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Anti-zika virus activity of polyoxometalates

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ABSTRACT

Zika virus (ZIKV) is an emerging infectious viral pathogen associated with severe fetal cerebral anomalies and the paralytic Guillain-Barrè syndrome in adults. It was the cause of a recent global health crisis following its entrance into a naïve population in the Americas. Nowadays, no vaccine or specific antiviral against ZIKV is available. In this study, we identified three polyoxometalates (POMs), the Anderson-Evans type $[TeW_6O_{24}]^{6-}$ (TeW_6), and the Keggin-type $[TiW_{11}CoO_{40}]^{8-}$ ($TiW_{11}Co$), and $[Ti_2PW_{10}O_{40}]^{7-}$ (Ti_2PW_{10}), that inhibit ZIKV infection with EC_{50}s in the low micromolar range. Ti_2PW_{10}, the POM with the greatest selectivity index (SI), was selected and the step of ZIKV replicative cycle putatively inhibited was investigated by specific antiviral assays. We demonstrated that Ti_2PW_{10} targets the entry process of ZIKV infection and it is able to significantly reduce ZIKV progeny production. These results suggest that the polyanion Ti_2PW_{10} could be a good starting point to develop an effective therapeutic to treat ZIKV infection.

ZIKV is an enveloped positive-strand RNA virus belonging to the *Flaviviridae* family and mostly transmitted by *Aedes aegypti mosquitos* (Saiz et al., 2016). Sexual, vertical and blood transmissions have also been reported (Musso et al., 2015; Mlakar et al., 2016; Motta et al., 2016). In symptomatic individuals (around 18% of cases), ZIKV causes a mild illness characterized by fever, rash, headache, conjunctivitis, joint and muscle pain (Paixão et al., 2016); this clinical presentation is similar to that of other arbovirus infections, such as chikungunya and dengue virus. However, unlike other flavivirus, ZIKV is associated to two main neurological complications: the Guillain-Barré Syndrome in adults and the now termed Zika Congenital Syndrome (CSZ), a variety of neurological impairments in fetus and infants of women infected during pregnancy. The main congenital manifestations, developed in nearly one third of these newborns, are severe microcephaly, resulting in a partially collapsed skull, intracranial calcifications, eyes abnormalities, redundant scalp skin, arthrogryposis and clubfoot (Mlakar et al., 2016; Rasmussen et al., 2016; Cao-Lormeau et al., 2016; Brasil et al., 2016). Specifically, the risk of microcephaly, with a catastrophic impact on the socioeconomic status of affected families, was reported to be 1–13% during the first trimester and negligible during second and third trimesters (McCarthy, 2016).

ZIKV can be classified into two lineages (African and Asian) and three genotypes (West African, East African, and Asian), differing in pathogenicity and virulence. The Asian-lineage ZIKV, responsible for the latest epidemics (on Yap Island and Micronesia in 2007, in French Polinesya in 2013 and in the Americas in 2016), is considered to be less virulent than the African one, because of the lower infection rate, the lower viral production, the poor induction of early cell death and the lower immuno-stimulation in different models. These characteristics allow the virus to cause a prolonged infection within the central nervous system of fetus that could be the cause of its association with neurological impairments. On the contrary, the African lineage-ZIKV can result in a more acute infection (Duffy et al., 2009; Cao-Lormeau et al., 2014; Simonin et al., 2017; Shao et al., 2017; Beaver et al., 2018).

The last major epidemic in the Americas, in 2016, counted 177614 confirmed ZIKV cases and 2552 cases of CSZ at the end of the year, driving the World Health Organization to declare a public health emergency of international concern (CDC; PAHO WHO,). Since then, great efforts have been carried out, but nowadays still no vaccine or specific antiviral against ZIKV is available (Richner and Diamond, 2018; Saiz and Martín-Acebes, 2017). The best way to prevent ZIKV infection is to avoid mosquito bites and the treatment of infected

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Table 1
Characteristics of POM aqueous solutions.

