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STUDY OF SOME POLYOXOMETALLATES OF KEGGIN'S TYPE AS POTENTIAL ANTITUMOUR AGENTS

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Summary: The antitumour action of three polyoxometallate compounds of Keggin's type: 12-molibdophosphoric acid (MoPA), 12-tungstophosphoric acid (WPA) and Mg salt of WPA (MgHWPA) was studied *in vitro*. For human cervix carcinoma (HeLa) cells survival, as well as for nonstimulated and stimulated peripheral blood mononuclear cells (PBMC), MTT test was applied and IC_{50} values of POMs were determinated. Index selectivity for WPA and MgHWPA are 1.9 and 1.8, calculated for nonstimulated, as well as 2.5 and 2.0, calculated for stimulated PBMS. Combination of studied POMs do not contribute to their lower IC_{50} values. Apoptosis detection implies mild cytotoxic effect of WPA and more cytostatical effect of MgHWPA. Combination of each of the studied POMs with caffeine decreases HeLa survival in dose dependent way. None of the studied POMs in the used concentrations (up to 100 μmol/L) damages blood cells and/or decreases their number.

Key words: polyoxometallates, antitumour effect, HeLa cells

Introduction

The earliest reports on the therapeutic use of metals and metal containing compounds in cancer and leukaemia date back to the sixteenth century. They were forgotten until 1960s when antitumour activity of inorganic *cis*-diamminedichloroplatinum(II) (cisplatin) was discovered (1). Numerous other metal compounds were shown to be effective against tumours up to date (2).

Polyoxometallates (POMs) are a huge group of complex inorganic compounds with a great variety of uses (analytical reagents, catalysts, superionic proton conductors, sensors, membranes in fuel cells, etc.). There are two main classes of POMs: the isopolycompounds that contain only d metal and oxygen atoms as anion and heteropoly compounds that contain one or more *p, d* or *f* block heteroatoms in addition (3). As heteropoly compounds are more numerous and their structural properties are easier to modify synthetically, this family of POMs dominates the medically oriented research and uses up to date (4). Several general attributes of POMs such as polarity, redox potential, surface charge distribution, shape and acidity, render them attractive for usage in medicine (4). Structurally, heteropoly compounds can be of different types, but most of them with biological issues have common Keggin's structure.

The first biological activity of POM was reported in 1971, when Raynaud noted that polytungstosilicate heteropoly compounds inhibited murine leukaemia sarcoma virus *in vitro* (5). Prior to 1990, *in vitro* studies conducted by various groups showed the efficiency of POMs against several viruses: vesicular stomatitis, polio, rubella, Rausher leukaemia, rhabdovirus, etc (4). Today, there is a lot of data about antiviral activity, especially agains HIV virus (6). Although the mode of antiviral action has been well documented, the primary mechanism remained elusive. The most likely of all the proposed mechanisms are inhibition of viral enzymes (reverse transcriptase and/or protease in retroviruses) or surface viral proteins, such as gp120 for HIV (4).

Contrary to numerous data on antiviral activity, there is a limited information about POMs antitumoral

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activity. Among POMs, only some complex derivates of 12-molibdophosphoric acid have been reported as agents with antitumour activity (4). The only mechanism of *in vivo* antitumour activity, proposed by Yamase (7), revolves around a single electron reduction/oxidation cycle, where POMs reoxidation and tumour cell reduction process kill the cells.

The aim of the present article was to study *in vitro* antitumour effects of 12-molibdophosphoric acid, $H_3PMo_{12}O_{40}$ (MoPA), and 12-tungstophosphoric acid, $H_3PW_{12}O_{12}$ (WPA), both with common Kegin's structure.

Another compound suitable for such an investigation was Mg salt of WPA, $MgHPW_{12}O_{40}$ (MgHWPA). Introducing Mg^{2+} in WPA structure, antitumour effects could be considerably improved. It is already known that magnesium defficiency can paradoxically protect against oncogenesis (8). Over 300 enzymes that influence the metabolism of carbohydrates, amino acids, nucleic acids and proteins, and also regulate ion transport, require Mg. It has been proposed that Mg is central in the cell cycle, and that its deficiency is an important conditioner in precancerous cell transformation (9 12).

Since the principal disadvantage of POMs is their high toxity (renal toxity, hepatotoxicity and thrombocytopenia), *in vitro* experiments of these POMs in blood cells in the periferial blood, were also performed.

