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Research paper

Polyoxoanions are cytotoxic to malignant glioma cells

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We studied the cytotoxicity of five polyoxoanions on two human malignant glioma cell lines (T98G and 86HG39), a rat glioma cell line (C6) and a human fibroblast cell line (NIH-3T3) using MTT tests to measure the drug concentration killing 50% of the cells (LC_{50}). Cisplatin was used as a reference agent. Cisplatin had the highest efficacy in three of the four cell lines. Only in T98G cells, one of the components (POA5) had a lower LC_{50} value (1.3×10^{-6} mol/l) than cisplatin (2.5×10^{-6}). POA5 was also the most cytotoxic polyoxoanion when the LC_{50} values of all four cell lines were averaged (6.6×10^{-6}). Average LC_{50} values of the other compounds were 10.9, 12.6, 19.0 and 19.2×10^{-6} mol/l in POA1, POA2, POA3 and POA4, respectively. When the benign fibroblasts were used to calculate a therapeutic index as LC_{50} in fibroblasts divided by LC_{50} in glioma cells, POA5 was superior to cisplatin. These results indicate that polyoxoanions are cytotoxic for malignant glioma cells and that the most promising compound investigated here was POA5.

Key words: Cell culture, chemotherapy, glioblastoma.

Introduction

Polyoxoanions are compounds containing molybdenum, tungsten or vanadium, oxygen and heteroatoms in a fixed ratio, building huge negatively charged molecules. Their synthesis is possible with almost all heteroatoms of the periodic system. The compounds were first synthesized in 1826 by Berzelius, but their chemical structure was not fully understood until 1933 when Keggin described an oktaendric structure using X-ray analysis.[1,2] The possibility of applying them in medicine was first discussed when they were found to inhibit the enzyme reverse transcriptase. Inhibition of DNA and RNA polymerases,[3] protection of cell cultures from viruses,[4] and protection of animals against viruses[5] supported the potential use of these molecules in the treatment of infectious diseases. They were also investigated for AIDS therapy.[6,7] Antitumor efficacy was shown in mice[1] using a heteromolybdate. Not much work has been done yet to clarify their molecular mechanisms as anticancer drugs or to compare the efficacy of various polyoxoanions in this respect. The large number of potential compounds and their straightforward synthesis suggests they should be further investigated as novel chemotherapy agents. In the present study we investigated their effect on glioblastoma multiforme, which is a highly malignant brain tumor of which the median survival of patients is only 12 months and 'cure' is rare. We evaluated the efficacy of five polyoxoanions on malignant glioma cells *in vitro* in comparison to cisplatin.

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Materials and methods

Synthesis of polyoxoanions

POA1 $[(NH_4)_{10}[Sb_2W_{20}Co_2O_{70}(H_2O)_6] \cdot 12H_2O]$ and POA2 $[Na_4(NH_4)_5[Sb_2W_{19}Mn_3O_{67}(OH)(H_2O)_6] \cdot 21H_2O]$ were synthesized as described previously.[8] These two compounds consist of two SbW_9 units combined by WO_6 and MO_6 units. POA1 contains cobalt and POA2 contains manganese as the metal atom inside the MO_6 units. The molecular weights are 5662.95 and 5658.97 g/mol for POA1 and POA2, respectively. POA3 $[(K_9Na[Cu_3Se_2W_{18}O_{66}] \cdot 18H_2O)]$ and the closely related molecule POA4 $(Na_{12}[(Cu($-

$H_2O))_3Sb_2W_{18}O_{66}]\cdot 51H_2O$) were newly synthesized using the same protocol with the adequate ratio of compounds. POA5 ($(Na_4(H_3O)_{14}[Fe_4Se_8 W_{36}O_{136}(OH)_6]\cdot 18H_2O$) was synthesized in a similar fashion, resulting in a molecule of about twice the molecular weight compared to the other four compounds. The molecular weights are 5412.96, 6048.16 and 10434.32 units for POA3, POA4 and POA5, respectively. The compounds were dissolved in 1 ml of sterile water and diluted with complete cell culture medium 1:9, resulting in a 2×10^{-3} molar stock solution, which was used for further dilutions in complete growth medium. The process of dissolving and diluting was controlled by checking the concentration of aliquots: 1 ml of polyoxoanion solution+2 μl of zinc suspension+10 μl of 1 M HCl heated to 80°C and measured 60 s later in a photometer at 650 nm using 440 nm as a reference.