POM sample	pH	Osmolarity (mOsm)	Zeta potential (mV)
TeW_6	5.65	324	- 6.06 ± 3.11
$TiW_{11}Co$	5.45	320	- 6.95 ± 3.49
Ti_2PW_{10}	6.25	316	- 5.31 ± 1.95

patients is palliative, involving analgesics and antipyretics. In this context, ZIKV infection presents a huge challenge to the global health system and the search for efficient antivirals is absolutely necessary. To this aim, we investigated *in vitro* the anti-ZIKV activity of a minilibrary of three polyoxometalates (POMs). POMs are discrete, anionic metal-oxo complexes of early *d* block metal ions in high oxidation states (e.g. W^{VI}, Mo^{VI}, V^{V}) with a very large structural and compositional variety and a multitude of associated physicochemical properties (Pope, 1983; Pope and Müller, 1991; Pope and Kortz, 2012). POMs are usually synthesized in aqueous acidic media, but some selected species are also stable at pH 7–8. In fact, POMs have been investigated for many years as potentially useful agents in medicine, mainly for their antiviral, antitumoral, and antibacterial properties (Sarafianos et al., 1996; Rhule et al., 1998; Hasenknopf, 2005; Mauracher et al., 2014; Giang et al., 2015; Yang et al., 2016; Selman et al., 2018). Here, we decided to investigate the following three solution-stable POMs, the Anderson-Evans type $[TeW_6O_{24}]^{6-}$ (TeW_6) (Schmidt et al., 1986), and the Keggin-type $[TiW_{11}CoO_{40}]^{8-}$ ($TiW_{11}Co$) (Chen and Liu, 1997), and $[Ti_2PW_{10}O_{40}]^{7-}$ (Ti_2PW_{10}) (Domaille and Knoth, 1983), which were all synthesized according to the published procedures. The size of all three polyanions is in the range of 1 nm diameter. The purity (≥95%) of the compounds was confirmed by NMR and IR (Data available in Supplementary info). Some of these POMs have already been used in biological studies. For instance, **Ti_2PW_{10}** showed interesting results in the inhibition of acetylcholinesterase activity while maintaining low toxicity levels (Čolović et al., 2017). On the other hand, **TeW_6** showed good activity against diabetes and Alzheimer's disease (Ilyas et al., 2014; Iqbal et al., 2013).

In order to perform *in vitro* biological assays, we first prepared aqueous solutions of **TeW_6**, **$TiW_{11}Co$**, and **Ti_2PW_{10}** and we determined their physico-chemical characteristics (pH, osmolarity, Zeta potential) (Table 1) and their biocompatibility. The POMs were stable in aqueous solution up to 6 months stored at 4 °C. Indeed, a concentration decrease of 3.25, 5.05 and 4.45% was observed for **TeW_6**, **$TiW_{11}Co$** and **Ti_2PW_{10}** respectively, after 6 months. In the hemolysis assay, no significant hemolysis caused by the POM solutions was observed, indicating good biocompatibility. (Data available in Supplementary info). The tonicity and pH values were suitable for the following cell experiments.

Therefore, to evaluate the anti-Zika virus activity of the three POMs, we performed virus inhibition assays against two Zika virus strains, the 1947 Uganda MR766 and the 2013 French Polynesia HPF2013, representing the African and the Asian lineage respectively. The cells were treated with decreasing concentrations of POMs before, during and after infection, in order to use a complete protection assay. As shown in Table 2, all three POMs were active against both ZIKV strains with half maximal effective concentrations (EC_{50}s) ranging from 0.63 to 2.52 μM. Moreover, in order to assess the specificity of the anti-ZIKV activity of the POMs, they were tested against the human rotavirus (HRoV), an unrelated RNA virus belonging to the Reoviridae family. Interestingly, we did not observe any inhibition. Next, to exclude the possibility that this antiviral activity was due to a cytotoxic effect of the POMs, viability assays were carried out on uninfected cells, challenged with the compounds under the same conditions as the virus inhibition assays. The CC_{50}s were different for all the three POMs (**TeW_6** CC_{50} = 210.1 μM, **$TiW_{11}Co$** CC_{50} = 97.08 μM, **Ti_2PW_{10}** CC_{50} > 225 μM), and demonstrated that they are not toxic at the concentrations used in the antiviral assays. The Selectivity Index (SI) of **Ti_2PW_{10}** was the most favorable one, so we decided to concentrate our research on the study of the mechanism of action of this polyanion. All the experiments were performed with the two Zika virus strains used for the initial screening. We first investigated whether the antiviral activity of **Ti_2PW_{10}** was exerted via direct inactivation of the viral particles. The ZIKV particles were incubated with a concentration of **Ti_2PW_{10}** that reduces almost completely the virus infection (EC_{90}) and then the viral titer was determined at high dilutions at which the polyanion was no longer active when added to cells. As depicted in Fig. 1A, there was no significant difference between the titer of treated virus and the titer of untreated control, demonstrating that **Ti_2PW_{10}** is not able to impair extracellular viral particles. Having excluded the viral particle as the target of the antiviral activity of **Ti_2PW_{10}**, further experiments were performed to investigate whether this polyanion acted directly on cells or on essential steps of the ZIKV replicative cycle. Vero cells were pretreated with decreasing dilutions of the polyanion for 2 h before virus infection; as reported in Fig. 1B, the infection of both ZIKV strains was not inhibited even at the highest tested concentration. Hence, we explored the possibility that **Ti_2PW_{10}** treatment could affect the early steps of the ZIKV replicative cycle. Binding assays were performed allowing the virus to bind host cell surface in the presence of a high concentration of **Ti_2PW_{10}**. The results (Fig. 2A) demonstrated that the treatment did not significantly reduce ($p > 0.05$) the titer of viral particles bound to the cell surface, thus suggesting that the inhibition occurs at a post-binding stage. To verify this hypothesis, we treated cells immediately after virus attachment, i.e. during virus entry into the host cell. In this case (Fig. 2B), we observed a marked antiviral activity of **Ti_2PW_{10}** against both, MR766 and HPF2013, ZIKV strains (EC_{50} = 1.11 and 1.25 μM respectively). To exclude an additional antiviral action of **Ti_2PW_{10}** on the last steps of the ZIKV replicative cycle, we executed focus reduction assays adding the polyanion to cells immediately after virus entry into the host cell (post-entry assay). We stopped the treatment at 24 h post-infection, i.e. at the end of the first replicative cycle, in order to avoid inhibition of the entry step of the upcoming viral progeny. As shown in Fig. 2C, the post-entry treatment did not reduce virus infectivity, suggesting that only the entry step is