Material and Methods

MoPA, WPA and MgHWPA were synthezided in the way described earlier (13). The thermal analysis, IR, and SEM examinations for compound characterization were performed. The content of Mg in MgHWPA was determined by AAS (14).

In order to study the antitumour effects, MoPA, WPA and MgHWPA were tested in vitro on human cervix carcinoma cells (HeLa cells) under the same conditions, and the obtained effects were compared.

Antitumuor-test

Chemicals. Stock solutions of examined compounds were made in water at concentrations of 10 mmol/L and afterwards diluted by nutrient medium to various final concentrations (in the range 25 200, or 0.01 100 mmol/L). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and RPMI 1640 cell culture medium were purchased from Sigma Chemicals (St. Luis, MO, U.S.A.). MTT was dissolved, 5 mg/mL in phosphate buffer saline of pH= 7.2, and filtered through millipore filter, 0.22 μm, before use.

Cell culture. Human cervix carcinoma, HeLa cells, were maintained as a monolayer culture, in the nutrient medium (RPMI 1640 medium supplemented with L-glutamine (3 mmol/L), streptomycin (100 mg/mL), and penicillin (100 IU/mL), 10% heat inactivated foetal bovine serum, FBS and 25 mmol/L Hepes, adjusted to pH= 7.2 by bicarbonate solution). The cells were grown at 37 ²C in humidified air atmosphere with 5% CO_2.

Treatment of HeLa cells. Target cells were seeded in 0.1 mL of nutrient medium into one group of 96-well microtiter plates, 2000 cells per well. After twenty hours of cell seeding, solutions of various concentrations of examined compounds were added to the wells, except to the control wells where only nutrient medium was added. All samples were done in triplicate. Nutrient medium with corresponding agents concentrations but without target cells, was used as blank, also in triplicate.

Preparation of peripheral blood mononuclear cells. PBMC were separated from the whole heparinised blood of healthy volunteers by LymphoprepTM gradient centrifugation. Interface cells, washed three times with Haemaccel˜ aqueous solution supplemented with 145 mmol/L Na^+, 5.1 mmol/L K^+, 6.2 mmol/L Ca^{2+}, 145 mmol/L Cl and 35 g/L gelatine polymers, pH=7.4, were counted and resuspended in nutrient medium.

Treatment of PBMC. Target cells were seeded (100,000 cells per well) into nutrient medium and in nutrient medium enriched with 5 mg/mL phytohaemagglutinin, PHA, (Welcome) in 96-well microtiter plates and two hours later, five different concentrations of compounds were added to PBMC in triplicates (to the same final concentrations used for treatment of malignant cells) except to the control wells where a nutrient medium was added to the cells. Nutrient medium with corresponding concentrations of compounds, but void of cells was used as blank.

Determination of target cell survival. Cell survival was determined by MTT test according to the method of Mosmann (15) and modified by Ohno and Abe (16), 72 hours after the drug addition. Briefly, 20 mL of MTT solution (5 mg/mL PBS) was added to each well. Samples were incubated for further four hours at 37 °C in humid-ified atmosphere with 5% CO_2. Then, 100 mL of 10% SDS was added to the wells. Absorbance was measured at 570 nm the next day. To get cell survival (%), absorbance at 570 nm of a sample with cells grown in the presence of various concentrations of agent (A), was divided with absorbance of control group (AC, the absorbance of cells grown only in nutrient medium), implying that absorbance of blank was always subtracted from absorbance of a corresponding sample with target cells.

Apoptosis detection. HeLa cells were seeded on cover slips (3×10^5 cells) in 2 mL of complete medium (RPMI-1640 supplemented with 10% heat inactivated FCS) and, after 24 h, were treated with 100 μmol/L of WPA or MgHWPA for 2 days. After 24 h or

48 h of continuous agents action, cells were stained with 15 mL of acridine orange/ethidium bromide (3 g/mL AO and 10 g/mL EB in PBS) and visualized under a fluorescence microscope using blue filter.

Effects of POMs on blood cells

Assessment was performed in citrate human peripheral blood from healthy donors. The standard routine procedure for determination of the number of blood cells on cell counter (HMX »Coulter«) was used. The volumes of 3 mL of blood and 0.3 mL of 100 μmol/L of each sample of POMs were mixed. The effect of dilution was eliminated by adding corresponding amounts of physiological solution instead of POMs.

