Cytotoxic effects of novel polyoxotungstates and a platinum compound on human cancer cell lines

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The cytotoxicity of a new platinum compound Pt1 [2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedichloroplatin(II)] and six polyoxometalates (POM1–6) on two neuroblastoma cell lines (SHEP-SF and KCN) and an Ewing's Sarcoma cell line (CADO–ES-1) was studied. Cisplatin [*cis*-diamminedichloroplatinum(II)] and carboplatin [*cis*-diammine(cyclobutanedicarboxylato) platinum(II)] were used as reference agents. Using MTT tests, the cytotoxicity (LD_{50}: lethal doses 50%) of the compounds were measured at different concentrations. After 72 h exposure, the LD_{50} data for the platinum-containing substances ranged between 4.47×10^{-6} and 1.91×10^{-4} M. The SHEP-SF cell line displayed the highest sensitivity to cisplatin. The novel platinum agent Pt1 had a similar cytotoxic effect to the reference agent cisplatin. Both cisplatin and Pt1 were more cytotoxic than carboplatin. The POMs reduced cell viability compared to untreated cells at concentrations between 8.4×10^{-7} and 3.47×10^{-5} M. POM1 ($[(CH_3)_4N]_2Na_{6.5}(NH_4)_2[Sn^{II}_{1.5}(WO_2(OH))_{0.5}(WO_2)_2(SbW_9O_{33})_2]\cdot 32H_2O$) was the most effective polyoxoanion with a mean LD_{50} value of 8.83×10^{-6} M in the three cell lines tested. With CADO-ES-1 and KCN cells, POM1 was found to be more effective than the platinum compounds cisplatin, carboplatin and Pt1.

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Introduction

Cisplatin and its less-toxic analog carboplatin are successfully used in the treatment of numerous cancers including head and neck cancer, ovarian cancer, neuroblastoma, osteosarcoma, germ cell, and brain tumors [1]. The platinum compounds inhibit the proliferation of tumor cells by forming various platinum inter- and intrastrand DNA cross-links [2]. The antitumor activity is supported by high mobility group domain proteins (HMG proteins) [3]. These hydrophobic proteins bind to platinated DNA and protect modified DNA against repair mechanisms, which inhibits DNA transcription and replication, and finally leads to apoptosis [4,5]. Apart from toxic side-effects, the clinical use of platinum agents is limited by a number of resistance mechanisms. They include an increased repair of platinum-induced DNA damage [increased nucleotide excision repair (NER) or loss of DNA mismatch repair], glutathione or metallothionein drug deactivation, reduced cellular uptake of the platinum and altered apoptosis [6–9].

To overcome cisplatin resistance, compound Pt1 [2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedichloroplatinum(II)] (Fig. 1) exhibits highly hydrophobic properties [10]. Hydrophobic platinum compounds are hypothesized to have an increased biological efficacy by an enhanced binding of DNA/HMG proteins and a reduction of enzymatic removal of the adducts by NER [8,9].

Polyoxometalates (POMs) are negatively charged molecules containing molybdenum, tungsten or vanadium in high oxidation states as well as oxygen and heteroatoms in a defined ratio. Apart from various other properties, POMs have been investigated in AIDS therapy, as they are able to protect cell cultures and animals against viruses [11–15]. The POMs tested represent different structural classes of tungsten–oxygen clusters. Two compounds (POM1 and POM5) belong to the so-called $M_2X_2W_{20}$ type ($M = Sn^{II}_{1.5}(WO_2(OH))_{0.5}$, Mn^{II}_2; $X = Sb^{III}$) consisting of two Keggin β–B–XW_9 subunits. POM2–POM4 are isostructural to the $M_3X_2W_{18}$ type ($M = Mn^{II}$, $(V^{IV}O)_3$, Mn^{III}; $X = Sb^{III}$, As^{III}) with two α-B-XW_9 subunits. POM6 is a polymer derivative of the $H_2W_{12}O_{40}$ anion. The structures of POM1–6 are shown in Figure 2.