Table 2
Antiviral activity of TeW_6, $TiW_{11}Co$ and Ti_2PW_{10}.

Compound	Virus	EC_{50} (μM) (95% CI)	EC_{90}(μM) (95% CI)	CC_{50} (μM) (95% CI)	SI
TeW_6	MR766	2.52 (1.87–3.39)	9.47 (4.41–20.35)	210.1 (161.3–273.6)	83.37
	HPF2013	0.71 (0.53–0.96)	6.12 (3.29–11.39)	210.1 (161.3–273.6)	295.91
	HRoV	n.a.	n.a.	> 75	–
$TiW_{11}Co$	MR766	1.04 (0.80–1.35)	5.19 (2.87–9.38)	97.08 (51.36–183.5)	93.34
	HPF2013	0.70 (0.57–0.87)	1.41 (1.02–1.94)	97.08 (51.36–183.5)	138.68
	HRoV	n.a.	n.a.	> 75	–
Ti_2PW_{10}	MR766	0.63 (0.51–0.78)	3.51 (2.19–5.63)	> 225	> 357.14
	HPF2013	0.70 (0.59–0.84)	2.78 (1.82–4.25)	> 225	> 321.42
	HRoV	n.a.	n.a.	> 75	–

EC_{50}: half maximal effective concentration; EC_{90}: 90% effective concentration; CC_{50}: half maximal cytotoxic concentration; SI: selectivity index; n.a.: not assessable; CI: confidence interval.