Results

Results of *in vitro* antitumour activity of investigated POMs on HeLa cells as well as on stimulated and nonstimulated PBMC is presented in *Table I*. Value IC_{50} presents the effective concentration of the agent (POM) required for inhibition of cell survival by 50% compared with control. The antitumour effect of different POMs was expressed through selectivity index obtained as ratio of IC_{50} for nonstimulated or stimulated PBM cells and of IC_{50} for HeLa cells.

Table I Values of IC_{50} and selectivity index for the activity of examined POMs on HeLa and PBMC, determined by MTT test, 72 h after the continuous agent action

Compound	IC_{50}, μmol/L			Selectivity index	
	HeLa cells	PBMC	PBMC + PHA	PMBC/HeLa	PMBC+PHA/HeLa
MoPA	169	>100	>100	/	/
WPA	74	141	183	1.9	2.5
MgHWPA	84	151	167	1.8	2.0

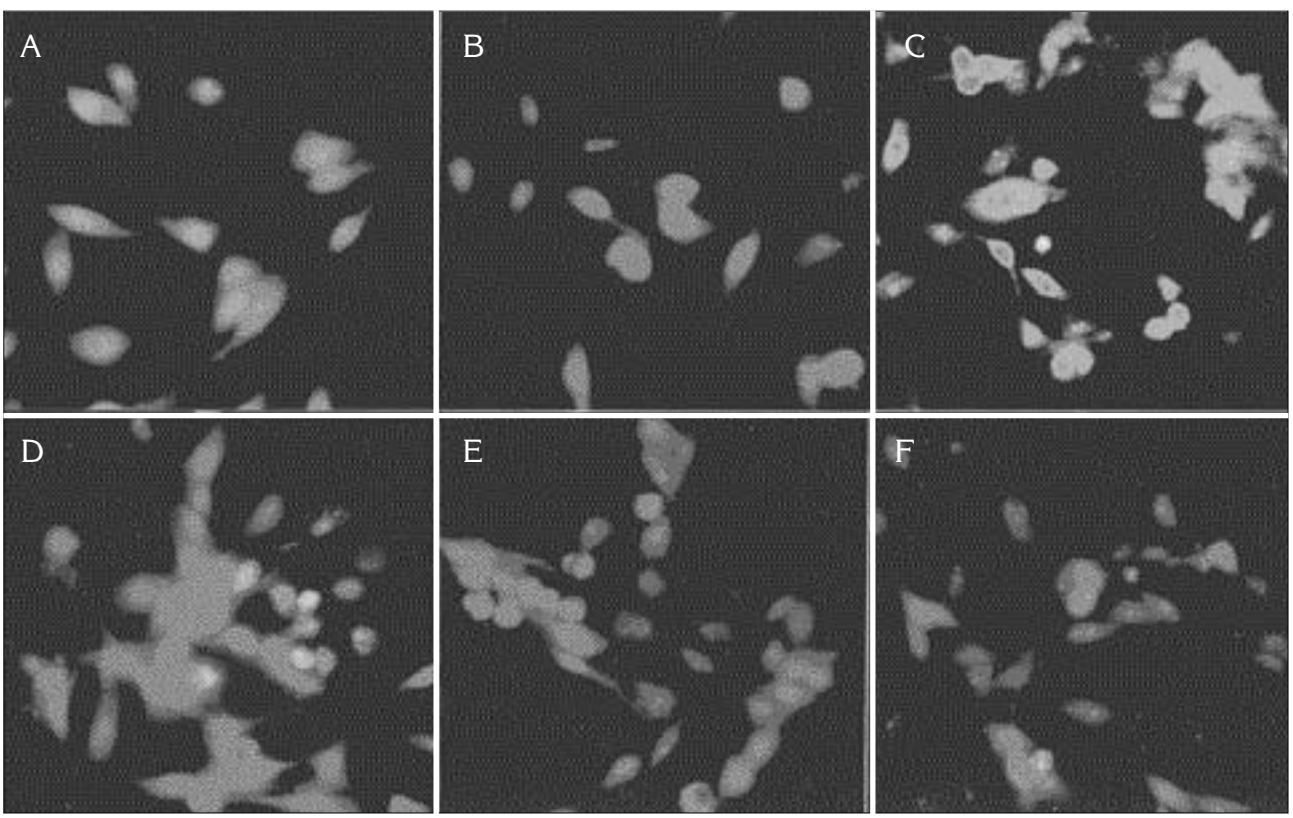

Figure 1. Acridine orange and ethidium bromide stained HeLa cells incubated in nutrient medium alone (A, B), or with 100 μmol/L of WPA after 24h (C) and 48h (D) or with 100 μmol/L of MgHWPA after 24h (E) and 48h (F).