Antitumor efficacy of POMs was shown *in vitro* with HeLa (human cervix uteri) and Pc-3 m (human prostate) cancer cell lines [16]. Moreover, cytotoxic screening of tungsten polyoxoanions showed cytotoxic activity on

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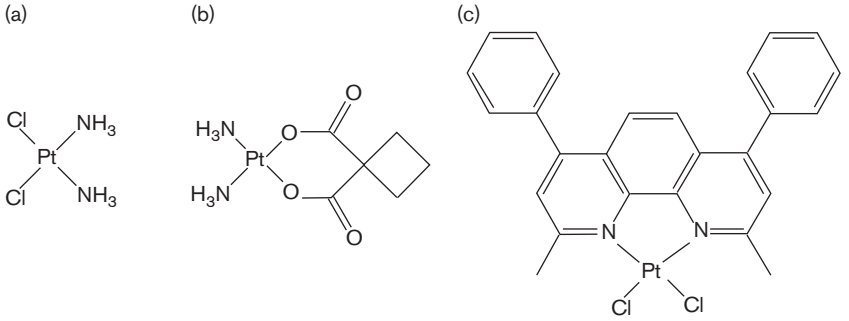
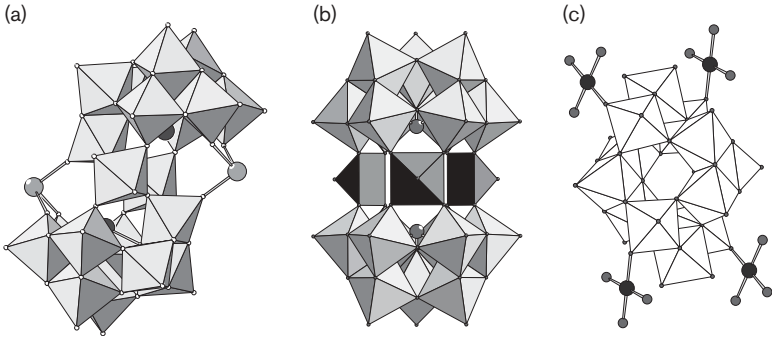
Fig. 1



Structure of cisplatin (a), carboplatin (b) and Pt1 (c).

Fig. 2



Structure of POM1 and POM5 (a), POM2–4 (b), and POM6 (c).

human glioma (T98G), rat glioma (C6) and human fibroblast cell lines (NIH-3T3) *in vitro* [17]. In addition to metal–oxygen clusters, experiments with organotin substituted heteropolytungsto-phosphates against HeLa and SSMC-7721 cancer cells were promising [18]. Cyclopentadienyltitanium substituted POMs showed remarkable inhibitory action on human cancer cells [19]. The *in vivo* activity of polyoxomolybdates was analyzed in athymic nude mice bearing Co-4 human colon cancer and in patients suffering from carcinoma of the intestinal tract [20,21].

Apart from the phenanthroline coordinated platinum compound (Pt1), the cytotoxicity of the six polyoxoanions POM1–6 was tested on two neuroblastoma and one Ewing's sarcoma cell lines, and their efficacy compared to the clinically used platinum compounds cisplatin and carboplatin.

Materials and methods

Preparation of compounds

POM1 ($[(CH_3)_4N]_2Na_{6.5}(NH_4)_2[Sn^{II}_{1.5}(WO_2(OH))_{0.5}(WO_2)_2(SbW_9O_{33})_2]\cdot 32H_2O$, $M = 6152$ g/mol), POM2 ($Na_{11}(NH_4)[(Mn^{II}(H_2O))_3(SbW_9O_{33})_2]\cdot 45H_2O$, $M = 5909$ g/mol) and POM5 ($Na_{10}[Mn^{II}_2(H_2O)_6(WO_2)_2(SbW_9O_{33})_2]\cdot 40H_2O$, $M = 6093$ g/mol) were synthesized as previously described in the literature [22–24].

