Enhanced Antileukemic Activity of the Novel Complex 2,5-Dihydroxybenzoate Molybdenum(VI) against 2,5-Dihydroxybenzoate, Polyoxometalate of Mo(VI), and Tetraphenylphosphonium in the Human HL-60 and K562 Leukemic Cell Lines

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We synthesized and studied the antitumor properties of the novel complex compound 2,5-dihydroxybenzoate molybdenum(VI) with tetraphenylphosphonium as counterion, which also acts as cancer cell growth inhibitor. A novel complex compound, the 2,5-dihydroxybenzoate molybdenum(VI) complex, $(PPh_4)_2[Mo_3O_6(\mu\text{-}O)_2\text{-}(2,5\text{-}DHBA)_2]$ was synthesized. ^{1}H NMR, ^{13}C NMR, IR, and UV−Vis analyses were used for its molecular characterization. The human leukemia cell lines HL-60 and K562 were tested for their viability by assessing the metabolic activity of cells (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT), the structural integrity of the cell membrane (Trypan blue assay), and cell proliferation ability (growth curves). We showed that both leukemia cell lines are induced to decreased proliferation efficiency after treatment with the novel complex, compared to 2,5-dihydroxybenzoate, tetraphenyl-phosphonium polyoxomolybdate, or tetraphenylphosphonium chloride as individual entities, in a time- and concentration-dependent manner. Our results suggest that the new 2,5-dihydroxybenzoate molybdenum(VI) complex may provide a valuable tool in cancer chemotherapy.

Introduction

Gentisic acid (2,5-dihydroxybenzoic acid), a second line metabolite of aspirin, is known for its antioxidant properties by trapping free radicals.[1,2] It is considered to be equally effective against arthritis and, like aspirin, also has anti-inflammatory properties.[3] It possesses inhibitory effects on the human hepatoma cell line, $HepG_2$,[4] while it is found to play a role, as a constituent of *Hibiscus rosa sinensis* extract, in the prevention of skin cancer.[5] The contribution of gentisic acid on leukemia cell death in phototherapy by enhancing the merocyanine-540 uptake has been reported.[6,7]

Polyoxometalates have been found to act antagonistically against HIV and a polyoxomolydenum cluster, $[NH_3i\text{-}Pr]_6[Mo_7O_{24}]\cdot 3H_2O$, was found to induce apoptotic cell death in human pancreatic cancer cells, AsPC-1,[8] and possesses potent antitumor activity against human Co-4 colon, MX-1 breast, and lung cancer.[9] An oxomolybdenum catechol complex demonstrated fairly good activity to DNA cleavage and against tumor S-180 in mice.[10] Tetrathiomolybdate is considered to be a copper-lowering agent with antiangiogenic action in patients with advanced kidney cancer.[11] Nitrosamine-induced tumors in the esophagus and forestomach of animals as well as *N*-nitroso-*N*-methylurea-induced mammary cancer in rats are mediated by sodium molybdate supplementation.[12]

Tetraphenyl-phosphonium, a lipophilic cation that can selectively accumulate in the mitochondria of carcinoma cells, displays a concentration-dependent mitochondrial toxicity and is the basis for selective cytotoxicity to carcinoma cells in vitro and in vivo.[13,14]

In this work, we examined the cytotoxic effect of a novel compound that contains three active moieties, a polymeric oxomolybdenum(VI) core, 2,5-dihydroxybenzoic acid (gentisic acid), and tetraphenylphosphonium as cation, on two leukaemia cell lines, HL-60 and K562, against 2,5-dihydroxybenzoate. We found that this compound could sensitize and potentiate the leukemia cells tested to cell death, in comparison to 2,5-dihydroxybenzoate alone.

