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Original article

In vitro and *in vivo* investigations on the antiviral activity of a series of mixed-valence rare earth borotungstate heteropoly blues

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Abstract

A series of mixed-valence rare earth borotungsto-heteropoly blues, $K_{15}H_2[Ln(BW_9W_2O_{39})_2]\cdot 28H_2O$ (Ln(2), Ln = La, Ce, Pr, Nd, Sm, Eu, Gd), have been prepared and characterized by IR, UV, XPS, ESR and electrochemistry. The cytotoxicity and antiviral activity of these rare earth borotungstate heteropoly blues were investigated against influenza A(FluVA) strain (A/H1N1/Jingfang/1/91 and A/H3N2/Jingfang/30/95) and influenza virus B(FluVB) (B/Hufang/1/87) in MDCK cells. The results show that $K_{15}H_2[Pr(BW_9W_2O_{39})_2]\cdot 28H_2O$ (Pr(2)) exhibits the highest antiviral activity against FluVA with EC_{50} of less than 4.0 μg/ml (A/H1N1/Jingfang/1/91) and 6.0 μg/ml (A/H3N2/Jingfang/30/95), respectively, and has the lowest toxicity with CC_{50} of more than 480 μg/ml against MDCK cells. Additionally, both $K_{15}H_2[Pr(BW_9W_2O_{39})_2]\cdot 28H_2O$ (Pr(2)) and $K_{15}H_2[Eu(BW_9W_2O_{39})_2]\cdot 28H_2O$ (Eu(2)) showed excellent antiviral activities against FluVB by inhibiting FluVB replication at an EC_{50} of less than 8.0 μg/ml. Furthermore, investigation on *in vivo* antiviral activity of Pr(2) against FluVA(FM1) in mice by giving the dosage either orally (p.o.) or intraperitoneally (i.p.), indicates that Pr(2) exhibits higher inhibitory activity than that of positive control, virazole, and that it's more effective when administered by i.p.

Keywords: Rare earth borotungstato-heteropoly blues; Influenza virus; Antiviral activity

1. Introduction

In recent years many efforts have been focused on the discovery and development of novel antiviral drugs to treat influenza. Although there are antiviral drugs now available for prophylaxis influenza, such as zanamivir, oseltamivir or virazole which were reported to be potent inhibitors of influenza A and B viral neuraminidases recently [1–4], they are ineffective against influenza B virus infections [5–7], and their utilization in clinic is further limited by the rapid emergence of resistant virus mutants [8–11].

Polyoxometalate (POM), which usually possesses a cryptate structure with an alkaline or alkaline-earth cation in the central cage and charges on all oxygen atoms, has been reported to possess antiviral or antitumor activity. The broad spectrum antiviral activity of these complexes against retro, herpes, orthomyxo and paramyxoviruses [12–18], as well as even RNA and DNA virus inhibitory activity have been reported [19–23], and POMs with Keggin-type structures have been reported to be good inhibitors of arenaviruses, human immunodeficiency virus types 1, 2, as well as herpes simplex viruses, influenza viruses and parainfluenza virus [24–34]. More recently, a heteropolyoxotungstate, $K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$, PM-19, exhibits excellent inhibitory activity on the replication of HSV at a non-cytotoxic concentration.

However, it's shown that heteropolymetalates are not stable at physiological pH value, and only short-lived effects have

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been observed. On the contrary, heteropoly blues which were prepared by reducing the corresponding heteropolymetalates exhibit significantly heat stability and acid–base stability compared to their prototype heteropoly acids [35–39]. But there are still no attention focused on the activity of heteropoly blues which may be used as potent antivirus drugs against influenza virus.

In this paper, a series of novel rare earth heteropoly blues, $K_{15}H_2[Ln(BW_9^{VI}W_2^{V}O_{39})_2]\cdot 28H_2O$, (Ln(2), Ln = La, Ce, Pr, Nd, Sm, Eu and Gd), as well as their prototype heteropoly acids, $K_{15}[Ln(BW_{11}O_{39})_2]\cdot 28H_2O$, (Ln(0), Ln = La, Ce, Pr, Nd, Sm, Eu and Gd), have been synthesized and characterized. The antiviral activity of these compounds against influenza virus was investigated and the results showed that Pr(2) exhibited excellent antiviral activities against both influenza A and B with almost no cytotoxicity.

2. Experimental section

2.1. Physical measurements and chemicals

Physical measurements: UV–vis absorption was recorded on a Shimadzu UVPC-3000 spectrophotometer. The elemental analysis of K was performed on PE-3030(USA). The other metal's Microanalyses were performed on a PLASMA-SPECI(I) ICP-AES Quantometer. IR spectra of the samples were recorded on a BIO-RAD FTS-7 IR spectrophotometer using KBr discs. The electrochemical study was performed by using a 348 polarographic analyzer equipped with 303A electrodes. The ESR spectra were recorded on a JES-FE 3AX spectrometer at 77 K. XPS spectra were recorded on a VG ESCALAB-MKII X-ray photoelectron spectroscopy (XPS).

Chemicals: $Na_2WO_4\cdot 2H_2O$, H_3BO_3, $Ln_2(CO_3)_3$ (Ln = La, Ce, Pr, Nd, Sm, Eu and Gd), KCl, HCl . All the chemicals obtained from commercial suppliers were used as received.

