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STUDIES ON THE ANTIVIRAL ACTIVITY OF INORGANIC HETEROPOLYANIONS AGAINST SEMLIKI FOREST VIRUS *IN VIVO* AND VACCINIA VIRUS *IN VITRO*

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Summary. — Seven new inorganic heteropolyanions were tested for their antiviral activity. One of these, sodium 12-tungstoborate, was found to protect mice from Semliki forest virus and mouse embryo fibroblast monolayers from vaccinia virus infections. In mice these heteropolyanions exhibited no synergistic antiviral activity with the interferon inducing mycoviral double-stranded RNA.

Key words: *inorganic heteropolyanions*; *sodium 12-tungstoborate*; *virus infection*; *interferon inducer*

Introduction

Heteropolyanions constitute a distinct category of inorganic compounds (Zonnvijlle *et al.*, 1982). Some of them are reported to have antiviral and anticancer activities. Mukherjee (1965) first described the effect of a complex preparation of phosphotungstic acid and caffeine in patients with intestinal tract cancer. Bonissol *et al.* (1972) showed that silicotungstate interferes with rubella virus multiplication *in vitro* which could not be attributed to a cytotoxic effect of the compound or to a direct inactivation of the virus. The compound was also not found to prevent virus adsorption at the cell surface. The antiviral spectrum of silicotungstate was later extended to murine leukaemia and sarcoma viruses (Haapala *et al.*, 1973). It was further shown that silicotungstate is a potent inhibitor of RNA-dependent DNA polymerase of RNA tumour viruses, DNA and RNA polymerases of *E. coli* and DNA polymerase extracted from mouse 3T3 cells (Haapala *et al.*, 1973).

Jasmin *et al.*, (1974) reported the antiviral activity of another heteropolyanion, 5-tungsto-2-antimoniate, which protected mice against Friend virus and plasma-variant-induced leukaemias. It could also delay the appearance of tumours in newborn mice inoculated with Moloney murine sarcoma virus. Ammonium-5-tungsto-2-antimoniate was shown to have antiviral activity against encephalomyocarditis and vesicular stomatitis viruses in mice (Wer-

ner *et al.*, 1976). This polyanion was also found to inhibit the *in vitro* multiplication of rabies virus (Tsiang *et al.*, 1978).

The inhibitory effect of heteropolyanions on sugar cane mosaic virus was reported by Srivastava *et al.* (1978). Later the inhibitory effect of several heteropolyanions on tobacco mosaic virus (Sharma *et al.*, 1984*a*) and potato virus × multiplication (Sharma *et al.*, 1984*b*) were described. This communication deals with the *in vivo* and *in vitro* antiviral effects of seven new heteropolyanions; of these one compound, sodium 12-tungstoborate, showed a promising antiviral activity.

Materials and Methods

Heteropolyanions and their preparation. 12-tungsto zincic acid, H_6 Zn $W_{12}O_{40} \cdot H_2O$, was synthesized using the method of Brown and Mair (1958). Potassium-13-vanado manganate (IV), K_7 Mn $V_{13}O_{38} \cdot 18\ H_2O$ and Potassium-13-vanado nickelate (IV), K_7 Ni $V_{13}O_{38} \cdot 16\ H_2O$ were prepared following the method of Flynn and Pope (1970), Potassium 11-tungsto cobalto silicate K_5 Si Co $W_{11}O_{40}H_2 \cdot 3H_2O$, Potassium-11-tungsto phospho nickelate (IV), K_5 P Ni $W_{11}O_{40}H_2$ $14\ H_2O$ and Ammonium-11-tungsto discobaltate, $(NH_4)_6$ $Co^{III}Co^{II}W_{11}O_{40}H_2 \cdot 13\ H_2O$ were made according to the procedure of Weakley and Malik (1967) and sodium-12-tungsto borate, Na_5 B $W_{12}O_{40} \cdot 6\ H_2O$ was synthesized according to the method of Brauer (1965).

Animals. Swiss albino mice weighing 14—15 g were randomly bred and housed at 25 °C. Each experimental group consisted of both male and female animals in the ratio 1 : 1. The animals were fed with standard pellet diet (Hindustan Lever Ltd., Bombay, India) and had access to water ad libitum.