Results

Preparation and Characterization of the Novel Compound $(PPh_4)_2[Mo_3O_6(\mu\text{-}O)_2(2,5\text{-}DHBA)_2]$. The trimeric molybdenum(VI) complex of gentisic acid was prepared according to the reaction scheme presented in Figure 1. In the ^{1}H NMR spectrum, it is shown that the ligand is coordinated to the metal by the carboxylate oxygen and the phenolate oxygen atom at the *ortho*-position as no peaks of the corresponding protons are observed given that the metal replaces them. The single peak at 8.94 ppm is characteristic of the second (protonated) hydroxyl group, the ligand peaks were observed at 6.85, 6.69, and 6.49 ppm, while the aromatic protons of the tetraphenylphosphonium cation in the region 7.6−8.2 ppm. The integral ratio of the tetraphenylphosphonium and the ligand protons was 1, as expected. In the ^{13}C NMR spectrum, the peak at 173 ppm corresponds to carboxylate carbon atom, and the peaks of the phenolate carbon atoms appeared at 155 and 149 ppm (Figure 2). Notable is that the NMR spectrum of the complex remains unchanged after a period of approximately 1 month, indicating the stability of the complex in dimethylsulfoxide solution.

The electronic absorption spectrum of the complex in acetonitrile solution demonstrates a ligand-to-metal charge-transfer band at 324 nm. The bands at 261 and 225 nm are characteristic of the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transition bands attributed to the catechol and the $Mo{=}O^{4+}$ chromophore band, respectively.

In the IR spectrum, the band at 1588 cm^{-1} is characteristic of the complex as it attributes to the asymmetric C−O vibration of the carboxylate group. The second most characteristic band of the complex appears at 940 and 908 cm^{-1}, corresponding to

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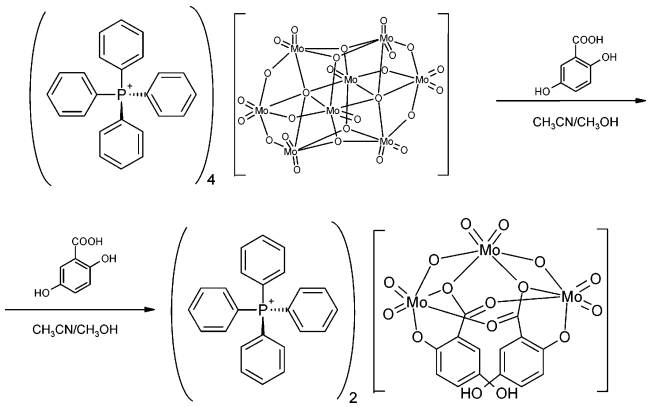

Figure 1. The synthetic scheme of the complex.

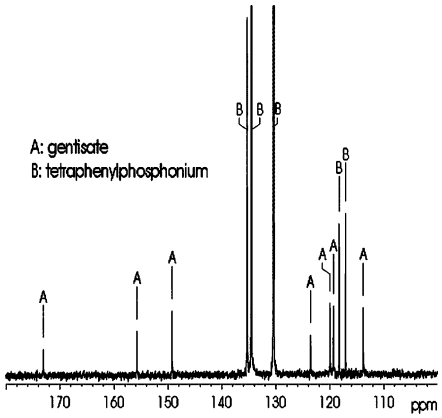

Figure 2. ^{13}C NMR spectrum of the complex in dimethylsulfoxide-d_6 solution.

the symmetric and asymmetric band of the *cis*-MoO_2^{2+} moiety, respectively.

Effect of the 2,5-Dihydroxybenzoate Molybdenum(VI) Complex on Leukemia Cells: Cell Growth, Cell Viability, and Necrosis. To determine whether the 2,5-dihydroxybenzoate molybdenum(VI) complex enhanced 2,5-dihydroxybenzoate, tetraphenyl chloride (PPh_4Cl), or tetraphenyl-phosphonium polyoxomolybdate ($(PPh_4)_4Mo_8O_{26}$) sensitivity, the cells were exposed to increasing doses of the 2,5-dihydroxybenzoate molybdenum(VI) complex, the 2,5-dihydroxybenzoate, the tetraphenyl-phosphonium chloride (PPh_4Cl), or the tetraphenyl-phosphonium polyoxomolybdate ($(PPh_4)_4Mo_8O_{26}$), as individual entities (10, 100, 1000, and 10 000 μg/mL), for increasing periods of time (24, 48, and 72 h), and cell viability was measured by the MTT[a] assay. As shown in Figure 3, both cell lines exhibited increased sensitivity to the 2,5-dihydroxybenzoate molybdenum(VI) complex, compared to 2,5-dihydroxybenzoate alone, in a dose- and time-dependent manner, but less sensitivity to the tetraphenyl-phosphonium chloride or the tetraphenyl-phosphonium polyoxomolybdate as individual entities. However, the sensitivity of cells to the cytotoxic effect of the 2,5-dihydroxybenzoate molybdenum(VI) complex was higher in HL60 rather than K562 cells, indicating cell-type dependence.