Cell culture

The human glioma cell line T98G, the rat glioma cell line C6 and human fibroblast cell line NIH-3T3 were purchased from ATCC (Rockville, MD). 86HG39 cells[9] were kindly provided by D Stavrou. The cells were grown in RPMI supplemented with 100 U/ml penicillin, 100 μmol/ml streptomycin and 100 mg/l 10% fetal calf serum (FCS; Hyclone, Logan, UT) in humidified atmosphere, 95% air+5% CO_2, 37°C, fed twice weekly and split 1:5 to 1:50 by trypsinization when 80% confluent.

MTT test

The cell number was determined as described by Hansen[10] with minor modifications. Two thousand cells/well were plated onto 96-well plates. After 24 h, 100 μl of polyoxoanion solutions in concentrations of 2×10^{-4}, 10^{-4}, 2×10^{-5}, 10^{-5}, 2×10^{-6} and 10^{-7} mol/l was added, resulting in final concentrations of 50% of the added concentrations. Controls included medium only and medium+solvent. A further 72 h later, the treatment medium was substituted by 100 μl of RPMI containing 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide; Sigma, St Louis, MO). Four hours later the MTT solution was substituted by 100 μl of 50% DMF/20% SDS in H_2O. Plates were cautiously vortexed for 50 min and the farmazan product measured using an ELISA reader (MR700 Dynatec), at 570 nm with 620 nm as reference. Percent of surviving cells was calculated by dividing the absorbance of the treated wells by the average of absorbance of the control wells. Key experiments were controlled by counting single cells (Coulter Counter; Coulter Hialeh, FL). The LC_{50} (lethal concentration 50%) is defined as the concentration of an agent that results in a cell number of 50% compared to the solvent-treated control.

Results

Growth during incubation

In the first experiments, the cell number during the incubation with the cytotoxic polyoxoanions was measured every 24 h. Figure 1 shows one of those curves using the effect of POA1 on 86HG39 cells as an example. As expected, exposure to cytotoxic concentrations of the polyoxoanions resulted in a decreased proportion of surviving cells. This effect was similar for all five new compounds and cisplatin. T98G cells and 86HG39 cells were also examined by morphology using experiments in flasks and phase-contrast microscopy. Under the influence of the toxic compound, the otherwise fibroblast-like appearing glioma cells stopped dividing, lost attachment and floated in the medium. Morphologic changes to large flat cells and the development of many processes and vacuoles, which are frequently observed in experiments with other chemotherapeutic agents, were not observed with polyoxoanions.

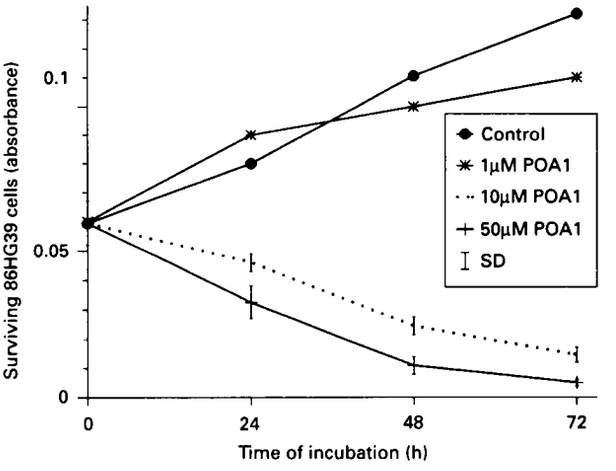

Figure 1. Time course of cytotoxicity of the polyoxoanion POA1 in 86HG39 human glioma cells. In experiments with effective concentrations of 10–50 μM the cell number continuously decreases with increasing incubation time. Error bars represent standard deviations; they disappear in the symbols in the datapoints of the control groups.

