VIRUS-INHIBITORY EFFECT OF A YEAST RNA – TILORONE MOLECULAR COMPLEX IN CELL CULTURES

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Summary. – The virus-inhibitory activity of a molecular complex (MC) of tilorone and yeast RNA was studied *in vitro* on three virus-cell systems: vesicular stomatitis virus (VSV) – murine fibroblast L929 cells, Venezuelan equine encephalitis virus (VEEV) – swine embryo kidney (SEK) cells and encephalomyocarditis virus (EMCV) – established piglet testicular (EPT) cells. In all these systems the MC exerted an antiviral effect similar to that of polynucleotide interferon (IFN) inducers such as poly(I)-poly(C), larifan and ridostin. The antiviral effect of the MC was similar when the compound was applied before or after virus adsorption to cells. The MC may be regarded as a perspective antiviral agent of common use.

Key words: yeast RNA; tilorone, molecular complex; antiviral effect, interferon; VSV, VEEV; EMC virus, cell cultures

Introduction

MCs formed during interactions of single-stranded yeast RNA and tilorone have been found to be able to cause induction of type I IFNs (a/b-IFNs) both ***in vivo*** and *in vitro* (Karpov and Zholobak, 1995; 1996). These MCs have been also demonstrated to inhibit the human immunodeficiency virus 1 (HIV-1) replication *in vitro* (Karpov *et al.*, 1997). The antiviral resistance developed in cells due to IFN induction is thought to be one of the most important properties of any IFN inducer. This triggering effect of IFN inducers have led to their use as antiviral drugs (Kleinshmidt, 1972; DeClercq, 1974).

In this study we compared the antiviral effect of a MC of yeast ssRNA and tilorone to those of its individual components and of several standard IFN inducers *in vitro* on three different virus-cell systems.

Materials and Methods

Cells Lines of EPT, SEK and murine fibroblast L929 cells were obtained from the Research Institute of Veterinary Medicine, Ukrainian Academy of Agriculture, Kiev, Ukraine EPT and SEK cells were cultivated in Medium 199 supplemented with 10% of calf embryo serum (Sigma) and 50 μg/ml kanamycin (Virion, Russian Federation) while L929 cells were cultivated in Eagle's Minimum Essential medium supplemented with the same components as above plus 300 μg/ml glutamine by standard techniques (Griffin, 1986).

Viruses VSV was grown in L929 cells to titers of about 10^6 ID_{50}/0.1 ml. VEEV strain 230 was grown in SEK cells to titers of about 10^8 ID_{50}/0.1 ml. EMC virus (EMCV) was grown in EPT cells. The infected cells were grown in the same media as those mentioned above except without any serum.

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Abbreviations: EMCV = encephalomyocarditis virus; EPT = established cell line of piglet testicles, IFN = interferon, MC = molecular complex; p.i. = post infection; SEK = established cell line of swine embryo kidney; VEEV = Venezuelan equine encephalitis virus; VSV = vesicular stomatitis virus

Table 1. Preventive effect of the MC, its components and standard IFN inducers on *in vitro* virus reproduction

Virus/cell system	Compound	Dose (μg/ml)	Virus titer (-log TCID50)	Drop of virus titer (D log TCID50)
VEEV/SEK	μC	25	3 86	3 01
	Tilorone	2.5	6.46	0.41
	Yeast RNA	22.5	6 85	0 02*
	Poly(I)-poly(C)	25	3 72	3 15
	Larifan	25	3.86	3.01
	Ridostin	25	3.84	3.03
	Control	–	6.87	
EMCV/EPT	μC	25	3.87	2 94
	Tilorone	2 5	5 95	0.86
	Yeast RNA	22 5	6 80	0.01*
	Poly(I)-poly(C)	25	3 82	2 99
	Larifan	25	3 88	2 93
	Ridostin	25	3 88	2 93
	Control	–	6 81	
VSV/L929	μC	25	2.93	2 98
	Tilorone	2 5	5 60	0 31
	Yeast RNA	22 5	5 88	0 03*
	Poly(I)-poly(C)	25	2 69	3.22
	Latifan	25	2 87	3.04
	Ridostin	25	2 88	3.03
	Control	–	5 91	

*Values marked by asterisk represent insignificant drops of virus titers. Values not marked by asterisk represent significant drops of virus titers

The MC was prepared from yeast RNA (Biochemreagent, Latvia) and tilorone hydrochloride (Sigma, USA) as described earlier (Karpov and Zholobak, 1995).