As the MTT method assesses quantitatively cell viability but cannot distinguish between growth arrest or necrosis, we also analyzed cell growth, as well as the percentage (%) of trypan blue +ve cells (necrosis) in the cell lines mentioned above. The

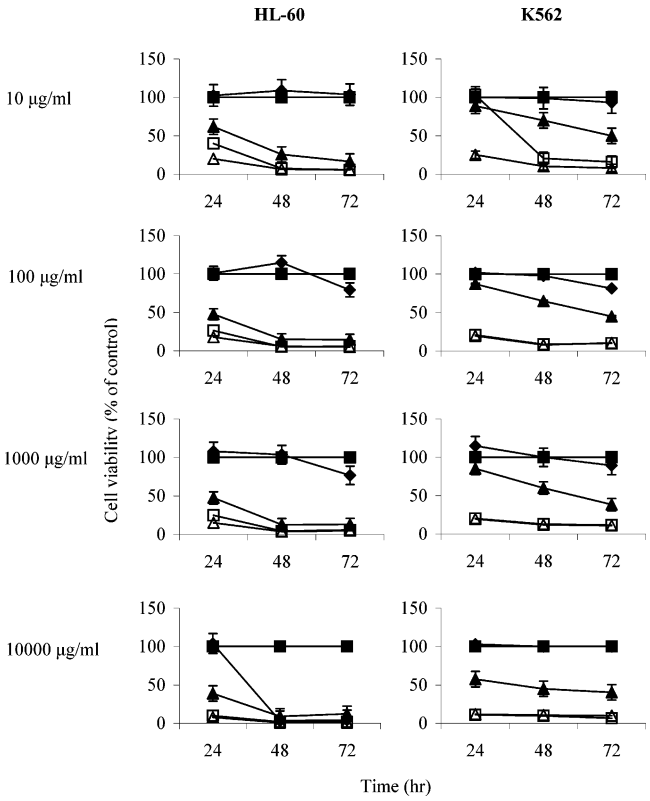

Figure 3. Treatment of HL-60 and K562 cell lines with the complex 2,5-dihydroxybenzoate molybdenum(VI) displayed enhanced cytotoxic effect compared to 2,5-dihydroxybenzoate, tetraphenyl-phosphonium chloride, but displayed decreased cytotoxic effect compared to tetraphenyl-phosphonium polyoxomolybdate. The cells were treated in the absence (−■−) and in the presence (−▲−) of various doses of the complex , as well as the 2,5-dihydroxybenzoate alone (−♦−), the tetraphenyl-phosphonium chloride (−△−), or the tetraphenyl-phosphonium polyoxomolybdate (−□−), as individual entities, for 24, 48 and 72 h, and the cell viability was measured by the MTT assay. Each data point represents the mean for three separate experiments (mean ± SD).

cell growth curves exhibited a concentration- and time-dependent inhibition of cell proliferation, which was much more evident and enhanced in the presence of the complex, tetraphenyl-phosphonium chloride, or tetraphenyl-phosphonium polyoxomolybdate, as individual entities (Figure 4). The trypan blue staining revealed that the complex displayed enhanced necrotic effects, in comparison to 2,5-dihydroxybenzoate alone, causing in some cases even 100% necrosis of the cells, whereas it displayed decreased necrotic phenomena in comparison to tetraphenyl-phosphonium chloride or tetraphenyl-phosphonium polyoxomolybdate as individual entities (Figure 5 and Tables 1 and 2). However, concentrations higher than 1000 μg/mL presented extensive toxicity for all the compounds under study in the case of HL-60 cells, which displayed much more sensitivity than K562 cells (Figures 3, 4, and 5 and Tables 1 and 2).

Discussion

Leukemia is one of the most frequent types of cancer, being characterized by more than 500 recurring translocations. In more than one-half of all cases of leukemia, recurrent chromosomal changes occur.[15] Heretofore, leukemia can be confronted mainly with chemotherapeutic regimens. Even if in the last years the means of its confrontation has been improved considerably, a

[a] Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; BCL2, B cell lymphoma gene 2; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulphate; PBS, phosphate-buffered saline.