From *Table I,* it can be seen that the antiproliferative effect of MoPA on HeLa cells is insignificant, while it was mild for WPA and MgHWPA. The toxicity of all examined POMs, followed through IC_{50} on PBMC and stimulated PBMC, is relatively low.

The combination of POMs does not significantly contribute to their better antitumour action. Namely, synergetic effect of WPA and MoPA results in IC_{50} = 133 μmol/L on HeLa cells while the same effect of combination of WPA and MgHWPA results in lower value of IC_{50} = 84 μmol/L.

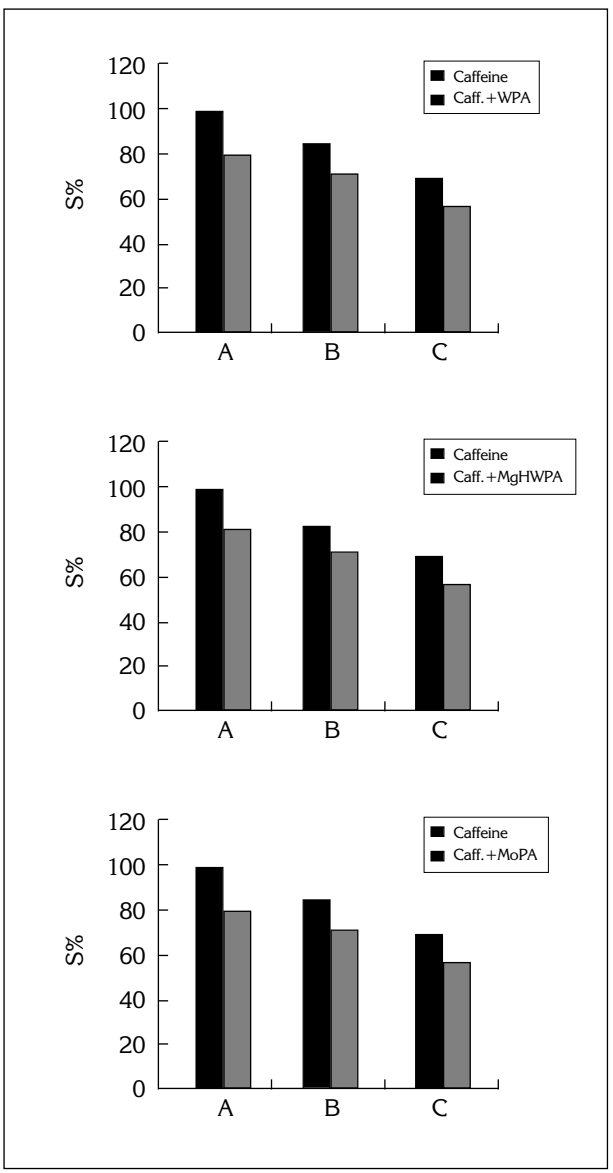

Figure 2. The effect of 40 μmol/L of WPA, MgHWPA, and MoPA on HeLa cells, pretreated with 0.0 μmol/L (A), 0.5 μmol/L (B) and 1 μmol/L(C) of caffeine for 72h. Cell survival was determined by MTT test, 72 h after the continuous agent action.

HeLa cells incubated in nutrient medium with 100 μmol/L of WPA and MgHWPA for 24 h and 48 h are presented in *Figure 1*. It can be seen that HeLa cells viability was not affected 24h after the continuous WPA action, although some flattened cells were seen. The appearance of few necrotic (orange) cells is observed 48h after the start of cells treatment. Continuous MgHWPA action did not change viability of examined cells, although some detachment of treated cells could be noticed.

