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Research article

DOI: 10.2478/rrlm-2014-0007

Antibacterial activity of some saturated polyoxotungstates

Activitatea antibacteriană a unor compuși polioxowolframici

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Abstract

Polyoxometalates are important inorganic compounds with a broad range of pharmacological properties, including antiviral, antibacterial, antiprotozoal or antitumoral activities, even that their molecular mechanism of action is poorly understood. Purpose: In this paper we evaluated the antibacterial activity of some saturated polyoxotungstates (POW) compounds, since nowadays, the increasing resistance of bacteria to drugs represents a major health problem. Materials and methods: The antibacterial activity was studied by disk diffusion method as a possible screening method and by successive micro-dilutions method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) have been calculated for each compound by successive dilutions. We also compared the reliability of each testing method for this particular POW evaluation. Results: The best antibacterial activity was expressed by $H_4[SiW_{12}O_{40}]_*nH_2O$ *and the lowest by* $Na_3[PW_{12}O_{40}]_*nH_2O$*, but with very good activity on Staphylococcus spp., especially on MRSA. The POW activity occurs only at relatively high concentrations, and it is dependent on bacterial strain, with very good activity on Staphylococcus spp. The most reliable method for assessing the antibacterial effects of POW is micro-dilutions. POWs could be practically applied in hospital decontamination and could have a possible in vivo antibacterial application.*

Keywords*: Polyoxometalates, Polyoxotungstates, Microbial Sensitivity Tests, Anti-Bacterial Agents, Inorganic Chemistry*

Rezumat

Polioxometalaţii sunt compuşi anorganici importanţi care prezintă o gamă largă de proprietăţi farmacologice,

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incluzând efecte antivirale, antibacteriene, antiparazitare sau antitumorale, chiar dacă mecanismul molecular de acţiune nu este pe deplin înţeles. Scop: În această lucrare am evaluat acţiunea antibacteriană a unor compuşi polioxowolframici saturaţi (POW), datorită faptului că dezvoltarea rezistenţei bacteriilor faţă de antibiotice reprezintă în prezent o problemă majoră de sănătate. Material şi metodă: Acţiunea antibacteriană a fost studiată prin metoda difuziei în agar ca posibilă metodă de screening şi prin metoda microdiluţiilor. Prin microdiluţii au fost calculate concentraţia minimă inhibitorie şi concentraţia minimă bactericidă pentru fiecare compus. Totodată am comparat fiabilitatea celor două metode de testare faţă de compuşii POW. Rezultate: Cea mai bună acţiune antibacteriană a prezentat-o $H_4[SiW_{12}O_{40}]*nH_2O$, iar cea mai slabă $Na_3[PW_{12}O_{40}]*nH_2O$, dar în schimb cu o acţiune foarte bună pe Staphylococcus spp., în special pe MRSA. POW îşi exercită acţiunea antibacteriană la concentraţii relativ mari, iar acţiunea lor este dependentă de specia bacteriană, având activitate foarte bună pe Staphylococcus spp. Cea mai fiabilă metodă pentru evaluarea acţiunii antibacteriene a POW este cea a microdiluţiilor. POW îşi pot găsi aplicaţie practică în decontaminarea spitalelor sau ca agenţi antibacterieni in vivo.

***Cuvinte cheie**: Polioxometalaţi, Polioxowolframaţi, Teste de sensibilitate bacteriană, Agenţi antibacterieni, Chimie anorganică*

Received: 30th September 2013; Accepted: 13th February 2014; Published: 16th February 2014.

Introduction

Polyoxometalates (POM) are negatively charged transition metal aggregates (mainly Vanadium, Molybdenum, and Tungsten – polyoxotungstates POW) with oxygen. They are composed of metal ions in their highest oxidation state, bridged by Oxo ligands (O^{2-}) (1,2). Almost any other element can be incorporated into POM framework and this leads to an overwhelming diversity of structures and properties (3,4). Recently, polyoxometalates (POMs) were investigated for their particular properties especially in the medical world (5–8).

All the compounds used for this study are saturated Keggin structures, with general formula $[XM_{12}O_{40}]^{n-}$ involving a XO_4 tetrahedron surrounded by twelve MO_6 octahedral that are arranged in four groups of three octahedral linked by common sides. These groups of triplet are linked by common peaks with central tetrahedron (9).

Unique structural and electronic properties of this class of compounds makes them to find their applications in many fields of science and technology, among which: chemical analysis, catalysis, biochemical applications, reagents for chemical analysis, ion exchange, thin-layer chromatography, etc (10–13). POMs' catalytic properties were described by Papaconstantinou and Mylonas in water decontamination of aliphatic and aromatic representative compounds (14) (i.e. $[SiW_{12}O_{40}]^{4-}$ and $[PW_{12}O_{40}]^{3-}$).

