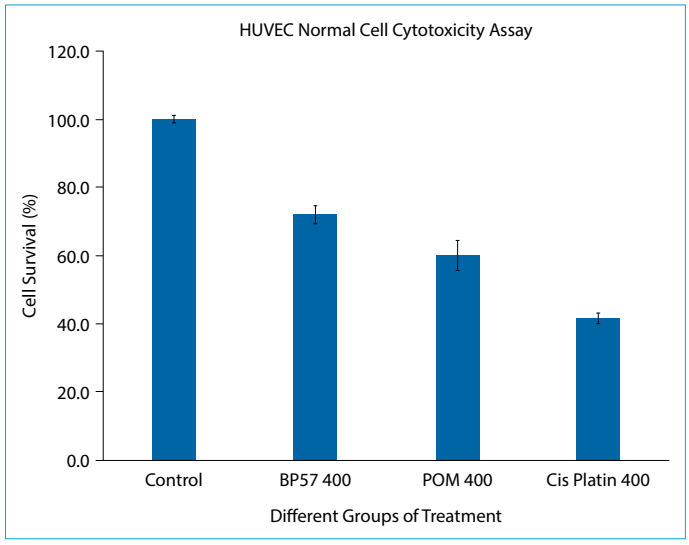

Figure 4. The cytotoxicity of BP57 and POM on the HUVEC cell line compared to the Cis-platin at the concentration of 400 µg/mL.

Cytotoxicity Assessments

To study the effect of biotinylation on the cytotoxicity profile of POM in BP57, we selected two cancer cell lines comprising MCF-7 and HepG2 due to their relatively high level of biotin receptor on them based on previous reports.[25] We treated the cells with different concentrations ranging from 50–500 µg/mL. We studied human umbilical vein endothelial cells (HUVEC) also for the assessment of normal cell cytotoxicity. The cell survival was assessed using MTT assay after 24 h of incubation.

Figure 2 shows the relative cell viability of MCF-7 treated with various BP57 concentration versus the POM. As shown in the figure, BP57 exhibited considerably better growth inhibition effect on MCF-7 cells compare to POM with no conjugated bio-ligand ($p<0.05$). The IC50 of the BP57 and POM on MCF-7 were 194.63 µg/mL (0.082 mM) and 390.50 µg/mL (0.207 mM) respectively. On the other hand, both of POM and BP57 showed somewhat less cytotoxic effects on HepG2 (Fig. 3), the IC50 of BP57 and POM on the HepG2 were respectively 214.22 µg/mL (0.091 mM) and 1196.31 µg/mL (0.636 mM) estimated. After the initial screening on cancerous cell lines, we examined the cytotoxicity of our compounds on the HUVEC cells at the concentration of 400 µg/mL, which was high enough to see the cytotoxic effects.

Interestingly, we did not get any considerable cytotoxicity on HUVEC in comparison to the positive control (cis-platin) at the same concentration for both BP57 and POM. As seen in Figure 4, both POM and BP57 have higher cell viability compared to the cis-platin at the same concentrations. This result always is promising for a new cytotoxic compound. Previous studies on organic functionalization of the manganese polyoxomolybdate have claimed that the modifica-

tion can decrease the side effects of POM beside the better biocompatibility.[31] Not surprisingly, the biotin-conjugated POM had lower cytotoxicity on the HUVEC.

The cellular cytotoxicity and cell internalization of POM has been reported in several studies.[32] Stephan and co-workers have discussed how extracellular target proteins as well as proteins integrated into the cell membrane with accessible extracellular binding sites, are the most possible sites for interaction with POMs and are the most critical issues affecting their pharmacological activity.[33]

The more cytotoxicity of BP57 compare to the POM, can be a result of the inherent toxicity of the POMs, with its facilitated entry through biotin receptors. The cytotoxicity of the BP57 improved by higher cell endocytosis of the conjugate through the biotin receptors. Although the cellular behavior of POM conjugates is not precisely apparent, we find the better activity of the conjugate against the MCF-7 cancerous cells than the HepG2 ones. This lower effect on HepG2 cell lines can be explained by the lower expression level of biotin-binding protein on HepG2 cells, or maybe, the lower sensitivity of the HepG2 cells compared to the MCF-7 cells in the culturing process or other intracellular mechanisms that predominant in MCF-7 cells relative to the HepG2 ones. Based on the evidence obtained from the Human Protein Atlas dataset, the breast tumor cells have more expression of the biotin-binding protein (HLCS gene) than the liver tumor cells; then, we can attribute the observed results to this fact.[34] Finally, as Zamolo et al.[35] have stated, this approach provides an efficient cytotoxic bioactive inorganic agent and paves the way to bio-functionalization of the POM for bio-recognition, cell internalization and biomimetic catalysis.

Conclusion

In conclusion, we have introduced the biotin bio-conjugate of an Anderson-type polyoxomolybdate (BP57) by amide bond formation between the carboxylic acid group of biotin and the amine moieties of POM-2. Our in-vitro cytotoxicity results on MCF-7 and HepG2 cells revealed that the biotin targeted POM (BP57) has excellent toxicity compare to the parent POM-2. So we can introduce a new anticancer agent from the POM derivatives, which offers an enhanced anti-cancer activity and selectivity in comparison with the parent POM and biotin. This synergistic effect can be related to the targeting ability of the biotin and the intrinsic cytotoxicity of the POM. This bio-conjugate has increased anti-cancer activity and lower normal cell cytotoxicity. Our preliminary findings in this study convinced us to continue the researches in this area. Our research team is focusing on the different aspects of the POMs abilities in the field of cancer treatment and will publish promising results soon.

Disclosures

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Ethics Committee Approval: The study was approved by the Local Ethics Committee and the ethics committee code is IR.MUI.REC.1396.1.230.

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – M.R.; Design – M.R., S.H.J.; Supervision – M.R., S.H.J.; Materials – M.R.; Data collection &/or processing – M.S.H., N.D., L.R.; Analysis and/or interpretation – M.S.H., A.A.H., N.D., M.R.; Literature search – M.S.H., A.A.H., L.R.; Writing – M.S.H., M.R., L.R.; Critical review – M.R.

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