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In vitro anti-hepatitis B and SARS virus activities of a titanium-substituted-heteropolytungstate

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ABSTRACT

A structural determined heteropolytungstate, $[K_4(H_2O)_8Cl][K_4(H_2O)_4PTi_2W_{10}O_{40}]\cdot NH_2OH$ **1**, has been synthesized and evaluated for *in vitro* antiviral activities against hepatitis B (HBV) and SARS virus. The identity and high purity of compound **1** were confirmed by elemental analysis, NMR, IR analysis and single-crystal X-ray diffraction. The compound **1**, evaluated in HepG 2.2.15 cells expressing permanently HBV, significantly reduced the levels of HBV antigens and HBV DNA in a dose-dependent and time-dependent manner. EC_{50} values were determined to be 54 μM for HBeAg, 61 μM for HBsAg and 2.66 μM for supernatant HBV DNA, as compared to 1671, 1570, 169 μM, respectively, for the commercially-available hepatitis B drug adefovir dipivoxil (ADV). Intracellular cccDNA, pgRNA and HBcAg were also found to be decreased by compound **1** in a concentration-dependent manner. Cytotoxicity results showed that compound **1** has low toxicity in HepG 2 cells with CC_{50} value of 515.20 μM. The results indicate that compound **1** can efficiently inhibit HBV replication in HepG 2.2.15 cells line *in vitro*. Additionally, compound **1** also shows high anti-SARS activity at an EC_{50} of 7.08 μM and toxicity with a CC_{50} of 118.6 μM against MDCK cells.

1. Introduction

Hepatitis B virus (HBV) infections continue to be a major public health problem worldwide (Barraud et al., 1999). More than 400 million people worldwide are currently infected with hepatitis B virus. Approximately 20% of HBV patients will develop chronic hepatitis, and are at significant risk of developing cirrhosis or liver hepatocarcinoma. HBV is the prototype of hepadnaviridae, a family of small enveloped hepatotropic DNA viruses that can infect the liver of human (Marion and Robinson, 1983). Chronic hepatitis B patients are commonly treated with either interferon alpha (INF-α), or the nucleoside analog lamivudine (3TC), adefovir, entecavir or telbivudine which are the synthetic reverse transcriptase inhibitors (De Clercq, 1999; Delmas et al., 2002; Buster and Janssen, 2006). However, none of these therapies are completely safe and effective. Although direct antiviral therapy with amivudine or adefovir could efficiently control chronic active hepatitis B, drug resistance or renal toxicity could develop progressively several months after the initiation of therapy. It is thus still urgently required to identify effective anti-HBV agents.

Polyoxometalates (POMs) are inorganic cluster-like complexes and constituted from oxide anion and transition metal cations. These complexes have shown potential applications in multitudinal fields such as catalysis, medicine and functional materials (Pope and Müller, 1991, 1994). Especially, the medicinal properties of POMs have been a subject of interest (Witvrouw et al., 2000; Judd et al., 2001; Shigeta et al., 2003; Yamase, 2005). These compounds have low toxicity for cultured cells, and relatively less expensive than the "chemical" antiviral drugs. Recently, POMs have been reported to inhibit the replication of RNA viruses and DNA viruses *in vitro* and *in vivo*, such as the human immunodeficiency virus, severe acute respiratory syndrome (SARS) virus, influenza virus and herpes simplex virus (Rhule et al., 1998; Dan et al., 2002). The activity of POMs against hepatitis B virus was also suggested by Zoulim (1999). The mechanism of action of POMs remains to be fully elucidated, but may occur at any of the life cycle stages, including viral adsorption, penetration, or reverse-transcription (Dan et al., 2002; Shigeta et al.,

Abbreviations: HBV, hepatitis B virus; INF-α, interferon alpha; 3TC, nucleoside analog lamivudine; POMs, Polyoxometalates; SARS, severe acute respiratory syndrome; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; Vero-E_6, African green monkey kidney cells; MTT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ADV, adefovir dipivoxil; CC_{50}, 50% cytotoxic dose; TI, therapeutic index; EC_{50}, 50% effective concentration; CC_0, maximal noncytotoxic concentration; EC_{90}, 90% effective concentration; MDCK, Madin–Darby canine kidney.

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2003). Among the various POMs, keggin-type heteropolyoxotungstates $[PW_{10}Ti_2O_{40}]^{7-}$ (Domaille and Knoth, 1983; Ozeki and Yamase, 1991) shows high antiviral activity. The interesting biological results of $[PW_{10}Ti_2O_{40}]^{7-}$ prompted us to explore the antiviral activity of compounds containing $[PW_{10}Ti_2O_{40}]^{7-}$ anions as potential anti-HBV agents.

It was also reported that the heteropolyoxotungstates $[PW_{10}Ti_2O_{40}]^{7-}$ have broad-spectrum anti-RNA virus activity (Dan and Yamase, 2006). Therefore, it is necessary to further explore the antiviral activity of compounds containing $[PW_{10}Ti_2O_{40}]^{7-}$. Severe acute respiratory syndrome (SARS), a disease seriously threatening human health caused by the single-stranded RNA coronavirus, spread in 29 countries in early 2003, presenting a worldwide public health concern (Fouchier et al., 2003; Drosten et al., 2003; Ksiazek et al., 2003). The research attention in exploitation of anti-SARS treatments has been mainly focused on vaccines, antiviral drugs, and their integration of traditional Chinese medicine and western therapy. However, no effective therapeutic drug is available to date (Stadler et al., 2003).