Forty years ago Chermann et. al. accidentally discovered silicontungstic acid effects as inhibitors for murine leukemia and sarcoma virus (15). Since then, there is a growing interest in POMs as potential drugs. Karlish and Shechter reported the antidiabetic efficacy of vanadium complexes, Yamase et. al. reported anticancer activities of some POMs, while antibiotic properties of polyoxotungstates derivatives were reported in the beginning of 90's (16–18). POM antiviral and antitumoral efficacy, as well as insulin-mimetic activity (19), were shown *in vitro* and *in vivo*. Recent studies suggest that the biological activities are due to the interaction of POMs with the cell surface, since they are highly negatively charged and large enough not to penetrate cell membrane; under certain circumstances, POMs can cross the barrier and penetrate inside a cell.

(20–22). Biological activities of POMs are mostly associated with enzyme inhibition, but the entire mechanism of action is poorly understood (23).

The main feature and advantage of POMs are that it almost any of their property can be changed, including properties that involve molecular recognition and reactivity with biological macromolecular target; this includes polarity, redox potential, surface load distribution, shape, and acidity (24).

Up-to-date, POMs have not yet been developed to the stage of a drug in clinical use, and there is a lack of similar studies that could clarify these aspects. Their toxicity and side effects are certainly major drawbacks (25,26). However, the studies opened the path for polyoxometalates administration without acute toxic effect. The gap between a useful drug and a toxic substance is very narrow, but possible derivatizations can change the properties of compounds dramatically (27,28). Therefore, further researches on polyoxometalates with possible application in medicine are encouraged.

Purpose

The main purpose of this paper was to study the antibacterial activity of some saturated polyoxotungstate compounds (all with Keggin structures), as the increasing resistance of bacteria to antimicrobial drugs, mainly antibiotics, represent a major health-related problem today. We also tried to establish the most reliable antibacterial testing method for these compounds.

Materials and methods

In order to study the antibacterial activity we used 3 saturated POW compounds: $H_4[SiW_{12}O_{40}]_*nH_2O$ (SiW_{12}), $H_3[PW_{12}O_{40}]_*nH_2O$ (PW_{12}) and $Na_3[PW_{12}O_{40}]_*nH_2O$ (Na_3PW_{12}). These compounds were commercially purchased and used without further purification. We created water solutions from each compound, in several concentrations that corresponded to the different antibiotics standard dosage, as they are commercialized. The water was purified using a Millipore filter (0.2 μ) and then sterilized.

The antibacterial activity was studied against four reference bacterial strains (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218) and 4 clinically isolated (Methicillin-resistant *Staphylococcus aureus* MRSA, multi-drug resistant (MDR) *Pseudomonas aeruginosa, Klebsiella pneumoniae,* extended spectrum beta-lactamase producing *Klebsiella pneumoniae*). The bacterial strains were provided by the Microbiology-Virology-Parasitology Department within the University of Medicine and Pharmacy Tg. Mures.

For analyzing the antibacterial activity we used two methods derived from EUCAST standards for antibiotic susceptibility testing: disk diffusion method (qualitative) and by successive dilutions (quantitative, with identification of minimum inhibitory and bactericidal concentrations – MIC and MBC respectively) (29).

The diffusion method was performed against 4 reference strains: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 35218, in order to appreciate the action of these compounds in the form of screening. Thus, the study was initiated by comparing our compounds' activity with the activity of few parenteral use antibiotics, from the most common classes, obtained from the pharmacy market: ceftriaxonum (CRO 1g), ciprofloxacinum (CIP 10 mg/ml), ampicillinum (AMP 1g), gentamicinum (GN 40 mg/ml) and cefuroximum (CXM 1.5g). We prepared water solutions of standard concentration for each compound according to their molecular mass (1x), similar to the concentrations referred by EUCAST for each antibiotic (30 μg/disk for CRO, 5 μg/disk

for CIP, 10 µg /disk for AMP, 10 µg /disk for GN, 30 µg /disk for CXM) (30). These solutions were tested against all four reference strains. Of the three POW compounds, 10x and 25x concentrated solutions were also made, their activity being tested against the reference *S. aureus* and *E. coli*, as members of Gram positive and negative classes.

For the diffusion method, we created 0.5 McFarland inoculum of each bacterial strain. Muller-Hinton plates were inoculated similar to antibiotic susceptibility testing standard. After 10 minutes, 4 µl of POM solution were inoculated on sterile Munktell Grade 005 filter paper disks (0.3 mm thickness and 6 mm diameter). After 10 minute pause, the media was incubated for 16-20h at 35°C.