The effect of examined POMs together with caffeine on HeLa cells, was determined, since it has been reported that this combination has antitumour effect *in vivo* (17). Results are presented in histogram *(Figure 2)*. *Figure 2* shows that pretreatment of HeLa cells with caffeine alone decreases their survival in dose dependent way. Combination of each of examined POMs with caffeine added 72 h after caffeine, contributes to further decrease of cell survival for next 48 h.

Results of the effect of examined POMs on number of blood cells (red-RBC, white-WBC and platelet-PLT) from peripheral blood are presented in *Table II*. From *Table II,* it is evident that none of the examined POMs causes decrease in the number and/or damages blood cells The last was confirmed by microscopic examination of the peripheral blood cells films.

Table II Effects of MoPA, WPA and MgHWPA (c=100 μmol/L) on various peripheral blood cells counts, determined by blood cell counter, 2h after the continuous agent action

	RBC $\times 10^{12}$/L	WBC $\times 10^{9}$/L	PLT $\times 10^{9}$/L
Blood (healthy donor)	4.58	9.26	171
Blank	4.10	8.24	153
MoPA	4.11	8.29	167
WPA	4.17	8.47	149
MgHWPA	4.13	8.22	166

Discussion

Examined POMs show no considerable effects on HeLa cells survival tested *in vitro:* effect of MoPA is insignificant, WPA has a mild cytotoxic effect and MgHWPA acted more cytostatically, what is evident from *Figure 1*. Evaluation of promising antitumour agent *in vitro* usually results in high therapeutic (selectivity) index (>5 for antiviral action) (4). In our study this index is the ratio of IC_{50} for normal (PBMC) and malignant (HeLa) cells, and ranges from 1.8 to 2.5. Although our examined POMs are not in compliance

with this ratio, their action *in vivo* could be expected to be more prominent. It was already confirmed that POMs antiviral activity *in vivo* differs from activity *in vitro,* because POMs can penetrate cell membranes and thus localize intracellulary (4).

In early sexteen it was reported, the combination of MoPA, WPA and caffeine in patients with non-metastatic carcinoma of the intestinal tract results in total tumour disappearance. From our results, the antiproliferative effect of caffeine alone is evident, and some enhancement of its action in the presence of POMs do exist. Therefore it could be supposed that the antiproliferative action of the mentioned agent combinations could be enhanced *in vivo,* taking into account that caffeine is a compound with known complex pharmacological activity.

It should be pointed out that POMs in concentrations which exert cytotoxic effect on HeLa cells do not modify the number and morfology of blood cells. This implies that this concentration range of POMs is suitable for their probable use.

Although the obtained *in vitro* results of antitumour effect of POMs are not remarkable, the further *in vivo* studies with WPA and MgHWPA should be reasonable. In order to improve the anticancer action of WPA and to decrease its toxicity, the modification of this compound with organic molecule (amino acid) is in progress.

ISPITIVANJE NEKIH POLIOKSALOMETALATA KEGINOVOG TIPA KAO POTENCIJALNIH ANTITUMORSKIH AGENASA

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Kratak sadržaj: Ispitivano je *in vitro* antitumorsko dejstvo tri jedinjenja iz grupe polioksometalata (POM-a) Keginovog tipa: 12-fosformolibdenska kiselina (MoPA), 12-fosforvolframova kiselina (WPA) i Mg so 12-fosforvolframove kiseline (MgHWPA). Primenom MTT testa na ćelije karcinoma cerviksa (HeLa ćelije), kao i na nestimulisane i stimulisane mononuklearne ćelije iz periferne krvi (PBMC), određene su IC_{50} vrednosti ispitivanih POM-a. Indeksi selektivnosti za WPA i MgHWPA su 1,9 i 1,8 računati za nestimulisane, odnosno 2,5 i 2,0 za stimulisane PBMC. Kombinacijom ispitivanih POMa ne postiže se smanjenje njihovih IC_{50} vrednosti. Detekcijom apoptoze utvrđeno je da WPA pokazuje blagi citotoksični efekat, dok MgHWPA ima blagi citotostatski efekat. U kombinaciji sa kofeinom, POM jedinjenja još više smanjuju preživljavanje HeLa ćelija. U ispitivanim koncentracijama (do 100 μmol/L), nijedno od POM jedinjenja ne utiče na broj i izgled krvnih ćelija pune krvi zdravih davalaca.

Ključne reči: polioksometalati, antitumorsko dejstvo, HeLa ćelije

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