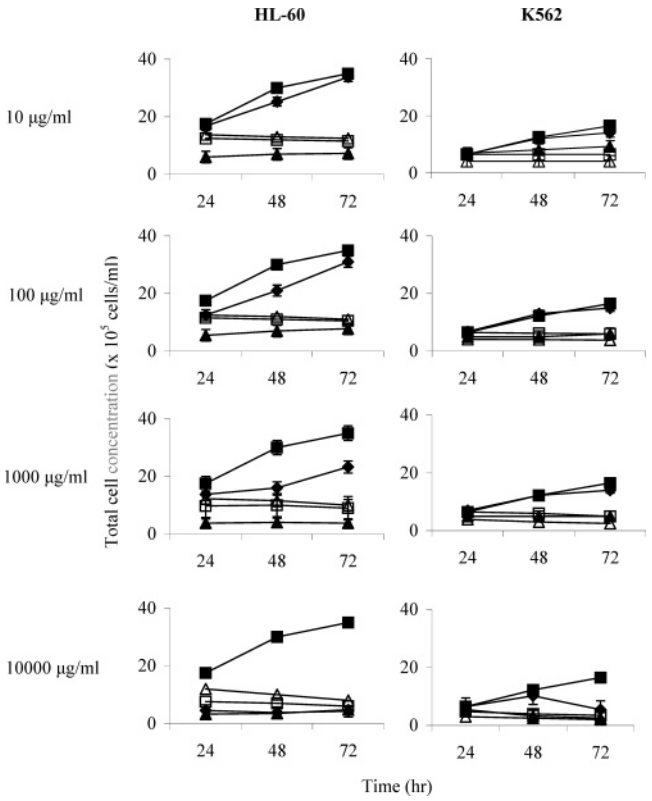

Figure 4. Treatment of HL-60 and K562 cell lines with the complex 2,5-dihydroxybenzoate molybdenum(VI) displayed enhanced antiproliferative effect compared to 2,5-dihydroxybenzoate, tetraphenyl-phosphonium chloride, or tetraphenyl-phosphonium polyoxomolybdate. The cells were treated in the absence (−■−) and in the presence (−▲−) of various doses of the complex, as well as the 2,5-dihydroxybenzoate alone (−♦−), the tetraphenyl-phosphonium chloride (−△−), or the tetraphenyl-phosphonium polyoxomolybdate (−□−), as individual entities, for 24, 48 and 72 h, and the total cell concentration was measured using a hemeocytometer. The results were expressed as the total cell concentration of cells. Each data point represents the mean for three separate experiments (mean ± SD).