We followed the development of inhibition zones, and we measured their diameter in mm, with one decimal precision, with a digital caliper. The activity of each POW was compared to each other; the biggest diameter represented a better activity for the specific concentration, as there are no standard interpretive criteria for POW evaluation. After reading the results, we have further incubated the plates for another 24 hours, in order to see whether there are differences in the inhibition zone diameter, as the bacteria can adapt or the compounds can lose their activity in time.

There were slightly differences between the used POW concentrations, because of their different molecular masses.

In *successive dilutions method*, from the three POW compounds, successive binary dilutions were created and 50 µl were distributed in sterile 96-well plates by rows using a multichannel automatic pipette. From 0.5 McFarland bacterial suspensions, 10 µl were transferred in 9,990 ml Muller-Hinton broth and homogenized. One hundred µl from the bacterial suspension were transferred in each well using a multichannel automatic pipette. On each plate we prepared one positive (growth control) and one negative control (non-inoculated culture medium, for sterility control). The plates were sealed and incubated for 16-20h at 35°C.

We followed the MIC for each compound in the first well that showed signs of bacterial growth. From each well that did not show any bacterial growth, we spot-inoculated 10 µl on Muller-Hinton agar, incubated for 16-20h at 35°C, and followed the bacterial growth. The MBC was considered in the lowest dilution from which bacteria did not grow.

All statistic tests were performed in spreadsheet software and by GraphPad InStat3, at significance level alpha 0.05.

Results and Discussions

Diffusion method

The values obtained for two of the three compounds at 25x and 10x concentrations on *S. aureus* and *E. coli* are presented in *Table I*. In 1x concentration, no compound showed any antibacterial effect. As Na_3PW_{12} is sparingly soluble and the desired concentrations could not be achieved, we worked with a saturated solution of this compound. At this maximum concentration (1x compared to the CRO, 0.29x compared to CIP, 0.53x compared to AMP, 0.38x compared to GN and 0.87x compared to CXM), Na_3PW_{12} compound showed no antibacterial activity.

After 48 hours of incubation, the diameters were slightly lower, with up to 14%, which may suggest either a slight bacterial adaptation or a decrease of POW's activity in time.

According to the diffusion method, both SiW_{12} and PW_{12} presented activity on *S. aureus* and *E. coli*, and SiW_{12} presented better activity than PW_{12}, especially on *E. coli*. Both PW_{12} and SiW_{12} inhibited the growth of bacteria at about the same concentration values (for PW_{12}= 206.18-972.6 mg/ml, and for SiW_{12}= 205.93-971.4 mg/ml). On *E. coli*, SiW_{12} had action only

Table I. Diameters of zones of inhibition produced by different concentrations of PW_{12} and SiW_{12} on *S. aureus* and *E. coli.*

		X times molar concentration of CXM for PW_{12}		X times molar concentration of AMP for PW_{12}		X times molar concentration of CRO for PW_{12}		X times molar concentration of CIP for PW_{12}		X times molar concentration of GN for PW_{12}	
		10X	**25X**	**10X**	**25X**	**10X**	**25X**	**10X**	**25X**	**10X**	**25X**
PW_{12} (mg/ml)		338.46	846.16	206.18	515.47	389.04	972.6	108.92	272.32	1.55	3.89
S. aureus	24h	11.4	15.6	9.9	13.3	12.7	15.6	7	10.5	6	6
	48h	11.24	14.7	9.43	12.6	11.9	15.07	7	10.15	6	6
	Δ	1.4%	5.8%	4.7%	5.3%	6.3%	3.4%	-	3.3%	-	-
E. coli	24h	7	7	7	7	6	7	6	6	6	6
	48h	6	6	6	6	6	6	6	6	6	6
	Δ	14.3%	14.3%	14.3%	14.3%	-	14.3%	-	-	-	-

		X times molar concentration of CXM for SiW_{12}		X times molar concentration of AMP for SiW_{12}		X times molar concentration of CRO for SiW_{12}		X times molar concentration of CIP for SiW_{12}		X times molar concentration of GN for SiW_{12}	
		10X	**25X**	**10X**	**25X**	**10X**	**25X**	**10X**	**25X**	**10X**	**25X**
SiW_{12} (mg/ml)		338	845	205.93	514.84	388.56	971.4	108.80	272	1.55	3.88
S. aureus	24h	11.3	15	9	13.4	12.5	17.1	7	10.6	6	7
	48h	10.9	14.4	9	12.9	12.16	16.9	6.4	10.6	6	6
	Δ	3.5%	4%	-	3.7%	2.7%	1.2%	8.6%	-	-	14.3%
E. coli	24h	7	11.2	7.3	8.5	8.2	13	8	7	6	6
	48h	7	11.1	7.3	8	7.8	13	7.7	7	6	6
	Δ	-	0.9%	-	5.9%	4.9%	-	3.8%	-	-	-

at high concentrations (from 845 to 971.4 mg/ml), whereas PW_{12} did not show any effect.