Viruses. Semliki forest virus (Smithburn and Haddow strain), originally obtained from ATCC, was used in the mouse-protection experiments. The virus has been maintained in our laboratory by intracerebral (i.c.) passage in adult swiss mice weighing 14—15 g. A 20% homogenate of infected mouse brain prepared in phosphate buffer saline (PBS) pH 7.2 containing 1% bovine serum albumin (Sigma, U.S.A.) and stored at −20 °C after lyophilisation served as stock virus. The virus was titrated before use by subcutaneous inoculation in adult mice and the LD_{50} was calculated according to Reed and Muench. Vaccinia virus (IHD strain), obtained from ATCC and maintained by i.c. passage in adult swiss mice (14—15 g), was used to infect mouse embryo fibroblast monolayers. A 20 % infected mouse brain homogenate prepared in PBS pH 7.2 containing 1% bovine serum albumin and stored at −20 °C after lyophilisation served as virus stock, it was diluted to give about 30 plaque forming units per 0.05 ml of the inoculum in a microplate well.

Toxicity assay. The compounds, dissolved in sterile PBS pH 7.2, were injected intraperitoneally into groups of adult mice (6 animals per group) at the rate of 100 mg/kg as a single dose. The animals were observed for 7 days for mortality or other adverse effects. All the compounds except two were safe and produced no visible toxicity. The animals which were given Potassium-13-vanado nickelate and Potassium-13-vanado manganate died within 24 hr. These two compounds were retested for toxicity; the dose of 20 mg/kg was found safe.

The *in vitro* toxicity was assessed from the effect of various concentrations of the compounds on treated mouse fibroblast monolayers compared to untreated control. The concentration that brought about a 50% destruction of cell monolayer was noted.

In vivo antiviral assay. The compounds were injected intraperitoneally in a single dose or in multiple doses at various intervals before and/or after challenge with Semliki forest virus (SFV). Animals were observed for 20 days post infection (p.i.) to record the appearance of symptoms and deaths. The significance of treatment was statistically analysed by the test of proportions (Hill, 1966).

In vitro antiviral assay. The *in vitro* antiviral activity was tested by plaque reduction according to Wagner (1961). Briefly, primary mouse embryo fibroblasts were grown to confluency in 96-well microtitre plates (Flow Lab., U.S.A.) in medium 199 (Flow Lab.) containig 10% goat serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Monolayers which were complete by 24 hr of incubation at 37 °C in 5% CO_2 atmosphere were challenged with vaccinia virus. After

adsorption for 60 min, the monolayers were washed and further incubated in maintenance medium (medium 199 plus 2% goat serum and antibiotics) for 36 hr. Plaques were counted after staining with 0.1% crystal violet. Various amounts of test compounds were incorporated in the maintenance medium which was allowed to remain in contact with cells throughout the post-challenge incubation period. Each dilution of the compound was tested in triplicate and the antiviral effect of the compound was judged by the number of plaques appearing in the treated monolayer compared to the untreated control.

Results

The data summarized in Table 1 show that sodium-12-tungsto borate was the most active among the heteropolyanions tested in mice against a mild SFV infection (2 LD_{50}). All animals treated with this compound survived infection while 90% of the untreated control mice succumbed (p value $<$ 0.01). Potassium-11-tungsto cobalto silicate and potassium-11-tungsto phospho nickelate protected 66% (p value $<$ 0.05) and 33%, respectively. Potassium-13-vanado nickelate and potassium-13-vanado manganate were highly toxic while non-toxic doses had no antiviral activity.