2.2. Cells and animals

Madin–Darby canine kidney (MDCK) cells were obtained from the Chinese Academy of Military Medical Sciences (Peking, China). Typing culture collection, the MDCK cells were grown in Eagle's minimum essential medium (MEM) containing 8% heat-inactivated fetal bovine serum (FBS, From Gibco). The MEM was supplemented with 10 U/ml of trypsin (Sigma), 1 μg/ml of EDTA, 0.18%$NaHCO_3$ and 50 μg/ml of gentamicin. The 96-well flat-bottomed microplates were used for *in vitro* antiviral experiments and virus titrations.

BALB/c mice were obtained from the Animal Institute, Peking Union Medical School, Chinese Academy of Medical Sciences (Beijing, China). They were 7 weeks old and weighed 14–16 g.

2.3. Viruses and subculture

Influenza viruses (A/H1N1/Jingfang/1/91, A/H3N2/Jingfang/30/95 and B/Hufang/1/87) were obtained from the Chinese Academy of Medical Sciences (Beijing, China). The virus was propagated in chorio-allantoic cavities of 10-day old embryonated hen eggs for 3 days at 35 °C. The infected allantoic fluids were clarified by centrifugation at 1000× for 20 min and stored as virus stock solution in small portions at −80 °C. The MDCK cells were cultured as monolayers in MEM supplemented with 8% fetal bovine serum.

Influenza virus FM1 (type A, H1N1) passage 12 times in mice, 11 times in chick embryos, and mice lung adaptable strains were obtained from the Institute of Medical Biotechnology, Chinese Academy of Medical Science. The virus was stored at −70 °C before use. Frozen virus was thawed rapidly and diluted with serum free E-MEM at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilutions.

2.4. In vitro antiviral assays

In vitro antiviral assays were carried out according to literature [40], and inhibition of virus-induced cytopathic effect (CPE) was determined by visual (microscopic) examination of cells. In general, eight concentrations of tested compounds, each varying by one-half $\log_{10}$ from the next, were evaluated in MDCK cells. Standard placebo treated virus controls, toxicity controls and normal medium controls were included in all assays. CPE inhibition data were expressed as the 50% effective (viral CPE-inhibitory) concentration (EC_{50}), 50% cytotoxicity (cell-inhibitory) concentration (CC_{50}) and selectivity index (SI), determined as the CC_{50}/EC_{50}.

2.5. Toxicity

Mice were divided into four groups at random with 10 mice in each group. Samples were given either orally or intraperitoneally as a single dose or twice daily for 4 days to assess acute and chronic toxicity. Control group was given i.p. or p.o. with physiological saline. Mice were observed for adverse events and death after 10 days. Autopsy was also carried out and the 50% lethal doses LD_{50} of acute and chronic toxicity were calculated.

2.6. In vivo antiviral activity

An appropriate non-toxic dose of sample was chosen according to the results of accumulated toxicity experiments by i.p. and p.o. administration. Samples were diluted by physiological saline. The dosage for p.o. is 100, 50, 20 and 10 mg/kg, respectively, and for i.p. 5, 2, 1 and 0.5 mg/kg. The control group of ribavirin is at a dosage of 100 mg/kg.

Mice were anaesthetised by diethylether and drip-nose infected with 4 LD_{50} (10^{-4} dilution) of FM1 at 0.04 ml per mouse. They were then administered twice daily of sample or ribavirin for 4 days, tested in parallel with a saline treatment and placebo group (injected i.p. or p.o. with physiological saline twice daily), a compound group (not infected with virus and administered i.p. or p.o. with the largest dose of compound twice daily) and a normal group. The mice were observed daily for 14 days. Death and body weight of mice in

each group were recorded. The 50% effective dose (ED_{50}) was calculated by Reed–Muench method and the therapeutic index TI was obtained.

2.7. Synthesis of $K_{15}[Ln(BW_{11}O_{39})_2]\cdot 28H_2O$

$Na_2WO_4\cdot 2H_2O$ (20 g, 60 mmol) and H_3BO_3 (2.5 g, 40 mmol) were added slowly to 100 ml H_2O at 80 °C. The pH value of the solution was then adjusted to 6.5 with HCl (3 mol L^{-1}), and the temperature kept constant at 80 °C during the whole course. After 30 min, the pH value of the mixture was readjusted to 6.5, and rare earth carbonate (4 mmol) in H_2O (10 ml) was added drop-wise under stirring, and the pH value of the mixture was readjusted to 6.5 again. After this, KCl (5 g, 67 mmol) in H_2O (10 ml) was added drop by drop under stirring, and the mixture was stirred at 80 °C for another 1 h. After filtering and cooling, the mixture was kept at 0–5 °C for several days, and the products $K_{15}[Ln(BW_{11}O_{39})_2]\cdot 28H_2O$ (Ln(0), Ln = La, Ce, Pr, Nd, Sm, Eu and Gd) were obtained and re-crystallized in water.

2.8. Synthesis of $K_{15}H_2[Ln(BW_9^{VI}W_2^{V}O_{39})_2]\cdot 28H_2O$

$K_{15}[La(BW_{11}O_{39})_2]\cdot 28H_2O$ (1.0 g) was dissolved in HOAc–NaOAc–dioxane buffer (50 ml, 0.1 mol dm^{-3}, pH = 6.5) electrolytically at a controlled potential (*ca.* −0.545 V) under N_2 atmosphere. The reduction process was monitored by polarography and UV spectra and the extent of reduction was checked by a copper coulometer. The solution was transferred to a vacuum drier with silica gel under nitrogen atmosphere. After 7 days, the dark blue crystals $K_{15}H_2[Ln(BW_9^{VI}W_2^{V}O_{39})_2]\cdot 28H_2O$ (Ln(2), Ln = La, Ce, Pr, Nd, Sm, Eu and Gd) were obtained and re-crystallized in water. Yield: 62.5–70.1%.