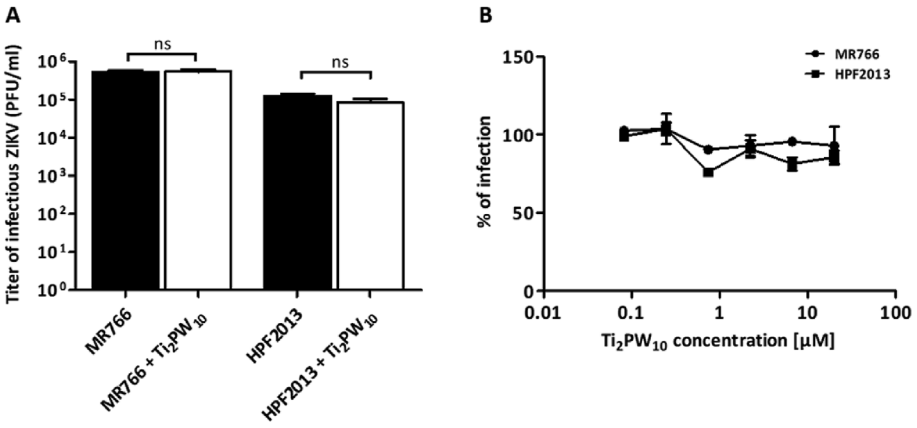

Fig. 1. Ti_2PW_{10} does not impair extracellular ZIKV particles and the cells pre-treatment does not affect viral infection. Panel **A** shows the evaluation of the virucidal effect of **Ti_2PW_{10}** on infectious ZIKV particles. Approximately 10^5 PFU of ZIKV (MR766 or HPF2013) plus EC_{90} of **Ti_2PW_{10}** were added to MEM and mixed in a total volume of 100 μL. The mixture was incubated for 2 h at 37 °C then diluted serially to the non-inhibitory concentration of the test polyanion; the residual viral infectivity was determined by viral plaque assay. Panel **B** displays the effect of cells pre-treatment with **Ti_2PW_{10}**. Vero cells were pre-treated with serial dilutions of **Ti_2PW_{10}** for 2 h before infection. After washing, cells were infected with ZIKV and the number of viral plaques was evaluated after 72 h. In panels A, the viral titers are expressed as PFU/ml and are shown as mean plus SEM for three independent experiments. In panels B, the number of viral plaques in the treated samples is expressed as a percentage of the untreated control and each point represents mean and SEM for three independent experiments. Experimental details are described in the Supplementary data file.