Comparison of the different cell lines

The sensitivity of each cell line differed to some extent among the various cytotoxic compounds (Figure 2). Using LC_{50} values as a measure of sensitivity, each cell line was arranged for each of the cytotoxic compounds according to the specific efficacy of the compound (Table 1). As shown in Table 1, these sequences varied with each cytotoxic compound. When all LC_{50} values were averaged, the most sensitive cell line was NIH-3T3 with an average LC_{50} of 5.3×10^{-6} M, followed by 86 HG39 (5.7×10^{-6}), C6 (10.1×10^{-6}) and T98G (25.13×10^{-6}), which was the most resistant cell line. The same sequence was obtained when only the LC_{50} values of the polyoxoanions were averaged. Again, NIH-3T3 was the most sensitive and T98G was the most resistant. However, this was different when only the LC_{50} to cisplatin was used, as in that case C6 was the most sensitive cell line to this compound.

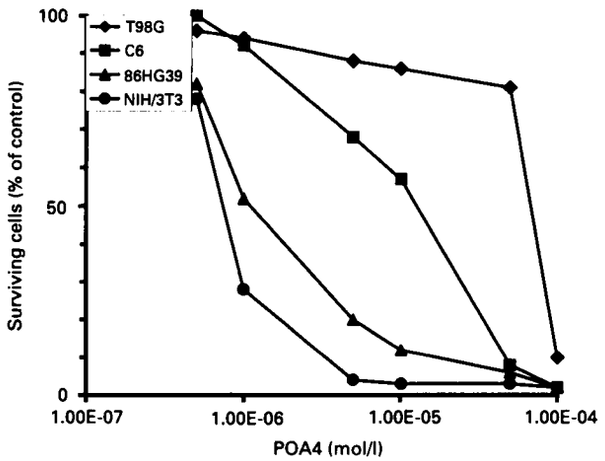

Figure 2. Cytotoxicity of POA4 on malignant glioma cell lines. Cell numbers were measured after 72 h of incubation and compared with the solvent-treated controls. Standard deviations were below 5%. The most resistant cell line was the human glioma cell line T98G; the most sensitive cells were NIH-3T3 fibroblasts.

Comparison of the various compounds

As shown by Table 1, the efficacy of the cytotoxic agents varied for each particular cell line. When all LC_{50} values were averaged, cisplatin was the most effective cytotoxic agent investigated, with an average LC_{50} of 10^{-6} M. In order of decreasing efficacy it was followed by POA5 (6.6×10^{-6} average LC_{50}), POA1 (10.9×10^{-6}), POA2 (12.6×10^{-6}), POA3 (19.0×10^{-6}) and POA4 (19.2×10^{-6}). The same sequence with cisplatin being the most potent and POA4 being least potent was true when LC_{50} values were averaged only in the malignant cell lines (T98G, 86HG39 and C6) or in the human malignant cell lines (T98G and 86HG39), but the sequence was different in the human fibroblast cell line. In NIH-3T3 cells, cisplatin was most toxic followed by POA4 and POA1, while POA5 was less toxic. Since the goal of chemotherapy is to harm the malignant cells more than the benign cells, a therapeutic index is generally calculated as the ratio of toxic dose divided by effective dose. In an analogy to this, an *in vitro* therapeutic index was calculated, dividing the LC_{50} concentration that killed 50% of the benign fibroblasts by the LC_{50} that killed 50% of the malignant cells. High values of this quotient indicate high efficacy against malignant cells. Considering human glioma cells, the most potent agent was POA5 with a therapeutic index of 4.2, followed by POA3 (0.83), cisplatin (0.36), POA2 (0.12) and POA4 (0.02). The same sequence was true when all three malignant cell lines were averaged:

Table 1. Sensitivity (measured by) LC_{50} (μmol/l) of cytotoxic agents in various glioma cell lines

Agent	Cell line				
	T98G	86HG39	C6	NIH-3T3	Average[a]
POA1	31	5.2	6.1	1.5	11
POA2	32	4	12	2.2	12.6
POA3	20	19	21	16	19
POA4	64	1.1	11	0.7	19.2
POA5	1.3	4	10	11	6.6
Cisplatin	2.5	0.8	0.5	0.6	1.1
Average	25.1	5.7	10.1	5.3	11.6

The cytotoxicity of the tested agents varied between different cell lines. The most cytotoxic polyoxoanion was POA5 as indicated by the lowest LC_{50} values. In addition this was the only agent more effective in the malignant cell lines compared to the benign NIH-3T3 fibroblasts.
[a]Average of all LC_{50} values within one particular cell line.

POA5 was the only compound with a value above 1, followed by POA3 and cisplatin; POA1, POA2 and POA4 were less beneficial. A more detailed comparison of POA5 and cisplatin in T98G cells is shown in Figure 3.

Discussion

All five investigated polyoxoanions were found to be cytotoxic to malignant glioma cells *in vitro*. The concentrations necessary to kill these tumor cells were in the order of magnitude of other cytotoxic agents. The concentrations were higher than those necessary for cisplatin *in vitro* and reached the concentrations necessary for carboplatin *in vitro*.[11] Furthermore, the therapeutic index of one of these (POA5) was superior to cisplatin. This confirms results of others[1] using other polyoxoanions.

The mechanism of action of polyoxoanions in malignant cells is unknown. In comparison to other cytotoxic molecules, their molecular weight is huge. Four of the compounds investigated here were between 5000 and 6000 Da; one of them (POA5) has a molecular weight over 10000. These compounds are water soluble, it is unlikely that they penetrate the cellular membrane or blood-brain barrier easily. An interaction with DNA inducing apoptosis as observed with cisplatin is unlikely. For example, agents which act through apoptosis, such as radiation or cisplatin, typically do not produce cell death after 24 h of treatment. Instead, after this DNA-damaging treatment, cells start dying later, as indicated by the morphologic findings of cytoplasmic vacuoles and nuclear fractionation.[2,12] Therefore, neither the morphological changes nor the time course of cell death we observed with polyoxoanion treatments (Figure 1) suggest these act by inducing apoptosis. The largest investigated compound (POA5) was the most effective cytotoxic agent against glioma cells. Among the different properties of these molecules, their capacity to reverse oxidation might explain their mechanism of action in this system. Polyoxoanions have been used as reversible oxidation mediators.[13,14] The size of the compounds might facilitate the transport of electrons between different organic molecules on the cellular membrane over large molecular distances interfering with cell adhesion molecules or signal transducing receptors. Interestingly, T98G cells, which were most resistant against polyoxoanions in other experiments (Table 1), are known to have high levels of intracellular glutathione which protects from redox damage.[12]

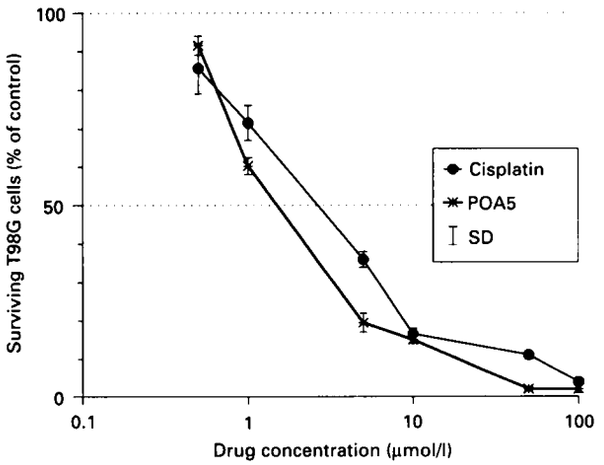

Figure 3. Comparison of the cytotoxicity of cisplatin versus POA5 in the human glioma cell line T98G. In this cell line POA5 was slightly more cytotoxic.