POM3 ($Na_5K_7[(V^{IV}O)_3(AsW_9O_{33})_2]\cdot 29H_2O$, $M = 5620$ g/mol) [25] and POM4 ($[(CH_3)_4N]_2Na_7[(Mn^{III}(H_2O))_3(SbW_9O_{33})_2]\cdot 24.5H_2O$, $M = 5578$ g/mol) [26] were obtained via a two-step synthesis, while POM6 ($Na_6[Cu^{II}_2(H_2O)_2(H_2W_{12}O_{42})]\cdot 26H_2O$, $M = 3637$ g/mol) [27] was obtained by a one-step synthesis using the adequate ratio of single metal compounds dissolved in water at the appropriate pH value.

For the synthesis of the novel platinum compound Pt1 [2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedichloroplatinum(II), $M = 626$ g/mol], 1 mmol of potassium tetrachloroplatinate(II) (K_2PtCl_4) was dissolved in 20 ml water, after stirring for 30 min, a 1 mmol solution of ligand (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) in 10 ml ethanol/water (1:1) was added. The solution then was stirred for another 2 h at 50°C. The product was filtered, washed with ethanol and dried.

All tested compounds have been fully characterized by elemental analyses, single crystal X-ray diffraction, UV-vis and IR spectroscopy.

Further details on the crystal structure investigation of the POMs may be obtained from the Fachinformationszentum Karlsruhe, Gesellschaft für wissenschaftlich-technische Zusammenarbeit, 76344 Eggenstein-Leopoldshafen, Germany, on quoting the CSD registration numbers: POM3 (391147), POM4 (391145) and POM6 (413336).

Crystallographic data for the structural analysis of the platinum compound Pt1 have been deposited with the Cambridge Crystallographic Data Centre (CCDC no. 193826). Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: + 44 1223 336033; e-mail: ccdc@cam.ac.uk or www.ccdc.cam.ac.uk).

Cell cultures and incubation

The human neuroblastoma cell lines KCN and SHEP-SF were kindly provided by Professor C. Poremba (Institute of Pathology of the Heinrich Heine University of Düsseldorf). The Ewing's Sarcoma cell line (CADO-ES-1) was purchased from ATCC (Rockville, MD). The cells were grown in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Life Technologies) and 2 mM L-glutamine (Life Technologies) in a humidified atmosphere with 5% CO_2 at 37°C. The cell culture medium was replaced twice a week and the cells were split 1:2 to 1:7 by trypsinization when 80% confluent.

MTT test

The antitumor activity of the compounds was tested by standardized MTT experiments [28]. The POMs were dissolved in sterile water, resulting in a 2×10^{-3} molar stock solution. Pt1 was dissolved in dimethylsulfoxide (DMSO) at a concentration of 4×10^{-3} M. For further dilutions complete growth medium was used. Two thousand cells resuspended in 100 µl of complete medium were seeded in each well of a 96-well plates. After 72 h, 100 µl of test compound solutions was added, resulting in final concentrations of 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5}, 2.5×10^{-5}, 5×10^{-4} and 1×10^{-4} mol/l. The highest DMSO concentration employed had no effect on the growth of KCN, SHEP-SF and CADO-ES-1 cells. Thus, controls included medium only. At 24, 48, 72 and 96 h after addition of the compounds, the number of living cells was measured by adding 10 µl of a 0.5% solution of MTT (Sigma, Deisenhofen, Germany) dissolved in PBS buffer (pH 7.4).

Surviving cells reduced the yellow MTT within 3 h to insoluble blue formazan, which was dissolved with *N*,*N*-dimethylformamide/sodium dodecylsulfate and measured at 570 nm with 620 nm as reference using a Dynatec MR700 plate reader. The percentage of surviving cells was calculated by dividing the average absorbance of the treated wells by the average of absorbance of the control wells. Based on these results the concentration of an agent, which reduces the cell viability by 50% compared to untreated controls (LD_{50}), was calculated.

Results

Each cell line was incubated with POM1–6, Pt1, cisplatin and carboplatin. The number of viable cells was measured every 24 h. The dose–response curves of Pt1 on KCN cells after 24, 48, 72 and 96 h can be seen in Figure 3. As expected, exposure to cytotoxic concentrations of Pt1 resulted in a decreased proportion of surviving KCN cells. The concentrations needed for decreasing cell viability declined with increasing incubation time. This effect shown in a sigmoidal dose–effect relationship was observed for each compound and cell line tested. The sensitivity of each cell line differed to some extent between the various cytotoxic compounds. Table 1 shows LD_{50} data of all cell lines incubated with each test substance after 72 h of incubation.

