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In vitro inhibition of Chikungunya and Semliki Forest viruses replication by antiviral compounds: synergistic effect of interferon-α and ribavirin combination

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Abstract

Chikungunya virus (CHIKV) and Semliki Forest virus (SFV) were used in our laboratory to screen active antiviral compounds against viruses of the *Alphavirus* genus. Antiviral activity was estimated by the reduction of the cytopathic effect of each alphavirus on infected Vero cells and by virus titer reduction. Cytotoxicity was evaluated by determining the inhibition of Trypan blue exclusion in confluent cell cultures and by the evaluation of the inhibitory effect on cell growth. With CHIKV and SFV, the selectivity indices of human recombinant interferon-α and iota-carrageenan were much higher than that of ribavirin, which has been previously investigated for its inhibitory effect on alphavirus infections. Compared to ribavirin, 6-azauridine was more effective against CHIKV and showed a similar antiviral activity against SFV. IFN-α2b, glycyrrhizin, 6-azauridine, and ribavirin caused a concentration-dependent reduction in the virus yield with CHIKV and SFV. Moreover, the combination of IFN-α2b and ribavirin had a subsynergistic antiviral effect on these two alphaviruses and should be evaluated for the treatment of these infections.

Keywords: Chikungunya virus; Semliki Forest virus; Alphavirus; Interferon; Ribavirin; 6-Azauridine

1. Introduction

Chikungunya virus (CHIKV) and Semliki Forest virus (SFV) are arthropod-borne alphaviruses, members of the *Togaviridae* family. Alphaviruses are enveloped viruses; their genome is made up of a single RNA strand of positive polarity. They are mainly responsible for encephalitis or polyarthritis, with high level of morbidity and important economic impact in many tropical countries, such as the extensive outbreak of O'nyong-nyong fever virus (a closely related virus of CHIKV), which occurred in East Africa between 1959 and 1962 when an estimated 2 million people were affected (Haddow et al., 1960). After an absence of almost 35 years, a new minor O'nyong-nyong fever epidemic appeared in Uganda during 1996 and 1997 (Sanders et al., 1999), pointing to the importance of alphaviruses as re-emerging infectious diseases.

SFV is widely distributed in Africa and human infection is relatively common (Lundstrom, 1999). This virus was used in non-microbiological laboratories and several seroconversions have been observed, and one scientist working with SFV developed an encephalitis (Willems et al., 1979). Likewise, during an outbreak in the Central African Republic in which patients had fever, arthralgia and headache, SFV was isolated from the blood of 20 patients (Mathiot et al., 1990), indicating a potential importance of this virus as a human pathogen.

CHIKV is responsible for an acute disease in man, characterized by a triad of fever, arthralgia, and maculopapular rash (Brighton et al., 1983; McGill, 1995). In a few cases, hemorrhagic forms of the disease have been recorded in South-East Asia and India (Hammon et al., 1960; Sarkar et al., 1965). First described in the Newala province of Tanzania (Robinson, 1955), outbreaks occurred in most areas of Africa and extensive epidemics have been described in India (Jadhav et al., 1965), Thailand (Nimmannitya et al., 1969), and Vietnam (Deller and Russell, 1968). Recently, two outbreaks have been reported in Senegal in 1996 and 1997 (Thonnon et al., 1999).

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SFV and CHIKV are viruses closely related to Venezuelan equine encephalitis virus (VEEV) which is considerably more pathogenic for man. This agent induces a severe disease which may be fatal. VEEV is present in Central America and in South America. VEE epidemics have occurred at approximately 10–20-year intervals. During epidemics, attack rates vary from 13 to 93%. The incidence of encephalitis in clinically ill humans is up to 5% and the mortality up to 1%. Essentially, all deaths occur in children. During 1995, a major VEE outbreak occurred in coastal areas of Venezuela and Colombia, causing disease in 75,000–100,000 people (Rivas et al., 1997). Moreover, this virus can be weaponized and is thus a potential biological weapon that could be used by terrorists (Paredes et al., 2003).

No treatment exists for this viral disease. A vaccine is only available against VEEV for horses.

A study has been undertaken in our laboratory to test drugs that could be used for treatment of hemorrhagic fever, arthralgia, and encephalitis infections caused by alphaviruses. We have investigated the inhibitory effects of several compounds on the replication of CHIKV and SFV in vitro. Ribavirin, which has been reported to have antiviral activity against several members of the genus *Alphavirus* (Huggins et al., 1984a), was included in this study.

2. Materials and methods

2.1. Virus

The SFV strain used was the Dakar B1418 strain, provided by the Reference and Research WHO collaborator Center for Arbovirus and Hemorrhagic Fever virus (Pasteur Institute of Dakar, Senegal). The CHIKV strain used was the Ross C347 strain, supplied by the National Reference Center for Hemorrhagic Fever virus (Pasteur Institute of Paris, France). These strains were adapted to Vero cells by serial passages. Virus titers were determined by the 50% cell culture infective dose ($CCID_{50}$) method in Vero cell cultures (Crance et al., 1997; Reed and Muench, 1938).

2.2. Cells

Vero cells were grown at 37 °C, in 5% CO_2, in 199 medium (M199) supplemented with 5% heat-inactivated fetal calf serum (FCS), 100 IU ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin. CHIKV- and SFV-infected cells were maintained, at 37 °C, in M199 supplemented with 0.4% FCS.