Standard IFN inducers Larifan, a therapeutic form of the phage f2 dsRNA received from the A Kirchenstain Institute of Microbiology, Vilnius, Latvia, ridostin, a commercial *Saccharomyces cerevisiae* dsRNA (Vector, Russian Federation), and a commercial poly(I)-poly(C) (Calbiochem) were used.

Assay of antiviral effect of compounds was based on evaluation of virus yields. Two experimental approaches were used. The tested compounds properly diluted in appropriate media were added to cell monolayers grown in 96-well microtiter plates (Falcon) at 37°C in 5% CO_2 (0.2 ml per well) either 6 hrs before ("preventive effect") or immediately after virus infection ("therapeutic effect"). Optimal concentrations of compoundes, estimated previously, were used. Triplicate wells were employed as a rule. The multiplicity of infection was 0.1 ID_{50}/cell for all viruses tested. Virus yields (infectious virus titers) were estimated 72 hrs post infection (p.i.).

Virus titration was performed in a standard way on cell monolayers in microtiter plate wells in quadruplicates on the basis of cytopathic effect (CPE) read 72 hrs p.i. For each virus was used the same type of cells as that employed for assay of antiviral effect.

Statistical evaluation of results was made by Student t-test. As significant were regarded differences with $P \leq 0.05$

Results and Discussion

Preventive antiviral effect

The comparative study of antiviral effect of the MC, individual components of the MC and several standard polynucleotide IFN inducers on *in vitro* virus reproduction was carried out by estimating virus yields (infectious virus titers). Doses of compounds tested as well as the time of their application in the case of the preventive effect (6 hrs before infection) were chosen to enable maximal IFN induction according to earlier results (Karpov and Zholobak, 1995).

The results obtained in our experiments concerning the preventive antiviral effect of the compounds tested are given in Table 1.

It is evident the cell culture pretreatment with the MC led to significant virus growth inhibition. In comparison with controls virus titers (-log $TCID_{50}$) dropped by very similar values in the three virus-cell systems, namely 3.01, 2.94 and 2.98. The effect of the MC was similar also to those of the polynucleotide IFN inducers tested, namely poly(I)-poly(C), larifan and ridostin. The similarities mentioned above mean that the small differences shown in Table 1 were not significant.

In testing the effect of the MC in comparison to its individual components, tilorone and yeast RNA (Table 1),

Table 2. Therapeutic effect of the MC, its components, and standard IFN inducers on *in vitro* virus reproduction

Virus/cell system	Compound	Dose (mg/ml)*	Virus titer (-log TC ID50)	Drop of virus titer (D logTCID50)
VEEV/SEK	μC	25	3 69	3.17
	Tilorone	2 5	5 86	1 00
	Yeast RNA	22 5	6 84	0.02*
	Poly(I)-poly(C)	25	4.03	2 83
	Larifan	25	4 16	2.70
	Ridostin	25	4 15	2.71
	Control	–	6.86	
EMCV/EPT	μC	25	3.64	3.17
	Tilorone	2 5	5.75	1 06
	Yeast RNA	22 5	6 79	0 02*
	Poly(I)-poly(C)	25	4 11	2.70
	Larifan	25	4 13	2 68
	Ridostin	25	4 14	2 67
	Control	–	6 81	
VSV/L929	μC	25	2.70	3.22
	Tilorone	2 5	4 89	0 03
	Yeast RNA	22 5	5.91	0 01*
	Poly(I)-poly(C)	25	2 97	2.95
	Latifan	25	3.16	2 76
	Ridostin	25	3 16	2 76
	Control	–	5 92	

For the legend see Table 1

the effect of tilorone was small in all the virus-cell systems tested; the titer drops were namely 0.41, 0,86 and 0.31. On the other hand the effect of yeast RNA was none, the drops were 0.02, 0.01 and 0.03. Analyzing possible additive or synergistic effect of the components of the MC it is obvious that the sum of titer drops caused by individual components was in each virus-cell system lower than the titer drops caused by the MC (3.01, 2.94 and 2.98). From these data it is clear that the components exerted synergism in the MC. A similar conclusion was earlier drawn for the MC in induction of IFN (Karpov and Zholobak, 1995).