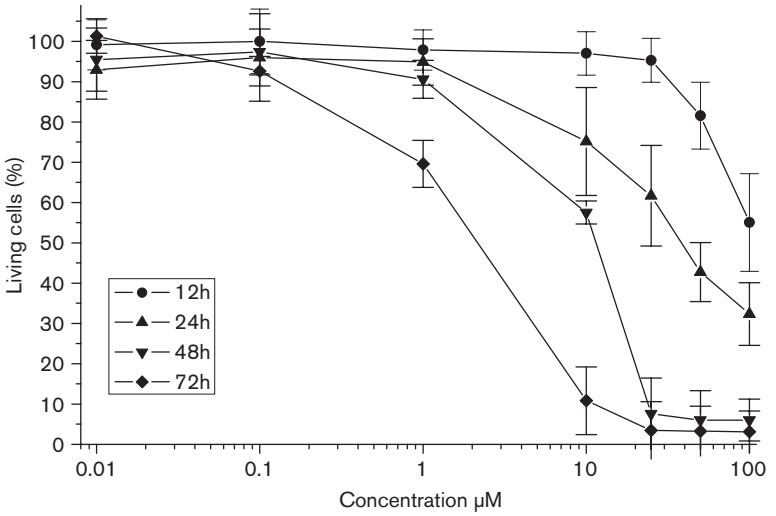
Comparison of the different cell lines

The LD_{50} concentrations ranged from 8.4×10^{-7} M calculated for POM1 in the KCN cells to 1.9×10^{-4} M determined for carboplatin in the KCN cell line (Table 1). Cisplatin was always more cytotoxic than carboplatin and the most cytotoxic substance concerning SHEP-SF cells ($LD_{50} = 4.47 \times 10^{-6}$ M). While cisplatin and, in the case of severe adverse reactions to cisplatin, carboplatin are effectively used in the treatment of neuroblastoma, they are considered less effective in the treatment of Ewing's sarcomas. In the cell lines examined the neuroblastoma cell line SHEP–SF, which displayed a less malignant phenotype without N-Myc amplification and 1p deletion, was highly sensitive to cisplatin, but about 13 times less sensitive to carboplatin. The KCN cell line, which are characterized by N-Myc amplification and 1p deletion, thus, would be associated with an unfavorable prognosis *in vivo*, was about 2 times less sensitive to cisplatin and about 17-times less sensitive to carboplatin compared to SHEP-SF. The Ewing's sarcoma cell line CADO-ES-1 showed similar sensitivities to cisplatin compared to KCN. However, in contrast to KCN, CADO-ES-1 showed a better response to carboplatin. After a 72-h exposure, the effects of carboplatin in CADO-ES-1 were comparable to those observed in SHEP-SF.

Comparison of the various compounds

In Pt1, two nitrogen atoms of the phenanthroline system coordinate the platinum atom. This compound has remarkably high hydrophobic properties caused by the phenanthroline system, the aromatic phenyl rings and

Fig. 3



Percentage of viable KCN cells exposed to Pt1 for 24, 48, 72 and 96 h.

Table 1 LD_{50} (µM) data of KCN, CADO-ES and SHEP-SF cells incubated with POM1–6, Pt1, carboplatin and cisplatin after 72 h

Compound	LD_{50}		
	SHEP-SF	KCN	CADO-ES-1
POM1	24.22	0.84	1.42
POM2	9.84	8.05	6.85
POM3	30.59	6.43	6.66
POM4	34.71	8.14	75.03
POM5	27.57	10.00	6.67
POM6	25.00	8.39	31.79
Pt1	6.97	12.25	6.24
Cisplatin	4.47	10.71	8.13
Carboplatin	60.31	191.41	68.03

aliphatic substituents [10]. This phenanthroline coordinated cisplatin derivative displayed higher cytotoxic activity compared to carboplatin in all cell lines tested. In both neuroblastoma cell lines, Pt1 was slightly less effective compared to cisplatin, while in the Ewing's sarcoma cell line it proved to be slightly more effective than cisplatin.