3. Results

3.1. Chemistry

3.1.1. Elemental analysis

Elemental analysis of heteropoly compounds is shown in Table 1.

3.1.2. Electrochemistry

Compounds Ln(2) were prepared by electrolytically reducing the corresponding Ln(0) under a controlled potential (*ca.* −0.5 V). The redox properties of Ln(0) and Ln(2) were investigated by cyclic voltammetric and polarographic methods. The half-wave potentials and CV data of Ln(0) and Ln(2) are listed in Table 2.

For all polyoxometalates, there are two half-wave potentials in polarographic diagram resulting from the conversion of W^{VI} to W^{V} and the conversion of W^{VI} to W^{IV}. From the slope of E versus pH, it's calculated that n, which stands for the number of electrons, is 2 for the reduction of tungsten, indicating that there are two protons added to gain two electrons while the polyanion is reduced. This prevents charge build-up on the anion, which would otherwise be destabilized. These data show that the title anion undergoes a two-step, two-electron tungsten reduction. The half-wave potential of Ln(2) changed little with different rare earth ions (Table 2), indicating rare earth ions do not take part in the redox process. In addition, the polarography of Ln(2) in the HOAc–NaOAc–dioxane buffer (pH = 6.3) exhibits two reduction waves with similar shape and height, indicating Ln(2) still keeps the Keggin structure as its prototype compound does.

3.1.3. XPS and ESR spectra

The XPS spectra of Pr(2) are shown in Fig. 1.

Compared to Ln(0), the peak shape of the Ln(2) was broader and deformed. This may be attributed to the additive result of the splitting peaks for both W^{VI} and W^{V} upon the reduction process. When deconvoluted (Fig. 1), the area ratio of these peaks was 9:2, demonstrating that the reduced Ln(2) have two W^{V} atoms as expected. The result suggests that the electron densities around the B atom in compounds I and II have decreased compared to those of the parent heteropoly blues.

The inner electron binding energy of Ln, O, B and W decreased in Ln(2) compared to the corresponding Ln(0) ascribed to the increase of charge density over the anions of Ln(2), and the extra electrons are, to a certain extent, delocalized in the whole molecule. These results suggest that Ln^{3+} still exists in the molecular structure and they are not affected during the whole reduction process.

The ESR spectrum of Ln(2) at 77 K shows two signals. All the heteropoly compounds exhibit the signal of W^{V}. Pr(2) exhibits hyperfine structure at low field intensity, which demonstrates that the extent of electron pairing is lower after introducing an even number electrons at low temperature, and each reduced electron mainly localizes in the $1b_2$ orbit of W^{V}. In addition, the ESR spectra of Pr(2) exhibits signal at

Table 1
Elemental analysis of heteropoly compounds

Compound	Ln	W	K	B	H_2O
$K_{15}H_2[La(BW_{11}O_{39})_2]\cdot 28H_2O$	2.12(2.12)	61.63(61.80)	8.97(8.96)	0.30(0.30)	7.61(7.63)
$K_{15}H_2[Ce(BW_{11}O_{39})_2]\cdot 28H_2O$	2.14(2.14)	61.81(61.78)	8.95(8.96)	0.33(0.33)	7.71(7.70)
$K_{15}H_2[Pr(BW_{11}O_{39})_2]\cdot 28H_2O$	2.15(2.15)	61.69(61.77)	8.97(8.96)	0.33(0.33)	7.72(7.70)
$K_{15}H_2[Nd(BW_{11}O_{39})_2]\cdot 28H_2O$	2.20(2.20)	61.67(61.76)	8.93(8.93)	0.33(0.33)	7.68(7.69)
$K_{15}H_2[Sm(BW_{11}O_{39})_2]\cdot 28H_2O$	2.29(2.29)	61.65(61.70)	8.93(8.92)	0.33(0.33)	7.67(7.69)
$K_{15}H_2[Eu(BW_{11}O_{39})_2]\cdot 28H_2O$	2.31(2.31)	61.62(61.68)	8.93(8.92)	0.32(0.32)	7.68(7.69)
$K_{15}H_2[Gd(BW_{11}O_{39})_2]\cdot 28H_2O$	2.40(2.40)	61.64(61.68)	8.92(8.91)	0.33(0.33)	7.70(7.69)

Calculated numerical value is in parentheses.

Table 2
Polarography and cyclic voltammetry data for heteropoly compounds

Compound	$E_{1/2}$ (V)		E_{pa} (V)	E_{pc} (V)	ΔE_p (mV)
La(0)	−0.404	−0.612	−0.406	−0.436	30
			−0.623	−0.652	31
La(2)	−0.302	−0.510	−0.308	−0.336	32
			−0.526	−0.556	30
Ce(0)	−0.406	−0.618	−0.405	−0.436	31
			−0.622	−0.652	30
Ce(2)	−0.300	−0.512	−0.306	−0.336	30
			−0.524	−0.556	28
Pr(0)	−0.404	−0.619	−0.408	−0.438	30
			−0.625	−0.656	31
Pr(2)	−0.305	−0.516	−0.305	−0.334	31
			−0.524	−0.554	30
Nd(0)	−0.402	−0.616	−0.407	−0.436	31
			−0.624	−0.652	32
Nd(2)	−0.304	−0.514	−0.306	−0.334	32
			−0.527	−0.555	32
Sm(0)	−0.405	−0.615	−0.408	−0.439	29
			−0.627	−0.656	31
Sm(2)	−0.304	−0.512	−0.306	−0.334	32
			−0.528	−0.557	31
Eu(0)	−0.401	−0.610	−0.405	−0.433	32
			−0.624	−0.654	30
Eu(2)	−0.305	−0.516	−0.308	−0.336	32
			−0.526	−0.556	30
Gd(0)	−0.405	−0.611	−0.404	−0.435	29
			−0.625	−0.656	29
Gd(2)	−0.304	−0.515	−0.305	−0.335	30
			−0.524	−0.555	29

$\overline{g} = 1.928$ besides $\overline{g} = 1.845$ (W^V), which is ascribed to ESR signal of rare earth ion Pr(2).