Table 1. Antiviral activity of some inorganic heteropolyanions in mice against challenge Semliki forest virus infection

Compound*	Treatment and dose schedule		Protection** (%)	p value
	-1 day	"0" day		
12-tungsto zincic acid	100 mg/kg	100 mg/kg	0.0	
Potassium-13-vanado nickelate	20 mg/kg	20 mg/kg	0.0	
Potassium-11-tungsto cobalto silicate	100 mg/kg	100 mg/kg	66.6	<0.05
Potassium-11-tungsto phospho nickelate	100 mg/kg	100 mg/kg	33.0	
Potassium-13-vanado manganate	20 mg/kg	20 mg/kg	0.0	
Ammonium-11-tungsto dicobaltate	100 mg/kg	100 mg/kg	10.0	
Sodium-12-tungsto borate	100 mg/kg	100 mg/kg	100.0	<0.01
Control	Buffer	Buffer	10.0	

* Compounds were dissolved in sterile phosphate buffered saline (PBS) pH 7.2 and were given intraperitoneally in 0.5 ml quantity.

** Against challenge with 2 LD_{50} of SFV given subcutaneously on "0" day.

Table 2. Antiviral activity of sodium-12-tungsto borate and potassium-11-tungsto cobalto silicate in mice against challenge with Semliki forest virus infection

Compound	Treatment schedule −1 day	Treatment schedule "0" day	Virus dose	Protection (%)	p value
Sodium-12-tungsto borate	100 mg/kg	PBS	10 LD_{50}	50.0	<0.05
Sodium-12-tungsto borate	PBS	100 mg/kg	10 LD_{50}	20.0	
Control	PBS	PBS	10 LD_{50}	0.0	
Sodium-12-tungsto borate	100 mg/kg	PBS	50 LD_{50}	40.0	<0.05
Sodium-12-tungsto borate	PBS	100 mg/kg	50 LD_{50}	10.0	
Control	PBS	PBS	50 LD_{50}	0.0	
Sodium-12-tungsto borate	100 mg/kg	PBS	100 LD_{50}	40.0	<0.05
Sodium-12-tungsto borate	PBS	100 mg/kg	100 LD_{50}	20.0	
Control	PBS	PBS	100 LD_{50}	0.0	
Potassium-11-tungsto cobalto silicate	100 mg/kg	PBS	10 LD_{50}	20.0	
Potassium-11-tungsto cobalto silicate	PBS	100 mg/kg	10 LD_{50}	10.0	
Potassium-11-tungsto cobalto silicate	100 mg/kg	100 mg/kg	10 LD_{50}	20.0	
Control	PBS	PBS	10 LD_{50}	0.0	

PBS — Phosphate buffered saline pH 7.2

Table 2 depicts the effect of sodium-12-tungsto borate and potassium-11-tungsto cobalto silicate administrations to mice challenged with a more lethal dose of SFV (10—100 LD_{50}). A single dose of sodium-12-tungsto borate (100 mg/kg) given 24 hr before challenge could protect 50% of infected animals challenged with 10 LD_{50} of SFV (p value < 0.05). The protection rate was 40% when the virus dose was further increased to 50 or 100 LD_{50}. The rate of protection could not be raised by administering a second dose of the compound simultaneously with virus challenge. These studies also showed that the compound was most effective when given before virus challenge; the antiviral effect was weaker when it was given at the time of challenge. Similarly, potassium-11-tungsto cobalto silicate could protect only 20% of the animals infected with 10 LD_{50} of SFV when a single dose (100 mg/kg) was given one day before challenge. A repeated treatment simultaneously with challenge could not enhance the protection rate beyond 20%. The results shown in Table 3 further confirm that sodium-12-tungsto borate was effective when given one day before challenge and there was no additional advantage of its continued administration after challenge. The data also show the ineffecti-

Table 3. Effect of treatment with sodium-12-tungsto borate and potassium-11-tungsto cobalto silicate in the course of Semliki forest virus infection in mice: repeated administration

Compound	Treatment schedule			Protection* (%)	p value
	−1 day	"0" day	−1 day		
Sodium-12-tungsto borate	100 mg/kg	100 mg/kg	100 mg/kg	40.0	<0.01
Sodium-12-tungsto borate	PBS	100 mg/kg	100 mg/kg	20.0	<0.1
Sodium-12-tungsto borate	100 mg/kg	PBS	PBS	40.0	<0.01
Control	PBS	PBS	PBS	0.0	
Potassium-11-tungsto cobalto silicate	100 mg/kg	100 mg/kg	100 mg/kg	10.0	
Potassium-11-tungsto cobalto silicate	PBS	100 mg/kg	100 mg/kg	0.0	
Potassium-11-tungsto cobalto silicate	100 mg/kg	PBS	PBS	0.0	
Control	PBS	PBS	PBS	0.0	

* Against 100 LD_{50} of SFV challenge on day "0".