The tin-containing polyoxoanion POM1 was found to be the most effective compound with CADO-ES-1 ($LD_{50} = 1.42 \times 10^{-6}$ M) and KCN cells ($LD_{50} = 8.4 \times 10^{-7}$ M). In these cells POM1 was even more cytotoxic than cisplatin. LD_{50} concentrations of the isostructural $M_2X_2W_{20}$-type compounds POM2–4 differed significantly within the cell lines. These results show that the structure of this substance is not the only determining factor for biological activity. This is proved by the fact that POM6, which represents a complete different structure type, has LD_{50} data comparable to POM2–4.

Comparing the influence of the secondary heteroatom M, it is striking that POM2 containing manganese in oxidation state II turns out to be always more active than isostructural POM4 with manganese in oxidation state III. In contrast to the other tested polyoxoanions and platinum compounds, POM2 shows a very similar LD_{50} value for all three tested cell lines in the range between 6.85 and 9.84 µM.

Regarding the primary heteroatom X, it is noticeable that POM3 with $X = Sb^{III}$ could not be put in a similar ranking for all examined cell lines with isostructural POM2 and POM4 with $X = As^{III}$. Apparently under physiological conditions the heteroatom X is enclosed by the rest of the polyoxoanion and therefore only indirectly affects the biological properties.

Discussion

All six investigated polyoxoanions POM1–6 and the platinum compound Pt1 were found to be cytotoxic to three human cancer cell lines *in vitro*. The concentrations of Pt1 needed in order to inhibit cell growth were comparable to the cisplatin concentrations. In the Ewing's sarcoma cell line, Pt1 was slightly more effective than cisplatin. In the case of Pt1 we assume that the mechanism of action is similar to the standard platinum anti-cancer drugs, which ultimately leads to cell death, most probably via apoptosis [29]. This remarkable

biological activity is combined with a reduced enzymatic removal of the platinum–DNA adducts. Comparing the LD_{50} data of Pt1 and cisplatin, it has to be taken into consideration that a lot of other properties like solubility and transport mechanisms in the cell significantly influence the toxicity of the substances.

In the cisplatin-resistant cell lines KCN and CADO-ES-1, the polyoxoanions were even more effective than cisplatin and Pt1. The exact mechanism of action of polyoxoanions in malignant cells is yet unknown. Correlation of LD_{50} data with simple properties such as total negative charge, molar weight or types of cations does not lead to further information. There are several ideas to clarify the molecular mechanism of POMs. Association between POMs and proteins or cell membranes, reversible single-electron oxidation/re-oxidation processes [30] and dephosphorylation of ATP [31] are discussed models for POM action. In the manganese-containing POM2 and POM4, the secondary heteroatom M has different oxidation states, and therefore affects the reversible redox potentials of the compounds. This might be a useful hint to help to explain the action mechanism of POM2 in comparison to POM4 using the redox reaction as a model.

The mechanism of anti-HIV action of substituted polyoxotungstates was analyzed: it affected the binding of HIV to the cell membrane and syncytium formation between HIV-infected and uninfected cells [32]. The interaction between POMs and cellular membranes might contribute to the cytotoxicity of POMs on tumor cells.

Conclusions

Although cisplatin is highly effective against numerous cancers, its use is limited by its oto- and nephrotoxicity and by cisplatin-resistant tumors. Thus, new effective platinum compounds with less organtoxicities which overcome cisplatin resistance are highly sought after. Pt1 displayed similar efficacy in all three cell lines examined. Because of its hydrophobic ligands, it is likely to display different pharmacokinetics, which again might result in different toxicity profiles due to different distributions. Thus, Pt1 might be a worthy candidate for follow-up studies.

The results indicate that the tested POMs are active against malignant cell lines, and especially POM1 and POM2 are promising in comparison to the standard anti-cancer drugs cisplatin and carboplatin. Although their mechanism of action on tumor cells is still unknown, their superior activity compared to the platinum compounds in the platinum-resistant cell lines indicates the polyoxoanions for further evaluation as anti-cancer drugs.

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