Successive dilutions method

In *Table II* are presented the MICs and MBCs of the three compounds against the tested strains. By these values, we have computed some statistical calculations that clearly highlight the antibacterial activity of these compounds.

SiW_{12} had a constant, good inhibitory activity on all bacterial strains. SiW_{12} had similar inhibitory activity with PW_{12} on MDR *Pseudomonas and Staphylococcus* spp.; in contrast, on the rest of the strains, the MICs were almost double for PW_{12}. Na_3PW_{12} had a very poor action, especially on *Enterococcus* and *Klebsiella*, where MIC equaled MBC. We must underline that although Na_3PW_{12} had the highest MIC, its activ-

Table II. MIC and MBC of the three compounds against all studied strains

	SiW_{12}		PW_{12}		Na_3PW_{12}	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S. aureus	1.88	3.77	1.96	7.85	2.75	11.03
P. aeruginosa	1.88	3.77	3.92	7.85	5.51	11.03
E. faecalis	1.88	15.1	3.92	15.7	11.03	11.03
K. pneumoniae	1.88	7.55	3.92	7.85	11.03	11.03
E. coli	1.88	1.88	3.92	7.85	5.51	11.03
MRSA	1.88	3.77	1.96	7.85	1.37	11.03
MDR *Pseudomonas*	1.88	3.77	1.96	7.85	5.51	5.51
ESBL *Klebsiella*	1.88	3.77	3.92	7.85	5.51	11.03

ity on *Staphylococcus* spp. occurred at very low concentrations, even lower on MRSA (1.37 mg/ml). The low MIC and MBC on MRSA could be because of the depression of penicillin-binding protein PBP2a formation (31).

Following the MBC, the situation is similar to MIC, the best antibacterial activity being shown by SiW_{12} and the lowest by Na_3PW_{12}, although the latter presented some activity on MDR *Pseudomonas*. The bactericidal activity of PW_{12} was identical on all strains except *Enterococcus faecalis*, where the highest MBC is seen. The MBCs were generally higher than MICs for most of the compounds, except SiW_{12} against *E. coli*, where MBC equaled MIC (1.88 mg/ml).

According to Kruskal-Wallis test, there is a statistical difference (p=0.0052) between the MBCs of SiW_{12} (median of 3.77) and the MBCs of Na_3PW_{12} (median of 11.03), but not between PW_{12} and the other two compounds.

Summarizing the MICs and MBCs for each bacterial strain, reveals the fact that most sensitive microorganisms are *Staphylococcus* spp., especially MRSA, and MDR *Pseudomonas*; the most resistant are the enterococci.

The antibacterial activity of compounds with Keggin structures on MRSA was studied before for the synergistic effect of POMs in combination with β-lactamic antibiotics (2,8,18,23,32). In this paper, we studied the antibacterial activity of POM compounds as drugs themselves. The results showed that they are potential drugs especially against hospital-resident strains.

There are some differences between the results obtained by diffusion method and by seriates dilutions. Considering MIC as independent variable and diameter as dependent variable, we obtained a negative, but not quite significant correlation (p=0.069, R^2=0,602). There is no correlation between MBC and diameter (p=0.098, R^2=0.535). As the dilution method is more specific and more reliable (33), it makes the diffusion method not to be proper for the screening of POW's antibacterial activity, but gave us some information about a possible adaptation of bacteria to these compounds. These findings shall be clarified in future studies.

Conclusions

The strains with resistance phenotypes present in this study, that are found especially in hospital environment, show great sensitivity to the polyoxotungstate compounds which suggest that they can be successfully used as a method of hospital decontamination.

The results obtained by the diffusion method show that these compounds are effective only at relatively high concentrations. However, the administration in these concentrations as a substitute to antibiotics is possible, but it requires further studies on pharmacokinetics, pharmacodynamics, and toxicology.

The three studied polyoxotungstate compounds exhibited a potent antibacterial activity with the MIC ranges of 1.88-6.02 mg/ml, although they were inferior to antibiotics. SiW_{12} had the most antibacterial activity. The Na_3PW_{12} had the worst effect, but instead it demonstrated the most powerful antibacterial activity on MRSA.

In further evaluation of polyoxotungstates' antibacterial activity, we recommend using the microdilution method. The bacterial capacity to adapt to POW must be clarified in further studies.

Acknowledgements

This paper is partly supported by the Sectorial Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU 80641.

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