As a part of our ongoing antiviral drug discovery program, a number of POM analogs have been synthesized and evaluated for their potential antiviral activity (Li et al., 2004a,b). Herein, the POM analog $[K_4(H_2O)_8Cl][K_4(H_2O)_4PTi_2W_{10}O_{40}]\cdot NH_2OH$ **1** was prepared and structurally characterized. The antiviral activities against hepatitis B virus and SARS virus of **1** were investigated *in vitro*. The results indicate that compound **1** exhibits strong antiviral activities against the HBV and SARS viruses with low cytotoxicity, indicating that it is a potential medicinal candidate against HBV and SARS viruses.

2. Experimental

2.1. General procedures

All the chemicals were of analytic grade and used without further purification. Compound **1**, was freshly prepared and characterized. W and Ti in **1** were determined by a Leaman inductively coupled plasma (ICP) spectrometer. Infrared spectrum was recorded in the range 400–4000 cm^{-1} on an Alpha Centaurt FT/IR Spectrophotometer using KBr pellets. The ^{183}W NMR spectrum was obtained on a Bruker Am-500 spectrometer operated at 500 MHz with D_2O as the solvent. Quantitative RT-PCR was performed with ABI 7300 Sequence Detection System (Roche, Germany).

2.2. Synthesis of $[K_4(H_2O)_8Cl][K_4(H_2O)_4PTi_2W_{10}O_{40}]\cdot NH_2OH$ *1*

Synthesis of compound **1**: 5.94 g (2.0 mmol) $K_7PTi_2W_{10}O_{40}\cdot 5H_2O$ (Domaille et al., 1983) was added into 50 mL distilled water with stirring. To this solution, 1.03 g (15.0 mmol) $NH_2OH\cdot HCl$ and 1.04 g (2.8 mmol) $LaCl_3\cdot 7H_2O$ was added in sequence. The solution was heated to 70 °C for more than 2 h in a water bath, then filtered and kept slow evaporation in an undisturbed place at room temperature. Colorless crystals of compound **1** were isolated in 1 week with the yield of 60% based on $K_7PTi_2W_{10}O_{40}\cdot 5H_2O$. Elemental analysis (%) calcd for **1** ($H_{27}ClK_8NO_{53}PTi_2W_{10}$): P, 0.97; K, 9.73; Ti, 2.99; W, 57.45; Found: P, 1.10; K, 9.71; Ti, 2.89; W, 56.55%. IR (KBr pellet, cm^{-1}): 3427(s), 1621(s), 1081(m), 1064(m), 1050(w), 959(s), 885(m), 788(s), 593(w), 488(s). ^{183}W NMR (25 °C, D_2O, ppm): −78.53, −111.79, −124.51, −126.03, −137.54.

2.3. X-ray crystallography

The measurement for compound **1** was collected on a Rigaku R-AXIS RAPID IP diffractometer with Mo-Kα monochromated radiation (λ = 0.71073 Å) at 150 K. Empirical absorption correction was applied. The structure was solved by the direct method and refined by the Full-matrix least-squares on F^2 using the SHELXL-97 software (Sheldrick, 1997). All of the non-hydrogen atoms except the disordered atoms O(1), OW(2), OW(3) and Cl(1) were refined anisotropically. All the crystallographic parameters are tabulated in Table 1. Images were created with the DIAMOND program.

2.4. Cell culture and treatment

HepG 2.2.15 cells (provided by the Department of Infectious Diseases of the 1st Hospital, Jilin University, PR China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing a 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, 100 U/mL streptomycin, and 200 μg/mL G418 (growth medium). During the experiments, the cells were grown in the media as described above without G418. The cell lines were incubated at 37 °C in 5% carbon dioxide atmosphere. Prior to exposures to drugs, the cell viability was verified to be >85% according to the standard trypan blue exclusion test.

HepG 2 (provided by the Department of Infectious Diseases of the 1st Hospital, Jilin University, PR China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing a 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin. The cell lines were incubated at 37 °C in 5% carbon dioxide atmosphere.

SARS virus (provided by Academy of Military Medical Sciences, Beijing, PR China) was propagated in African green monkey kidney cells (Vero-E_6). Vero cells were propagated in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin and bicabouate. The cell lines were incubated at 37 °C in 5% carbon dioxide atmosphere.

Drugs were sterilized by filtration prior to use. Each agent was dissolved in DMEM to generate the appropriate doses for experimentation. Non-treated cells (DMEM alone) were used as negative controls.

2.5. Anti-HBV activity of compound *1*

2.5.1. Cytotoxicity analysis

The cytotoxicity of compound **1** was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

Table 1
Crystal data and structure refinements for **1**.

Compound	**1**
Formula	$H_{27}ClK_8NO_{53}PTi_2W_{10}$
Fw	3202.75
T (K)	293(2)
Crystal system	Tetragonal
Space group	P4/mnc
a (Å)	14.200(2)
b (Å)	14.200(2)
c (Å)	12.418(3)
α (°)	90
β (°)	90
γ (°)	90
V (Å^3)	2503.8(7)
Z	2
ρcalcd (g cm^{-3})	4.248
μ (mm^{-1})	24.041
Rint	0.0562
Reflections collected	16,509
Independent reflections	1501
Goodness-of-fit on F^2	1.103
R_1[a] [$I > 2\sigma(I)$]	0.0456
wR_2[b] [$I > 2\sigma(I)$]	0.1235

[a] $R_1 = \sum||F_0| - |F_C||/\sum|F_0|$.
[b] $wR_2 = \sum[w(F_0^2 - F_C^2)^2]/\sum[w(F_0^2)^2]^{1/2}$.