Therapeutic antiviral effect

As shown in Table 2, the MC caused a marked drop of virus titers (3.17, 3.17 and 3.22) in all virus-cell systems tested. In comparison to its preventive antiviral effect (3.01, 2.94 and 2.98) the therapeutic effect of the MC was a little higher but not significantly. The antiviral therapeutic MC effect might be due partly to the action of the MC-induced α/β-IFN and IFN-induced antiviral proteins (Baglioni, 1984; Staeheli, 1990) and partly to an IFN-independent antiviral effect of tilorone as the MC component. The latter effect might be higher due to the prolonged MC degradation by nucleases, the tilorone being released gradually from the complex. So if we use the MC according to our prophylactic scheme the majority of the MC are already degraded at the time of virus inoculation. So a significant number of tilorone molecules come out of cells due to cell metabolism and its antiviral effect might be in this case lower.

In comparing the antiviral therapeutic effect of the MC to those of its components a significant effect of tilorone was found in all the three virus-cell systems (the drops of virus titers were 1.00, 1.06 and 1.03) in contrast to absence of any antiviral preventive effect. As tilorone has been previously shown to possess no IFN-inducing activity *in vitro* (Kleinshmidt, 1972; DeClercq, 1974), the observed antiviral therapeutic effect might be due to some IFN-independent mechanism (Chandra *et al.*, 1972; Giron *et al.*, 1972; DeClercq, 1974).

No antiviral therapeutic effect was observed with yeast RNA similarly to the preventive scheme. Thus it may be concluded that also in the case of use of the therapeutic scheme a synergistic behavior of the MC components was observed.

The standard IFN inducers exerted also a marked inhibitory effect (the drops of virus titers were from 2.68 to 2.95), however, in comparison to their preventive effect the therapeutic effect was by about 10% lower, but not significantly.

It has been postulated that an *in vitro* drop of virus infectious titer by at least 1.78 log caused by a compound indicates that it is a perspective antiviral which should be investigated *in vivo* (Luria *et al.*, 1978).

References

Baglioni C (1984): The molecular mediators of interferon action. In Came PE, Carter WA (Eds). *Interferons and Their Applications*. Springer-Verlag, Berlin-Heidelberg-New York-Tokyo, pp. 153–168.

Balesina LE, Korneeva LE, Zemskov VM (1977) Comparative study of interferonogenic activity and antiviral action of the tobacco mosaic virus, tilorone, and sodium nucleinate *Acta Virol* **21**, 338–343

Chandra P, Zunino F, Gotz A (1972). Bis-DEAE-fluorenone: a specific inhibitor of DNA polymerases from RNA tumor viruses. *FEBS Lett.* **22**, 161–164.

DeClercq E (1974): Synthetic interferon inducers. *Top Curr Chem* **52**, 173–208.

Giron DJ, Schmidt JP, Pindak FF (1972): Tilorone hydrochloride. lack of correlation between interferon induction and viral protection. *Antimicrob. Agents Chemother.* **1**, 78–79

Griffin B (1989): Animal cells grafting in a culture. In Freshney RI (Eds)· *Animal Cell Culture. A Practical Approach.* IRL Press, Oxford-Washington, pp 56–107

Holper J (1969): Monolayer and suspension cell cultures In Habel K, Salzman NP (Eds): *Fundamental Techniques in Virology* Academic Press, New York-London, pp. 9–19.

Karpov AV, Zholobak Nμ (1995): Study of interferonogenic properties of yeast RNA and tilorone complexes in cell culture. *Antibiotiki i Khemoterapia* **40**, 20–23 (in Russian).

Karpov AV, Zholobak NM (1996) Production of type I interferons under the effect of yeast RNA-tilorone molecular complexes *Vopr Virusol.* **41**, 13–16 (in Russian).

Karpov AV, Antonenko SV, Barbasheva YV, Spivak NY (1997): Study of anti-HIV effect of yeast RNA-tilorone molecular complex. *Vopr Virusol* **42**, 17–19 (in Russian).

Kleinschmidt WJ (1972): Biochemistry of interferon and its inducers. *Ann. Rev Biochem* **41**, 517–539.

Luria SE, Darnell JE Jr, Baltimore D, Campbell A (1978). *General Virology*. John Wiley and Sons, Inc , New York-Santa Barbara-Chichester-Brisbane-Toronto, pp. 36–50.

Reed L, Muench H (1938) A simple method of estimating 50% endpoints. *Am J Hygiene* **27**, 493–497.

Staeheli P (1990): Interferon-induced proteins and the antiviral state *Adv. Virus Res* **38**, 147–200.