Patients with malignant glioma desperately need novel therapeutic approaches. The compounds investigated here have promising activity in malignant glioma cell lines *in vitro*. The comparison with fibroblast cell lines showed that POA5 was more toxic to the malignant glioma cells than the benign cells. In this respect it appears to be more promising than the commonly used chemotherapeutic agent cisplatin. More work needs to be done concerning the mechanism of action, pharmacokinetics and tissue distribution of these compounds. Their large size might reduce their central nervous system penetration and therapeutic efficacy. On the other hand, manipulations, which selectively increase blood-brain barrier permeability, might be potentially useful in delivering these novel compounds to malignant glioma cells, while sparing normal brain.

Acknowledgments

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References

1. Yamase T, Fujita H, Fukushima K. Medical chemistry of polyoxometalates. 1. Potent antitumor activity of polyoxomolybdates on animal transplantable tumor and human cancer xenograft. *Inorg Chim Acta* 1988; **151**: 15-8.
2. Wolff JEA, Boos J, Kühl J. (HiT-GBM: Multicenter study for children with malignant glioma). HIT-GBM: Multizentrische Studie zur Behandlung von Kindern mit malignen Gliomen. *Klin Päd* 1996; **208**: 193-6.

3. Haapala DK, Jasmin C, Sinoussi F, Chermann JC, Raynaud M. Inhibition of tumor virus RNA-dependent DNA polymerase by the heteropolyanion, silicotungstate. *Biomedicine* 1973; **19**: 7-11.
4. Jasmin C, Raybaud N, Chermann JC, *et al. In vitro* effects of silicotungstate on some RNA viruses. *Biomedicine* 1973; **18**: 319-27.
5. Jasmin C, Chermann JC, Herve G, *et al. In vivo* inhibition of murine leukemia and sarcoma viruses by the heteropolyanion 5-tungsto-2-antimoniate. *J Natl Cancer Inst* 1974; **53**: 469-74.
6. Moskovitz BL. Clinical trial of tolerance of HPA-23 in patients with acquired immune deficiency syndrome. *Antimicrob Agents Chemother* 1988; **32**: 1300-3.
7. Burgard M, Sansonette P, Vittecoq D, *et al.* Lack of HPA-23 antiviral activity in HIV-infected patients without AIDS. *AIDS* 1989; **3**: 665-8.
8. Krebs B, Klein R. *Synthesis and structural chemistry of novel heteropolymolybdates and -tungstates. Polyoxometalates: from platonic solids to anti-retroviral activity*. Dordrecht: Kluwer 1994.
9. Bilzer T, Stavrou D, Dahme E, *et al.* Morphological, immunocytochemical and growth characteristics of three human glioblastomas established *in vitro*. *Virchows Arch A Pathol Anat Histopathol* 1991; **418**; 281-93.
10. Hansen M, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989; **119**: 203-10.
11. Wolff JEA, Bilzer T, Stark M, Göbel U, Wechsler U. Effects of cisplatinum and carboplatinum on human glioma cells *in vitro*. *J Cell Pharmacol* 1994; **1**: 99-101.
12. Poppenborg H, Münstermann G, Knüpfer M, Hotfilder M, Hacker Klom U, Wolff JEA. Cisplatinum induces radioprotection in human T98G glioma cells. *Anticancer Res* 1997; **17**: 1131-4.
13. Ahmed S, Moffat JB. Reactant composition and concentration effects in the oxidation of methane on supported 12-molybdophosphoric acid. *Appl Catal* 1988; **40**: 101-18.
14. Bregeault JM, El Ali B, Mercier J, Martin J, Martin C. Novel carbon-carbon bond catalytic cleavage of alphadiols in the presence of vanadium precursors. *CR Acad Sci Ser II* 1989; **309**: 459-62.

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