3.1.4. IR spectra

The observed frequencies and tentative assignments of IR spectra for Ln(0) and Ln(2) are given in Table 3.

The IR spectra of Ln(2) exhibit four characterized asymmetric stretching vibrations for heteropolyanions at 930, 832, 784 and 700 cm^{-1}, assigned to W═Od, (Od, terminal oxygen), B−Oa (Oa, central oxygen atom), and W−Ob−W (Ob, bridging oxygen), W−Oc−W (Oc, bridging oxygen atom), respectively. The equivalent bands for Ln(0) appeared at 937, 840, 788 and 704 cm^{-1}, respectively. These data indicated that Ln(2) retains the Keggin structure of their prototype compounds but with slight distortion. This indicates that the Keggin structure of the heteropoly anion is not destroyed after the formation of the heteropoly blue compounds.

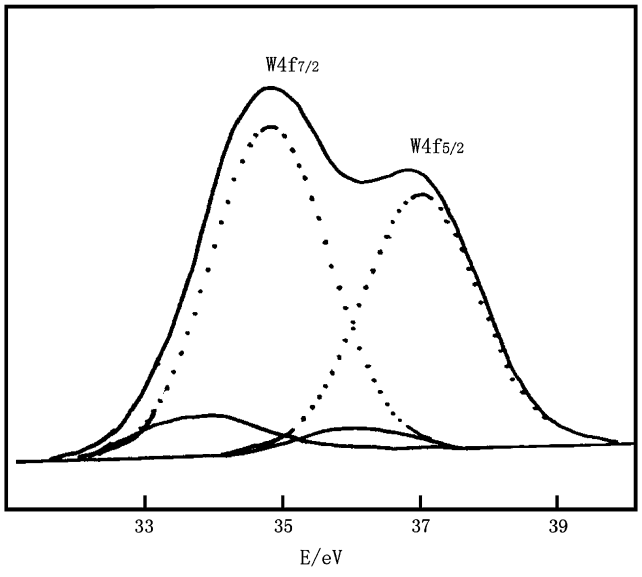

Fig. 1. XPS spectra of Pr(2).

3.1.5. UV−vis spectra

The observed frequencies and tentative assignments of the main UV−vis bands of the Ln(0) and Ln(2) are listed in Table 3.

There are two absorption transitions for Ce(0) at 198 and 247 nm in UV spectra, assigned to the Od → W charge transfer and the Ob/Oc → W charge transfer, respectively. For heteropoly blues Pr(2) under the same conditions, the absorptions ascribed to Od → W and Ob(Oc) → W were weakened, and shifted to 190 nm ($\Delta\lambda = 8$ nm) and 236 nm ($\Delta\lambda = 11$ nm), respectively. A similar change was observed for other heteropoly blues.

In the visible range, there is non-absorption observed for Ln(0). As for Ln(2), there are two intense absorptions at about 730 nm, which can be attributed to IVCT transition of charge transfer between W^V and W^{VI} with Ob acting as bridging oxygen [38].

3.2. Antiviral activity against influenza virus by heteropolymetalates

3.2.1. The anti-influenza virus efficacy and cytotoxicity of heteropolymetalates by CPE

The results of anti-influenza virus efficacy in MDCK cells by CPE methods are listed in Table 4.

MDCK is sensitive to influenza virus. In the absence of heteropolymetalates, there are small pathological changes observed for MDCK cells in microscope after 8 h infected by influenza virus A/N1H1/Jingfang/1/91, A/H3N2/Jingfang/30/95 or B/Hufang/1/87. The pathological changes observed to reach 80% 18 h later and 100% 72 h later. The pathological changes of MDCK were apparently at larger dosage ($p < 0.05$) of heteropolymetalates, whereas almost the same at small dosage ($p > 0.05$). The experiment *in vitro* showed that there were no cytotoxicity observed for Pr(2) in either resting or rapidly dividing cells at dosages as high as 256.8 μM.

It's shown that all heteropolymetalates except Sm(0), Sm(2), Gd(0) and Gd(2) exhibit perfect antiviral activity against influenza A(A/N1H1/Jingfang/1/91, A/H3N2/Jingfang/30/95) compared to that of control $K_5[BW_{12}O_{40}]\cdot 10H_2O$. Meanwhile, Pr(2) and Eu(2) exhibit perfect antiviral activity against influenza viruses A and B compared with the positive controls, virazole. The EC_{50} of Pr(2) was 4, 6 and 8 μg/ml for A/N1H1/Jingfang/1/91, A/H3N2/Jingfang/30/95 and B/Hufang/1/87, respectively, and the EC_{50} for Eu(2) was 5, 6 and 7 μg/ml for A/N1H1/Jingfang/1/91, A/H3N2/Jingfang/30/95 and B/Hufang/1/87, respectively.