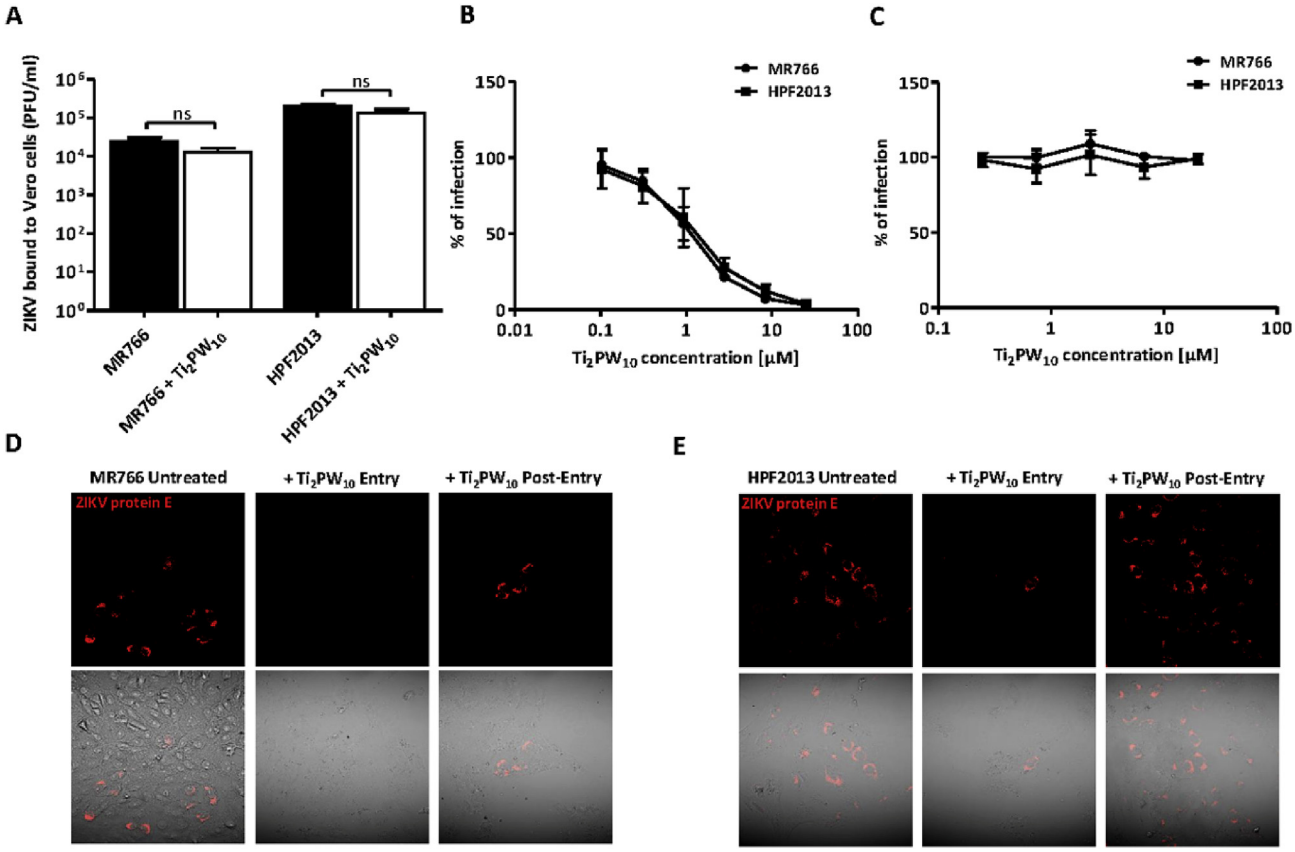

Fig. 2. Ti_2PW_{10} hampers the entry process of ZIKV into the host cell. In the binding assay (**2A**), ZIKV particles (MR766 or HPF2013, MOI = 3) were allowed to attach to cells in presence of **Ti_2PW_{10}** (EC_{90}) for 2 h on ice. Cells were then washed to remove the unbound virus and subsequently subjected to three rounds of freeze-thawing to release bound virus. The lysate was clarified and the cell-bound virus titer was determined by viral plaque assay. Here, the viral titers are expressed as PFU/ml and are shown as mean plus SEM for three independent experiments. For the entry assay (**2B**), ZIKV (MR766 or HPF2013) was adsorbed for 2 h at 4 °C on pre-chilled Vero cells. After the removal of the unbound virus, the temperature was shifted to 37 °C to allow the entry of pre-bound virus in presence of serial dilutions of **Ti_2PW_{10}**. Subsequently, unpenetrated virus was inactivated with an incubation with citrate buffer followed by 3 washes. The number of viral plaques was evaluated after 72 h. For the post-entry assay (**2C**), the same protocol of the entry assay was performed, but adding the polyanion after the incubation with citrate buffer for 24 h. The number of infected cells was assessed by indirect immunostaining after 24 h, in order to avoid the inhibition of the entry step of the upcoming viral progeny. In panels B, C, the number of viral plaques or infected cells in the treated samples is expressed as a percentage of the untreated control and each point represents mean and SEM for three independent experiments. In Fig. **2D** (MR766) and **2E** (HPF2013), the entry and the post-entry assays were performed with a concentration of **Ti_2PW_{10}** corresponding to EC_{99}. After 30 h of infection, cells were fixed and subjected to immunofluorescence. The ZIKV protein E is visualized in red. All experimental details are described in the Supplementary data file.

targeted by **Ti_2PW_{10}**. To confirm the inhibition of the ZIKV entry step, immunofluorescence experiments were performed by adding the polyanion (EC_{99}) during the virus entry step or immediately after the entry phase (post-entry). As reported in Fig. 2D (experiments with MR766) and Fig. 2E (experiments with HPF2013), it was possible to detect a strong red signal of ZIKV protein E only in the untreated and in the post-entry treated samples. The number of red infected cells in the post-entry treated samples was comparable to the one of the untreated control. On

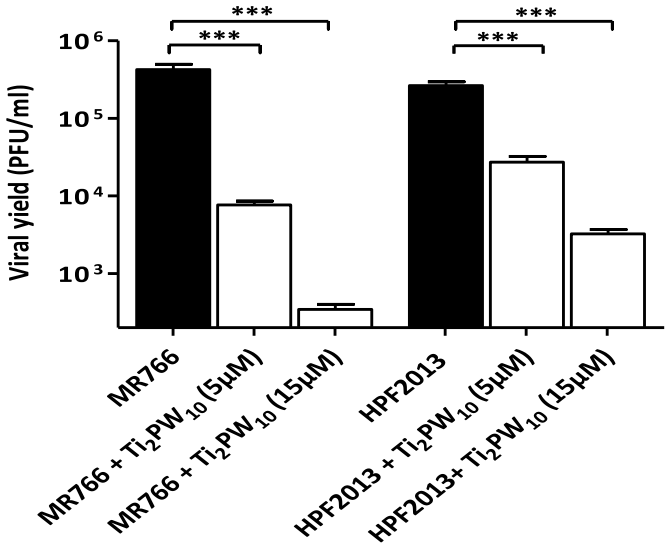

Fig. 3. Ti_2PW_{10} reduces ZIKV progeny production. To test the ability of **Ti_2PW_{10}** compound to inhibit multiple cycles of ZIKV replication, Vero cells were treated and infected with a mixture of Ti_2PW_{10} (5 μM or 15 μM) and ZIKV (MR766 or HPF2013, MOI = 0.001) for 2 h at 37 °C. The virus inoculum was then removed and cells were incubated with medium containing the compound (5 μM or 15 μM) until control cultures displayed extensive cytopathology. Supernatants were clarified and cell-free virus infectivity titers were determined by the plaque assay. The viral titers are expressed PFU/ml and are shown as mean plus SEM for three independent experiments. (***$P_{Tstud} < 0.001$).