Table 4. Antiviral activity of sodium-12-tungsto borate and potassium-11-tungsto cobalto silicate injected intracerebrally into Semliki forest virus infected mice

Compound*	Dose	Protection** (%)
Sodium-12-tungsto borate	18 mg/kg	10.0
Sodium-12-tungsto borate	9 mg/kg	0.0
Sodium-12-tungsto borate	4.5 mg/kg	0.0
Potassium-11-tungsto cobalto silicate	18.0 mg/kg	0.0 (toxic)
Potassium-11-tungsto cobalto silicate	9.0 mg/kg	0.0
Potassium-11-tungsto cobalto silicate	4.5 mg/kg	0.0
Control	Buffer	0.0

* In 0.03 ml of phosphate buffered saline pH 7.2 intracerebrally immediately after subcutaneous challenge.

** Against 10 LD_{50} of SFV challenge.

Table 5. Effect of heteropolyanions on the antiviral activity of an interferon inducing mycoviral dsRNA in mice

Compounds*	Protection*** (%)
12- tungsto zincic acid + dsRNA**	0.0
Potassium-13-vanado nickelate + dsRNA	50.0
Potassium-11-tungsto cobalto silicate + dsRNA	33.0
Potassium-11-tungsto phospho nickelate + dsRNA	33.0
Potassium-13-vanado manganate + dsRNA	33.0
Ammonium-11-tungsto dicobaltate + dsRNA	50.0
Sodium-12-tungsto borate + dsRNA	50.0
PBS + dsRNA	50.0
Control (PBS)	0.0

* The compounds were given intraperitoneally one day before challenge. Potassium-13-vanado nickelate and potassium-13-vanado manganate were given at the rate of 20 mg/kg while the rest of the compounds were given at the rate of 100 mg/kg.

** Against 100 LD_{50} of SFV challenge.

*** dsRNA (double-stranded RNA) was prepared from purified mycoviruses concentrated from 9-day-old stationary cultures of the fungus *Aspergillus ochraceus* ATCC 28706. The dsRNA was administered at the rate of 0.6 mg/kg intraperitoneally 1 hr before challenge. The dsRNA induces about 3000 I.U. of interferon per ml serum in treated mice (George *et al.*, 1986).

veness of potassium-11-tungsto cobalto silicate in protecting mice challenged with high lethal doses of the virus even when the compound was administered in three successive doses — one day before, along with and one day after infection.

Table 4 summarizes the results of experiments where the compounds were administered directly into the brain (mortality of SFV infected mice is due to multiplication of the virus in the brain) at the time of subcutaneous challenge with the virus. In these studies, both sodium-12-tungsto borate and potassium-11-tungsto cobalto silicate were found ineffective. Intracerebrally, 18 mg/kg of sodium-12-tungsto borate was well tolerated by mice while the same dose of potassium-11-tungsto cobalto silicate was toxic.

The results presented in Table 5 show the effect of the heteropolyanions on SFV infected mice when given in combination with an interferon inducer (mycoviral dsRNA preparation from fungus *Aspergillus ochraceus* ATCC 28706). Here, none of the tested heteropolyanions could enhance the protective activity of dsRNA. On the contrary, 12-tungsto zincic acid totally abrogated the antiviral activity of dsRNA in mice.