Table 3
IR and UV–vis spectral data of heteropoly compounds

Compound	IR (ν_{as}/cm^{-1})				UV (λ/nm)		Vis (λ/nm)
	W=Od	W–Ob–W	W–Oc–W	B–Oa	Od → W	Ob(Oc → W)	
$K_5BW_{12}O_{40}$	961	813	–	904	–	–	–
La(0)	947	847	784	888	199	246	–
La(2)	940	840	780	882	190	239	732
Ce(0)	947	847	784	889	198	247	–
Ce(2)	941	843	780	885	190	236	731
Pr(0)	950	791	785	886	198	246	–
Pr(2)	942	782	780	880	189	237	733
Nd(0)	952	831	782	886	198	246	–
Nd(2)	945	825	776	881	191	235	732
Sm(0)	951	832	781	886	199	247	–
Sm(2)	945	827	776	882	192	238	734
Eu(0)	950	831	781	886	198	246	–
Eu(2)	943	826	776	882	191	238	733
Gd(0)	951	830	780	885	199	247	–
Gd(2)	945	825	775	881	191	239	732

3.2.2. Toxicity of Pr(2)

Groups of 10 mice in each group were used in toxicity studies. Different doses of Pr(2) were administered by i.p. and p.o. routes. The mice were observed daily for 10 days, and deaths were recorded. The results of acute toxicity of Pr(2) in the mice are – p.o. group: $LD_{50} = 3850$ mg/kg; i.p. group: $LD_{50} = 530$ mg/kg; and accumulated toxicity are – p.o. group: $LD_{50} = 1845$ mg/kg; i.p. group: $LD_{50} = 168$ mg/kg. The difference was not significant ($p < 0.05$). Regardless of the route of administration (i.p. or p.o.), all mice were dead within 24 h, and examined microscopically for changes in the large organs of the abdominal and thoracic cavities. The 50% lethal dose of i.p. administration was far less than that of p.o. administration, indicating that the compound was more toxic when given by i.p. Survivors increased in weight slowly compared to the normal group.

3.2.3. In vivo antiviral activity of Pr(2) against influenza virus

The results of *in vivo* antiviral activity of Pr(2) against influenza virus are shown in Table 5.

The selectivity of drugs is usually related to its toxicity. The assessment index is expressed by the ratio of 50 lethal dose (LD_{50}) to 50 effective dose (ED_{50}) [41]. This ratio is the therapeutic index TI, $TI = LD_{50}/ED_{50}$. The results calculated by the Reed–Muench method were as follows: p.o. Pr(2) group: $ED_{50} = 15.7$ mg/kg, $TI = 117.5$; i.p. Pr(2) group: $ED_{50} = 0.77$ mg/kg, $TI = 218$.

Table 4
The anti-influenza virus activity of heteropolymetalates in MDCK cells

Compound	CC_{50}[a] (μg ml−1)	Virus					
		A/N1H1/Jingfang/1/9		A/H3N2/Jingfang/30/95		B/Hufang/1/87	
		EC_{50}[b,c] (μg ml^{-1})	SI[d]	EC_{50} (μg ml^{-1})	SI[d]	EC_{50} (μg ml^{-1})	SI[d]
La(0)	410	–[e]	–	–	–	–	–
La(2)	440	–	–	–	–	–	–
Ce(0)	430	–	–	–	–	–	–
Ce(2)	450	–	–	–	–	–	–
Pr(0)	460	5.0	92.0	8.0	57.5	11.0	41.8
Pr(2)	480	4.0	120.0	6.0	80.0	8.0	60.0
Nd(0)	450	12.0	37.5	14.0	32.0	10.0	45.0
Nd(2)	450	15.0	30.0	9.0	50.0	7.0	64.3
Sm(0)	430	–[e]	–	–	–	–	–
Sm(2)	440	–	–	–	–	–	–
Eu(0)	470	5.0	94.0	7.0	67.1	6.0	78.3
Eu(2)	490	5.0	98.0	6.0	81.7	7.0	70.0
Gd(0)	400	–	–	–	–	–	–
Gd(2)	420	–	–	–	–	–	–
Virazole	2877	26.2	108.2	26.2	108.2	34.8	82.6

[a] CC_{50}: 50% cell toxicity concentration determined by MTT assay.
[b] Mean of CPE inhibition and NR uptake data.
[c] EC_{50}: concentration reducing by 50% the optical density values (or the virus-induced CPE) in PC-treated virus-infected cells in relation to virus control. The value of EC_{50} is the mean of three experiments.
[d] SI: selectivity index (CC_{50}/EC_{50}).
[e] –: Lower than 50% inhibitory cytopathic induced by virus.

Table 5
Therapeutic effect of Pr(2) on the mice infected with influenza virus FM1[a]

Group	Dose (mg/kg)	Number of deaths	Survival rates (%)[b]
Pr(2) (p.o.)	100	0	100
	50	2	80
	20	3	70
	10	4	40
Virazole (p.o.)	100	2	80
Saline treatment		9	10
Pr(2) group (u.i.)	200	0	100
	5	10	90
Pr(2) (i.p.)	2	2	80
	1	2	80
	0.5	6	40
Virazole (p.o.)	100	3	70
Saline treatment		9	10
Pr(2) group (u.i.)	20	0	100
Placebo group		0	100

[a] BALB/c mice (10 per group) were infected by influenza virus strain FM1 and treated for 4 days either with Pr(2) (administered p.o. or i.p.) or ribavirin (administered p.o. once daily). Control groups (10 mice per group) were infected and untreated, or uninfected and either untreated or treated with the top dose of Pr(2). Mice were observed daily and the survival rate was determined at 14 days post-infection.
[b] *T*-test was adopted for data processing.

3.2.4. Survival rates

The survival rates of mice infected with influenza administered Pr(2) by p.o. and i.p. are shown in Fig. 2.