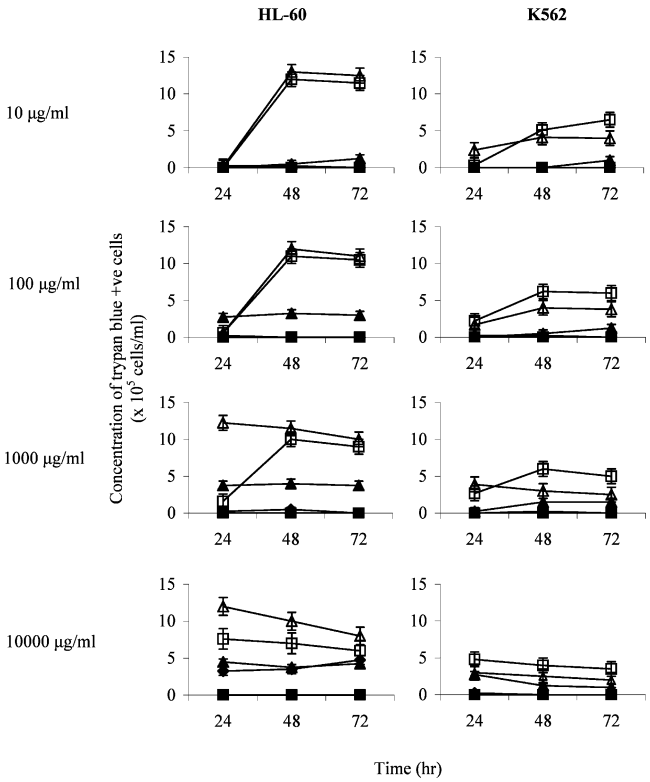

Figure 5. Treatment of HL-60 and K562 cell lines with the complex 2,5-dihydroxybenzoate molybdenum(VI) displayed enhanced necrotic phenomena compared to 2,5-dihydroxybenzoate, but displayed decreased necrotic phenomena compared to tetraphenyl-phosphonium chloride or tetraphenyl-phosphonium polyoxomolybdate. The cells were treated in the absence (−■−) and in the presence (−▲−) of various doses of the complex, as well as the 2,5-dihydroxybenzoate alone (−♦−), the tetraphenyl-phosphonium chloride (−△−), or the tetraphenyl-phosphonium polyoxomolybdate (−□−), as individual entities, for 24, 48 and 72 h, and the necrotic phenomena were detected by trypan blue staining. The results were expressed as the concentration of trypan blue +ve cells. Each data point represents the mean for three separate experiments (mean ± SD).

big percentage of patients relapse and finally die. It is, therefore, necessary to investigate better therapeutic interventions and new drugs.

An important parameter in the growth, prevention, and confrontation of leukemia, as well as other types of cancer, constitutes the balance between cell proliferation and cell death that is essential for the regulation of cellular homeostasis. The unsuccessful action of many chemotherapeutic drugs reflects, at the cellular level, the disability of specific drugs to induce cell death.[16−24] Furthermore, leukemia cells are characterized by the deregulated expression of genes acting as survival or cell death factors, having as a result apoptosis inhibition. According to recent work in our laboratory, important modulations in the expression levels of apoptosis related genes, along the induction of apoptosis, such as members of the BCL2 family of genes were observed that were dependent on both the apoptosis inducer (anticancer drug) and the specific apoptotic pathway induced.[25−29]

In this study, the response of two different leukemia cell lines (HL-60 and K562) to the novel 2,5-dihydroxybenzoate molybdenum(VI) complex as well as to 2,5-dihydroxybenzoate, tetraphenyl-phosphonium polyoxomolybdate, or tetraphenylphosphonium chloride, as individual entities, was evaluated. The two leukemia cell lines were selected because they cover all the spectrum of correspondence to chemotherapy, from the endogenous tendency for cell death (HL-60) until the resistibility to it (K562).

The novel complex, 2,5-dihydroxybenzoate molybdenum(VI), was prepared and its structure was confirmed. It contains three subunits, all of which possess anticancer properties, a polymeric oxomolybdenum(VI) core, 2,5-dihydroxybenzoic acid (gentisic acid), and tetraphenylphosphonium, a lipophilic cation.

Our results revealed that molybdenum(VI) with 2,5-dihydroxybenzoate could potentiate and sensitize both HL-60 and K562 human leukemia cells to a more pronounced decrease of cell viability, as assayed by MTT, in comparison to 2,5-dihydroxybenzoate alone, accompanied by increased necrotic phenomena and decreased cell proliferation. However, in comparison to tetraphenyl-phosphonium polyoxomolybdate or tetraphenylphosphonium chloride, as individual entities, the complex displayed higher cell viability, accompanied by much less necrotic phenomena and a more pronounced decrease in cell proliferation. This overall intense cytotoxic activity of the complex seems to be due to the cell proliferation inhibitory activity of the complex, as a synergistic effect of all three antitumor factors that exist in the complex, the polymeric oxomolybdenum(VI) core, the 2,5-dihydroxybenzoic acid (gentisic acid), and the tetraphenylphosphonium, and to a much lesser extent due to necrotic phenomena. In addition, the induction of cell growth inhibition or cell death with delay in K562 cells,

Table 1. Percentage (%) of Trypan Blue + Cells versus Total Number of Cells in the Cell Line HL-60