Table 6 presents the results of *in vitro* experiments with the heteropolyanions against vaccinia virus infection. These tests employing the plaque reduction method showed that sodium-12-tungsto borate had maximum antiviral activity. A dose of 25 μg/ml could completely protect cells from vaccinia virus infection. It had no toxic manifestations on the monolayers at the highest tested concentration of 400 μg/ml. Both potassium-11-tungsto

Table 6. Antiviral activity of heteropolyanions in primary mouse embryo fibroblast monolayers against vaccinia virus infection

Compound	Minimum effective concentration* (μg/ml)	Cytotoxic concentration** (μg/ml)
12-tungsto zincic acid	inactive	50
Potassium-13-vanado nickelate	inactive	<10
Potassium-11-tungsto cobalto silicate	50	200
Potassium-11-tungsto phospho nickelate	50	200
Potassium-13-vanado manganate	inactive	<10
Ammonium-11-tungsto dicobaltate	100	200
Sodium-12-tungsto borate	25	>400

* The dose that can completely protect the cell monolayer from virus infection (no plaques).
** The concentration of the compound that causes 50% destruction of the cell monolayer compared to the untreated uninfected control.

cobalto silicate and potassium-11-tungsto phospho nickelate protected cells at 50 μg/ml concentration; however, a dose of 200 μg/ml in each case was toxic to the cells. The 12-tungsto zincic acid was toxic at 50 μg/ml concentration and the lower doses were antivirally inactive. Ammonium-11-tungsto dicobaltate was antivirally active at 100 μg/ml but was toxic to the cells at a dose of 200 μg/ml. Potassium-13-vanado nickelate and potassium-13-vanado manganate were highly toxic even at 10 μg/ml concentration, and hence no antiviral action of these compounds could be assessed under these conditions.

Discussion

Several inorganic heteropolyanions have been reported to show inhibitory effect *in vivo* and *in vitro* against plant and animal virus infections (Bonissol *et al.*, 1972; Jasmin *et al.*, 1974; Werner *et al.*, 1976; Tsiang *et al.*, 1978; Srivastava *et al.*, 1978; Sharma *et al.*, 1984*a*, *b*). One interesting aspect of the heteropolyanions is that they exhibit antiviral properties at non-cytotoxic doses (Tsiang *et al.*, 1978). This means that these compounds have the ability to interfere with some specific functions of the virus, particularly certain steps in its multiplication cycle, without significantly affecting the host cell metabolism. These inorganic polyanions are known to differ in their antiviral spectrum. Thus, ammonium-5-tungsto-2-antimoniate could protect mice from vesicular stomatitis virus and encephalomyocarditis virus infections but not from Semliki forest virus infection (Werner *et al.*, 1976). Similarly, while this compound was inhibitory to rabies virus multiplication *in vitro*, another polyanion, silico tungstate, was not effective though it is known to be inhibitory to other RNA viruses like vesicular stomatitis virus (Raynaud

et al., 1971; Raybaud *et al.*, 1972). This difference in the antiviral spectrum of heteropolyanions may be due to differences in their mode of action and the stage of virus multiplication responding to it. This may emphasize the need to synthesize and assay newer heteropolyanions.

Our experiments on these lines with seven new heteropolyanions in mice against SFV infection have picked out sodium-12-tungsto borate as the safest and most active heteropolyanion tested. The *in vivo* results showed that sodium-12-tungsto borate is active when given one day before challenge. It was very effective against mild infections, but a decline (up to 60%) in its activity was observed with increasing doses of the challenge virus. Repeated administration of the compound could not stop this decline in the protection rate. Sodium-12-tungsto borate was not effective against SFV when administered intracerebrally. This observation and the finding that the compound was significantly active only when given sufficiently before infection suggests an indirect effect on virus multiplication, perhaps through modulation of the defense mechanisms of the host. However, the *in vitro* activity of this polyanion also indicates a more direct interference with the multiplication of the virus.

In our second trial, we tested the heteropolyanions to assess their ability to enhance the antiviral activity of interferon inducers. Such an action was suggested by the work of Werner *et al.* (1976) who reported synergistic activity of ammonium-5-tungsto-2-antimoniate with mouse interferon. We failed to observe any such synergistic activity with these heteropolyanions when tested in SFV infected mice in combination with a mycoviral dsRNA preparation having the ability to induce interferon and antiviral state. On the contrary, one of the heteropolyanions, namely 12-tungsto zincic acid totally abolished the antiviral activity of mycoviral dsRNA in mice. As to how this compound interferes with interferon activity should be further elucidated.

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