the contrary, the number of infected cells in the entry-treated samples was considerably reduced. All together these data indicate that the entry step is the target of the **Ti_2PW_{10}** antiviral activity. Finally, to complete the *in vitro* analysis of the antiviral potential of **Ti_2PW_{10}** against ZIKV strains, virus yield reduction assays were performed by treating cells during and after infection and allowing multiple cycles of viral replication to occur before measuring the production of infectious viruses. The results (Fig. 3) demonstrated that **Ti_2PW_{10}** significantly reduces the viral progeny production of both ZIKV strains ($p < 0.001$).

Previously, researchers focused on the antiviral properties of POMs because they are generally nontoxic to normal cells. Indeed, several studies reported the broad spectrum antiviral activities of POMs against different types of respiratory-viruses, as RSV, FluV A, FluV B, PfluV and SARS (Barnard et al., 1997; Shigeta et al., 2006), against HCV and DENV (Shigeta et al., 2003, 2006; Qi et al., 2013), belonging to the same family of ZIKV, and against others, as HIV, HSV-1, HSV-2 and HBV (Rhule et al., 1998; Shigeta et al., 2003; Wang et al., 2014). Herein, we showed that three heteropolytungstates, never tested before as antiviral agents, are endowed with a strong antiviral activity against ZIKV and we demonstrated their good biocompatibility. For the first time, POMs have been tested against two ZIKV strains and we can now include ZIKV in the list of pathogens targeted by the wide spectrum of action of POMs. Of note, we did not observe any inhibition against the human rotavirus, a taxonomically unrelated RNA virus. All together these results indicate that **TeW_6**, **$TiW_{11}Co$** and **Ti_2PW_{10}** exert a specific and not strain-restricted anti-ZIKV effect. In future experiments, we will investigate the antiviral action of **TeW_6**, **$TiW_{11}Co$** and **Ti_2PW_{10}** against other RNA and DNA viruses.

Some other POMs have already been investigated for their mechanism of action, which commonly depends on their shape, size and composition. Various studies reported on the inhibition of the early steps of an infection: for instance, Shigeta et al. (2003), demonstrated that the tri-vanadium-containing sandwich-type polyanion $[(VO)_3(SbW_9O_{33})_2]^{11-}$ affects the binding of HIV to the cell membrane and the syncytium formation between HIV-infected and uninfected cells; another biochemical study (Wang et al., 2014), reports that the ability of the tri-niobium-containing Keggin ion $[SiW_9Nb_3O_{40}]^{7-}$ to prevent the binding and fusion process of different viruses is mainly due to its localization on the cell surface; finally, Barnard et al. (1997), indicate the alteration of the attachment step as the primary mode of RSV inhibition by POMs of several structural classes. Consistent with these findings, we demonstrated that **Ti_2PW_{10}** acts as inhibitor of the entry process of ZIKV into the host cell. By contrast, no inhibition was observed at the binding stage. Further experiments are necessary to identify the cellular localization of this polyanion and to clarify its molecular mechanism of action.

In conclusion, we have discovered that the Keggin-type POM **Ti_2PW_{10}** inhibits ZIKV infection by hampering the entry process of the virus into the host cell. Since specific antivirals against ZIKV are not available, this polyanion could be a good starting point for the development of novel and efficient antiviral pharmaceuticals.

Declaration of interest

None.

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Abbreviations

ZIKV	zika virus
HRoV	human rotavirus
RSV	respiratory syncytial virus
FluV A	influenza virus type A
FluV B	influenza virus type B
PfluV	parainfluenza virus
SARS	severe acute respiratory syndrome
HCV	hepatitis C virus
DENV	dengue virus
HIV	human immunodeficiency virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HBV	hepatitis B virus
POMs	polyoxometalates
EC_{50}	half maximal effective concentration
EC_{90}	90% effective concentration
CC_{50}	half maximal cytotoxic concentration
SI	selectivity index
n.a.	not assessable
CI	confidence interval
PFU	plaque forming unit
PFU/ml	plaque forming unit per ml

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.01.005.

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