	Sample (10 μg/mL)					
	%	SD[a]	%	SD[a]	%	SD[a]
control	0	0.001	0	0.0001	0	0.002
2,5-dihydroxybenzoate molybdenum(VI)	0	0.0003	7.1	0.05	33.3	0.0004
2,5-dihydroxybenzoate	1.5	0.004	1	0.001	0	0.001
tetraphenyl-phosphonium polyoxomolybdate	0	0.0001	100	0.004	100	0.005
tetraphenyl-phosphonium chloride	1	0.001	100	0.006	100	0.003
	Sample (100 μg/mL)					
control	0	0.001	0	0.0001	0	0.002
2,5-dihydroxybenzoate molybdenum(VI)	50	0.005	46.4	0.05	38.7	0.0004
2,5-dihydroxybenzoate	2	0.001	0	0.0002	0	0.0003
tetraphenyl-phosphonium polyoxomolybdate	5	0.004	100	0.005	100	0.001
tetraphenyl-phosphonium chloride	4.5	0.003	100	0.001	100	0.008
	Sample (1000 μg/mL)					
control	0	0.001	0	0.0001	0	0.002
2,5-dihydroxybenzoate molybdenum(VI)	100	0.05	100	0.0001	100	0.005
2,5-dihydroxybenzoate	1.8	0.002	3.1	0.0001	0	0.0002
tetraphenyl-phosphonium polyoxomolybdate	16	0.004	100	0.003	100	0.001
tetraphenyl-phosphonium chloride	100	0.001	100	0.004	100	0.002
	Sample (10 000 μg/mL)					
control	0	0.001	0	0.0001	0	0.002
2,5-dihydroxybenzoate molybdenum(VI)	100	0.05	100	0.005	100	0.001
2,5-dihydroxybenzoate	100	0.003	100	0.003	100	0.005
tetraphenyl-phosphonium polyoxomolybdate	100	0.001	100	0.004	100	0.008
tetraphenyl-phosphonium chloride	100	0.003	100	0.001	100	0.001

[a] SD = standard deviation.

Table 2. Percentage (%) of Trypan Blue + Cells versus Total Number of Cells in the Cell Line K562

	Sample (10 μg/mL)					
	%	SD[a]	%	SD[a]	%	SD[a]
control	0	0.0001	0	0.005	0	0.001
2,5-dihydroxybenzoate molybdenum(VI)	0	0.0001	0	0.001	10.8	0.005
2,5-dihydroxybenzoate	0	0.0001	0	0.0005	0	0.0008
tetraphenyl-phosphonium polyoxomolybdate	4.7	0.003	78.7	0.04	100	0.05
tetraphenyl-phosphonium chloride	56.8	0.005	100	0.001	100	0.001
	Sample (100 μg/mL)					
control	0	0.0001	0	0.005	0	0.001
2,5-dihydroxybenzoate molybdenum(VI)	0	0.001	10	0.05	20.8	0.005
2,5-dihydroxybenzoate	3.7	0.005	1.9	0.003	0	0.001
tetraphenyl-phosphonium polyoxomolybdate	34	0.08	98	0.01	100	0.001
tetraphenyl-phosphonium chloride	41.8	0.04	100	0.002	100	0.005
	Sample (1000 μg/mL)					
control	0	0.0001	0	0.005	0	0.001
2,5-dihydroxybenzoate molybdenum(VI)	5	0.05	30	0.05	30	0.04
2,5-dihydroxybenzoate	0	0.0001	2	0.001	0	0.001
tetraphenyl-phosphonium polyoxomolybdate	41.3	0.05	90	0.05	100	0.001
tetraphenyl-phosphonium chloride	100	0.001	100	0.03	100	0.003
	Sample (10 000 μg/mL)					
control	0	0.0001	0	0.005	0	0.001
2,5-dihydroxybenzoate molybdenum(VI)	50	0.03	38.5	0.05	40	0.05
2,5-dihydroxybenzoate	3.8	0.03	0	0.0008	0	0.001
tetraphenyl-phosphonium polyoxomolybdate	100	0.001	100	0.001	100	0.004
tetraphenyl-phosphonium chloride	100	0.001	100	0.003	100	0.001

[a] SD = standard deviation

relative to HL-60 cells, could be interpreted by the more resistant nature of K562 cells to drug-induced cell death compared to that of HL-60 cells.

Taken together, the above results demonstrate that both leukemia cell lines examined are induced to decreased proliferation efficiency after treatment with the novel complex 2,5-dihydroxybenzoate molybdenum(VI) compared to 2,5-dihydroxybenzoate, tetraphenyl-phosphonium polyoxomolybdate, or tetraphenylphosphonium chloride, as individual entities, in a time- and concentration-dependent manner. This may suggest that the new 2,5-dihydroxybenzoate molybdenum(VI) complex may provide a valuable tool in cancer chemotherapy, although further work is required.