Mice without any treatment died within 4–8 days and the survival rates were 0–10%, and the survival rate for the positive control of ribavirin was 80% when administered p.o. at a dose of 100 mg/kg. On the other hand, the survival rate of mice treated by Pr(2) were 100% and 80% at doses of 100 and 50 mg/kg (p.o.), respectively. (Fig. 2), and the survival rate was 90% for mice administered (i.p.) Pr(2) at doses of 5 and 2 mg/kg while the survival rate was 70% when administered (i.p.) ribavirin at a dose of 100 mg/kg. Furthermore, when the dosage of Pr(2) was reduced to 2 and 1 mg/kg (ip), the survival rates decreased obviously. These results indicated that the survival rates of mice infected with influenza virus were significantly improved by treatment of Pr(2), and the survival rates were clearly dosage dependent.

3.2.5. Effects of Pr(2) on the weight of mice in treatment group

The relationship between the dosage of Pr(2) administration by p.o. and i.p. on the weight of mice is shown in Fig. 3

Compared with normal mice, mice treated with Pr(2) either by i.p. or by p.o. had no significant difference in weight.

After 12 days, the weight of mice treated by ribavirin increased to 20 g (p.o.) and 18 g (i.p.), respectively, while the weight of mice treated by Pr(2) increased to 25 g (p.o.) and 23 g (i.p.), respectively. These data showed that mice treated by ribavirin grew more slowly than those treated by Pr(2), indicating that ribavirin had some toxic effects at this dosage range investigated.

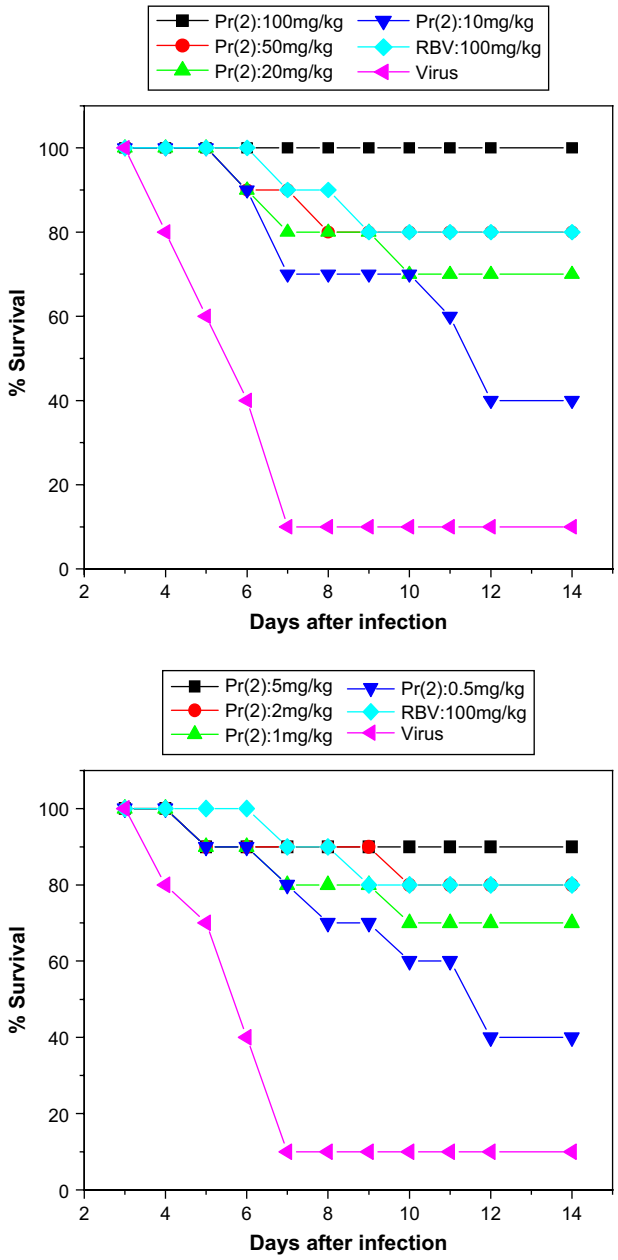

Fig. 2. Effect of Pr(2) given by oral (up) and intraperitoneal (down) on the survival of mice.

4. Discussions

In this paper, a series of novel heteropoly blues, $K_{15}H_2[Ln(BW_9^{VI}W_2^{V}O_{39})_2]\cdot 28H_2O$, (Ln = La, Ce, Pr, Nd, Sm, Eu and Gd), as well as their prototype heteropoly acids, have been prepared and characterized by elemental analysis, IR, UV, CV, XPS, ESR and NMR. The results showed that heteropoly blues (Ln(2)) keep α-Keggin structure but with a subtle distortion compared with their corresponding heteropoly acid (Ln(0)).

The inhibitory ability against influenza A and B viruses of Ln(2) and Ln(0) were evaluated by MTT method and a plaque reduction assay using MDCK cells. The results showed that