Experimental Section

Chemistry. The reagents 2,5-dihydroxybenzoic acid, ammonium molybdate ($(NH_4)_2MoO_4$), tetraphenyl-phosphonium bromide (PPh_4Br), as well as the solvents acetonitrile, methanol, and ether, were purchased from Aldrich Chemical Co. and used without purification. All the reactions took place under aerobic conditions and room temperature. The compound $(PPh_4)_4Mo_8O_{26}$ was produced dissolving tetraphenylphosphonium bromide (209.7 mg, 0.5 mmol) and ammonium molybdate (98.0 mg, 0.5 mmol) dissolved in 30 mL of water. A white solid was produced, filtered, and dried overnight in vacuum over $CaCl_2$, giving $(PPh_4)_4Mo_8O_{26}$, which was characterized by IR and elemental analysis. Anal. ($C_{96}H_{80}O_{26}P_4Mo_8$) C, H.

Synthesis of the 2,5-Dihydroxybenzoate Molybdenum(VI) Complex, $(PPh_4)_2[Mo_3O_6(\mu\text{-}O)_2(2,5\text{-}DHBA)_2]$. A white solid of $(PPh_4)_4Mo_8O_{26}$ was prepared from aqueous solution after the addition of PPh_4Br (209.7 mg, 0.5 mmol) to an aqueous solution of $(NH_4)_2MoO_4$ (98 mg, 0.5 mmol) and was filtered and dried overnight in vacuum over $CaCl_2$. The solid (71.6 mg, 0.03 mmol) was added to an acetonitrile/methanol stirred solution of 2,5-dihydroxybenzoic acid (2,5-DHBA; 34.5 mg, 0.22 mmol) giving a yellow solution. The addition of diethyl ether solution resulted amorphous orange crystals of the complex $(PPh_4)_2\cdot[Mo_3O_6(\mu\text{-}O)_2(2,5\text{-}DHBA)_2]$. The complex was filtered and dried overnight in vacuum over $CaCl_2$. Anal. ($C_{62}H_{48}O_{16}P_2Mo_3$) C, H.

NMR Analysis. ^1H and ^{13}C NMR spectra were recorded on a Varian UNITYplus spectrometer operating at 300 MHz in DMSO-d_6 using the solvent peak as an internal reference (2.50 ppm). NMR data: (^1H NMR, δ values in ppm) 8.94 (s), 6.85 (s), 6.69 (d), 6.49 (d); (^{13}C NMR, δ values in ppm) 173.1, 155.7, 149.2, 123.6, 120.0, 119.3, 113.8.

IR Analysis. The infrared spectral data were recorded on a on a Perkin-Elmer 880 IR spectrophotometer. The solid was taken in a KBr pellet, and the frequency range was 4000−250 cm^{-1}. IR data (ν, cm^{-1}): 1588, 1369, 1352, 1476, 1435, 940, 908, 881, 854, 640, 564.

UV−Vis Analysis. The UV−vis spectrum was recorded on a Hitachi U-2000 spectrophotometer. The complex was dissolved in acetonitrile and was transferred to a 1-cm cuvette. UV−vis data (λ_{max}, nm): 324, 261, 225.

Cell Cultures. HL-60 (peripheral blood human promyelocytic leukemia) and K562 (human chronic myelogenous leukemia) cell lines were maintained in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 200 U/mL penicillin, 100 μg/mL streptomycin,

0.3 g/mL l-glutamine, and 2 mM $NaHCO_3$ in an atmosphere of 95% air/5% CO_2, with 100% humidity, at 37 °C. Cells were seeded at 4×10^5 cells/mL, incubated at 37 °C, and 24 h later they were treated with the 2,5-dihydroxybenzoate molybdenum(VI) complex, 2,5-dihydroxybenzoate, tetraphenyl-phosphonium chloride, or tetraphenyl-phosphonium polyoxomolybdate, as individual entities, while in exponential growth phase for the indicated time periods. All compounds used were prepared as a stock in DMSO immediately before use. During experiments, control cultures received equivalent solvent treatment (DMSO; 0.03%). The compounds were added to the cell medium, where they remained constantly for the indicated time periods.