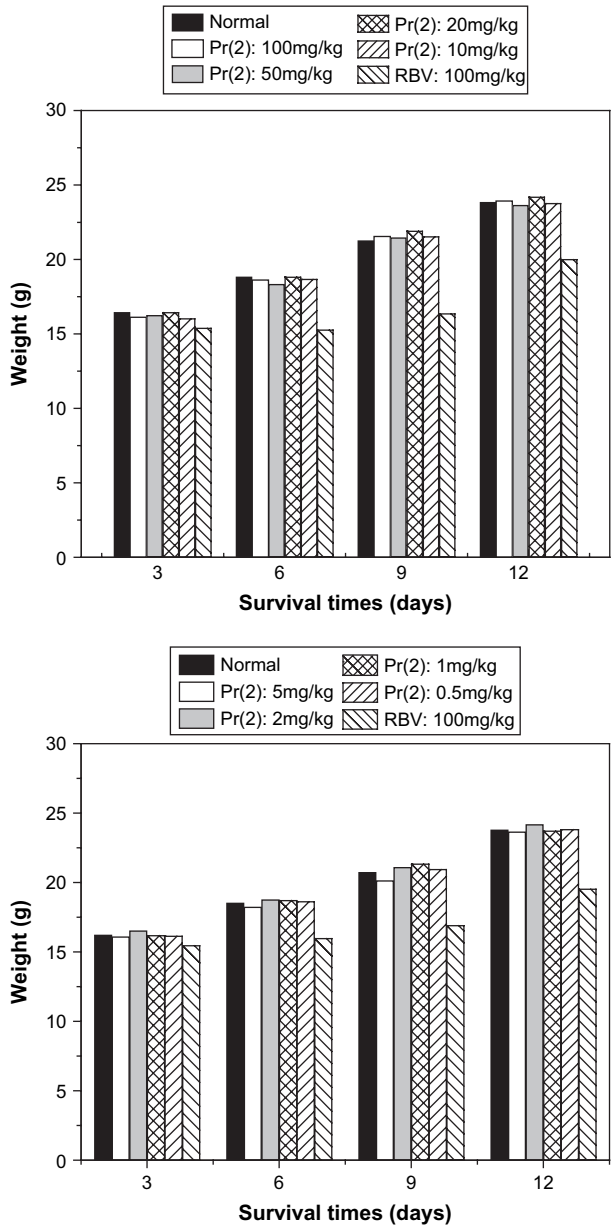

Fig. 3. Effect of dosage of Pr(2) administration by oral (up) or intraperitoneal (down) on the weight of mice.

Pr(2) exhibits the highest inhibition activity against influenza virus A and Eu(2) exhibits the highest inhibition activity against influenza virus B, while the heteropolyoxometalates containing Ln(0) showed almost no antiviral activity against both influenza viruses A and B. This may be attributed to the lower heat stability of Ln(0) compared to Ln(2), resulting in a relatively large concentration of rare earth ions Ln^{3+} and WO_4^{2-} released by the decomposition of Ln(0) within a short period to make the concentrations of rare earth ion Ln^{3+} and WO_4^{2-} so high enough to exhibit high cytotoxicity. On the contrary, heteropoly blues decompose more slowly and the concentrations of metal ions dissociated are moderate due to their higher heat stability. Furthermore, heteropoly blues containing Ce(2) or Pr(2) have a suitable molecular size to interfere and destroy the virus or its chemical components. Furthermore, the antiviral activity of these heteropolyoxometalates was determined by their anionic structures [41,42], the peripheral rare earth ions [43,44], the substituted metal ions and the number of the anionic negative charges [26].

In vivo antiviral activity was investigated on BALB/c mice, and the results show that the antiviral activity of Pr(2) was superior than that of positive control ribavirin which is more toxic on mice. On the other hand, the 50% effective doses (ED_{50}) of Pr(2) when given orally was 15.7 mg/kg and the therapeutic index (TI) was 117.5. The ED_{50} when given i.p. was 0.77 mg/kg and the TI was 117.5 mg/kg, indicating that i.p. administration showed better curative effects than p.o. administration.

In conclusion, Pr(2) and Eu(2) are shown to be potent POM inhibitors against the replication of influenza virus A type and influenza virus B type at a non-cytotoxic concentration.

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References

[1] J.H. Huffman, R.W. Sidwell, D.L. Barnard, A. Morrison, M.J. Otto, C.L. Hill, R.F. Schinazi, Antiviral Chem. Chemother. 8 (1997) 75–83.
[2] P.M. Colman, Vaccine 20 (2002) S55–S58.
[3] J.L. McKimm-Breschkin, Antiviral Res. 47 (2000) 1–17.
[4] R.W. Sidwell, J.H. Huffman, D.L. Barnard, K.W. Bailey, M.H. Wong, A. Morrison, T. Syndergaard, C.U. Kim, Antiviral Res. 37 (1998) 107–120.
[5] S.Z. Wilson, V. Knight, P.R. Wyde, S. Drake, R.B. Couch, Antimicrob. Agents Chemother. 17 (1980) 642–648.
[6] H. Heider, B. Adamczyk, H.W. Presber, C. Schroeder, R. Feldblum, M.K. Indulen, Acta Virol. 25 (1981) 395–400.
[7] J. Serkedjeva, A.J. Hay, Antiviral Res. 37 (1998) 121–130.
[8] S.K. Wray, B.E. Gilbert, V. Knight, Antiviral Res. 5 (1985) 39–48.
[9] S.K. Wray, R.H.A. Smith, B.E. Gilbert, V. Knight, Antimicrob. Agents Chemother. 29 (1986) 67–72.
[10] S. Mori, J. Watanabe, S. Shigeta, Antiviral Res. 30 (1996) A53.
[11] R.A. Burger, J.L. Billingsley, J.H. Huffman, K.W. Bailey, C.U. Kim, Immunopharmacol. 47 (2000) 45–52.
[12] C.X. George, C. Singh, S.N. Ram, K.D.S. Yadav, B. Sharma, Acta Virol. 34 (1990) 330–338.
[13] S. Ikeda, J. Neyts, N. Yamamoto, B. Murrer, B. Theobald, G. Bossard, G. Henson, M. Abrams, D. Picker, E. De Clercp, Antiviral Chem. Chemother. 4 (5) (1993) 253–262.
[14] S. Shigeta, S. Mori, J. Watanabe, T. Yamasi, C.L. Hill, R.F. Schinazi, Antiviral Res. 26 (1995) A298.
[15] L. Ni, F.D. Boudinot, S.G. Boudinot, G.W. Henson, G.E. Bossard, S.A. Martellucci, P.W. Ash, S.P. Fricker, M.C. Darkes, B.R.C. Theobald, C.L. Hill, R.F. Schinazi, Antimicrob. Agents Chemother. 38 (1994) 504–510.
[16] L. Ni, F.D. Boudinot, Eur. J. Drug Metab. Pharm. 20 (1995) 209–217.
[17] T. Yamase, N. Fukuda, Y. Tajima, Biol. Pharm. Bull. 19 (3) (1996) 459–465.
[18] K. Dan, K. Miyashita, Y. Seto, H. Fujita, T. Yamase, Pharm. Res. 46 (2002) 357–362.