Cytotoxicity Assay by MTT. The MTT assay was used to determine the cell viability as an indicator for the relative sensitivity of the cells to the 2,5-dihydroxybenzoate molybdenum(VI) complex, the 2,5-dihydroxybenzoate, the tetraphenyl-phosphonium chloride (PPh_4Cl), or the tetraphenyl-phosphonium polyoxomolybdate ($(PPh_4)_4Mo_8O_{26}$), as individual entities, as described previously,[30] by determining whether the compound concentrations used were toxic to at least 50% of the cells after the indicated time periods of treatment. Briefly, exponentially growing human leukemia cells were seeded at a density of 4×10^5 cells/mL, in triplicate, and 24 h later they were treated with the 2,5-dihydroxybenzoate molybdenum(VI) complex, the 2,5-dihydroxybenzoate, the tetraphenyl-phosphonium chloride (PPh_4Cl), or the tetraphenyl-phosphonium polyoxomolybdate ($(PPh_4)_4Mo_8O_{26}$), as individual entities, at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time periods. This was followed by the addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co, St. Louis, MO) at a final concentration of 0.5 mg/mL, in the presence of which they were further incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere, to allow MTT to form formazan crystals in metabolically active cells. The formazan crystals were solubilized overnight at 37 °C in a solution containing 12.5% SDS (Sigma Chemical Co, St. Louis, MO) and 45% (v/v) formamide (Acros Organics, Geel, Belgium). The absorbance of each cell lysate solution was measured at 545 nm, with a reference wavelength of 690 nm. The results were expressed as the percentage (%) of treated cells versus untreated cells.

Cytotoxicity Assay by Trypan Blue Staining. Exponentially growing, human leukemia cells were seeded at a density of 4×10^5 cells/mL, in triplicate, and 24 h later they were treated with the 2,5-dihydroxybenzoate molybdenum(VI) complex, the 2,5-dihydroxybenzoate, the tetraphenyl-phosphonium chloride (PPh_4Cl), or the tetraphenyl-phosphonium polyoxomolybdate ($(PPh_4)_4Mo_8O_{26}$), as individual entities, at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time periods. Then 50 μL from each sample was diluted 10× in PBS (1×) and 2 μL of 0.4% (w/v) trypan blue stain (Sigma Chemical Co, St. Louis, MO) was added to 18 μL of 10× diluted cell suspension. They were mixed thoroughly and allowed to stand 5 min at room temperature. The cell exposure time to trypan blue did not exceeded 5 min because extensive exposure is possible to cause an increase in the dead cell population (trypan blue positive) as a result of the trypan blue toxicity. The total number of cells and the number of blue-stained cells were counted on a hemeocytometer by observing under a microscope. Then the concentration of viable cells per mL of culture was calculated and results were finally expressed as the concentration of trypan blue +ve cells and as the percentage of the total cell number of cells. Each data point represents the mean for three separate experiments (mean ± SD).

The reactivity of trypan blue is based on the fact that this chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable. The well-known dye penetrates the cellular membrane, only in the case of damage of the structural integrity of the cells (necrosis or secondary necrosis), enters in the interior, and becomes attached to intracellular proteins, giving in the cell a blue pigmentation. As a consequence, dead cells stain blue, while live cells exclude trypan blue. However, it cannot distinguish necrotic versus apoptotic cells.[31]

Cell Proliferation. The proliferative capacity of leukemia cells was studied, which concerned the determination of the number of cells that were divided in the culture in the presence/absence of the compounds under study, as well as the creation of growth curves. For that purpose, exponentially growing, human leukemia cells were seeded at a density of 4×10^5 cells/mL, in triplicate, and 24 h later they were treated with the 2,5-dihydroxybenzoate molybdenum(VI) complex, the 2,5-dihydroxybenzoate, the tetraphenyl-phosphonium chloride (PPh_4Cl), or the tetraphenyl-phosphonium polyoxomolybdate ($(PPh_4)_4Mo_8O_{26}$), as individual entities, at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time periods. Then 50 μL from each sample were diluted 10× in complete medium without serum, they were mixed thoroughly, and the total cell number was counted on a hemeocytometer by observing under a microscope. The results were expressed as the total cell concentration of cells, with each data point representing the mean of three separate experiments (mean ± SD).

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Supporting Information Available: The data of all compounds examined for their purity by elemental analysis. This material is available via the Internet at http://pubs.acs.org.

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