[19] S. Ikeda, S. Nishiya, A. Yamamoto, T. Yamaser, C. Nishimura, E. De Clercq, J. Med. Virol. 41 (1993) 191–195.
[20] M. Fukuma, Y. Seto, T. Yamase, Antiviral Res. 16 (1991) 327–339.
[21] A. Yamamoto, N.D. Schols, E. De Clercq, Mol. Pharm. 42 (1992) 1109–1117.
[22] Y. Inouye, Y. Tokutake, T. Yoshida, A. Yamamoto, T. Yamase, S. Nakamura, Chem. Pharm. Bull. 39 (1991) 1638–1640.
[23] R.Y. Zhang, Z.H. Yu, D. Ouyang, Bioorg. Med. Chem. Lett. 16 (2006) 4300–4304.
[24] Y. Take, Y. Tokutake, Y. Inouye, T. Yoshida, A. Yamamoto, T. Yamase, S. Nakamura, Antiviral Res. 15 (1991) 113–124.
[25] C.L. Hill, M.S. Weeks, R.F. Schinazi, J. Med. Chem. 33 (1990) 2767–2772.
[26] S. Shigeta, S. Mori, J. Watanabe, M. Baba, A.M. Khenkin, C.L. Hill, R.F. Schinazi, Antiviral Chem. Chemother. 6 (1995) 114–122.
[27] T. Jeffrey, C.L. Hill, D.A. Judd, Chem. Res. 98 (1998) 327–357.
[28] G.S. Kim, D.A. Judd, C.L. Hill, R.F. Schinazi, J. Med. Chem. 37 (1994) 816–820.
[29] M.S. Weeks, C.L. Hill, R.F. Schinazi, J. Med. Chem. 35 (1992) 1216–1221.
[30] D.L. Barnard, C.L. Hill, T. Gage, J.E. Matheson, J.H. Huffman, R.W. Sidwell, M.I. Otto, R.F. Schinazi, Antiviral Res. 34 (1997) 27–37.
[31] D.L. Barnard, C.L. Hill, T. Gage, R.W. Sidwell, R.F. Schinazi, Antiviral Res. 26 (1995) A349.
[32] S. Shigeta, S. Mori, E. Kodama, J. Kodama, K. Takahashi, T. Yamase, Antiviral Res. 58 (2003) 265–271.
[33] Y. Inouye, Y. Tokutake, J. Kunihara, T. Yoshida, T. Yamase, A. Nakata, S. Nakamura, Chem. Pharm. Bull. 40 (1992) 805–807.
[34] W.L. Wang, D.Y. Yao, M. Gu, M.Z. Fan, J.Y. Li, Y.C. Xing, F.J. Nan, Bioorg. Med. Chem. Lett. 23 (2005) 5284–5287.
[35] J. Liu, W.J. Mei, A.W. Xu, C.P. Tan, L.N. Ji, Trans. Metal Chem. 28 (2003) 500–505.
[36] J. Liu, E.B. Wang, Z.P. Wang, C.W. Hu, Chem. J. Chin. Univ. 19 (1998) 1200–1204.
[37] J. Liu, W.J. Mei, Y.G. Li, E.B. Wang, L.N. Ji, P.Zh Tao, Antiviral Chem. Chemother. 11 (2000) 367–372.
[38] J. Liu, W.J. Mei, A.W. Xu, C.P. Tan, Sh. Shi, L.N. Ji, Antiviral Res. 62 (2004) 65–71.
[39] N. Casañ–pastor, L.C.W. Baker, J. Am. Chem. Soc. 114 (1992) 10384–10394.
[40] D.F. Smee, J.H. Huffman, A.C. Morrison, D.L. Barnard, R.W. Sidwell, Antimicrob. Agents Chemother. 45 (2000) 743–748.
[41] S. Ikeda, S. Nishiya, A. Yamamoto, T. Yamase, C. Nishimura, E. De Clercq, Antiviral Chem. Chemother. 5 (1) (1994) 47–50.
[42] L. Ni, P. Greenspan, R. Gutman, C. Kelloes, M.A. Farmer, F.D. Boudinot, Antiviral Res. 32 (1996) 141–148.
[43] Y. Inouye, Y. Take, Y. Tokutake, T. Yoshida, A. Yamamoto, T. Yamase, S. Nakamura, Chem. Pharm. Bull. 38 (1) (1990) 285–287.
[44] Y. Inouye, Y. Tokutake, T. Yoshida, Y. Seto, H. Hujita, K. Dan, A. Yamamoto, S. Nishiya, Y. Yamase, S. Nakamura, Antiviral Res. 20 (1993) 317–331.