2.3. Compounds

Eight compounds were tested. Ribavirin, 6-azauridine, glycyrrhizin, iota-carrageenan, fucoidan, and dextran sulfate were purchased from Sigma-Aldrich Chimie SARL (L'Isle d'Abeau, France). IFN-α2a and IFN-α2b were provided, respectively, by Produits Roche (Neuilly sur Seine, France) and by Schering-Plough (Levallois-Perret, France).

2.4. Inhibition of virus-induced cytopathogenicity

Vero cells in 96-well culture plates were used when confluent. Culture medium was removed and the cells were washed with M199. Then, 0.1 ml of diluted viral suspension (multiplicity of infection of 0.001 $CCID_{50}$ per cell for CHIKV and multiplicity of infection of 0.0001 $CCID_{50}$ per cell for SFV) and 0.1 ml of medium containing an appropriate concentration of the test compound were added. Eight wells were used for each concentration of the test compounds. Eight wells were used as virus controls (virus-infected non-drug-treated cells) and eight wells were used as cell controls (non-infected non-drug-treated cells). The culture plates were incubated at 37 °C in 5% CO_2 for 2 days until maximum cytopathic effect (CPE) of CHIKV or SFV was obtained in controls. Then, the CPE of each virus was microscopically recorded in each well. Antiviral activity was expressed as the 50% effective concentration (EC_{50}), i.e. the concentration of compound required to inhibit the cytopathic effect to 50% of the control value.

2.5. Cytotoxicity of the compounds

Vero cells grown to confluence in 24-well plates were exposed to different concentrations of the antiviral compounds (four wells per compound concentration) in maintenance medium for 4 days at 37 °C, in parallel with the virus-infected cell cultures. For each antiviral compound, four wells were used as controls (non-drug-treated cells). After 4 days of incubation, cytotoxicity was evaluated by the Trypan blue exclusion test as previously described (Crance et al., 2003). The concentration of antiviral compound that reduced the viability of Vero cells to 50% of the control was estimated as the 50% cytotoxic concentration (CC_{50}).

The inhibitory effect of the compounds on cellular proliferation was also studied: the cells were seeded at a rate of 2×10^4 cells per well in a volume of 1.0 ml into 24-well plates and allowed to proliferate for 24 h in M199, containing 5% FCS. The next day, 1.0 ml of medium containing two-fold increasing concentrations of the test compounds were added (four wells per concentration). After 3 days of incubation at 37 °C in 5% CO_2, medium was aspirated from the wells and cells were removed from monolayer culture by incubation with 0.5 ml of trypsin-EDTA in normal saline for 5 min at 37 °C. After addition of 0.5 ml of FCS, 0.05 ml of 2.5% Trypan blue was added to the cell suspension. Cell number and viability were determined by counting the cells in the presence of Trypan blue. The concentration of compound that reduced cell growth by 50% was estimated as the 50% inhibitory concentration (IC_{50}).

2.6. Inhibitory effect of compounds on CHIKV and SFV yield in infected cells

When the cells in 24-well culture plates were confluent, culture medium was removed and then 0.1 ml of diluted viral suspension (0.0001 $CCID_{50}$ for CHIKV and 0.00001 $CCID_{50}$ per cell for SFV) and 2 ml of maintenance medium containing the test compounds at an appropriate concentration were added (four wells per concentration). For each compound, four wells were used as controls (virus-infected non-drug-treated cells).

After a 37 °C incubation period ranging from 12 to 18 h (12 h for SFV and 18 h for CHIKV), when a maximum virus titer was reached in cell controls at the beginning of CPE appearance, the maintenance medium was removed, cells were washed and 1.0 ml of M199 was added. The virus was extracted by freezing and thawing. The homogenates were used for virus titer determination in cell culture.

2.7. Antiviral activity of the combination of IFN-α2b and ribavirin

Vero cells in 96-well tissue culture plates were used when confluent. Culture medium was removed and the cells were washed with M199. Then, 0.1 ml of diluted viral suspension (multiplicity of infection of 0.001 $CCID_{50}$ per cell for CHIKV and multiplicity of infection of 0.0001 $CCID_{50}$ per cell for SFV) and 0.1 ml of medium containing an appropriate concentration of the combination of IFN-α2b and ribavirin were added. In a 96-well culture plate, eight wells were used for each concentration of IFN-α2b (15, 10, 7.5, 5, 3.8, 2.5, 1.9, 1.3, 0.9, and 0.6 IU ml^{-1} for CHIKV and 20, 15, 10, 7.5, 5, 3.8, 2.5, 1.9, 1.3, and 0.9 IU ml^{-1} for SFV) and a fixed concentration of ribavirin was added to each well. Eight wells were used as virus controls (virus-infected non-drug-treated cells) and eight wells were used as cell controls (non-infected non-drug-treated cells). Eight plates were used with eight different concentrations of ribavirin (150, 100, 75, 50, 37.5, 25, 18.75, and 12.5 μg ml^{-1}) for CHIKV and eight plates were used with eight different concentrations of ribavirin (100, 75, 50, 37.5, 25, 18.75, 12.5, and 6.25 μg ml^{-1}) for SFV in order to obtain all possible combinations of IFN-α2b and ribavirin. Two plates for each virus were used as control with IFN-α2b alone and ribavirin alone. After 2 days of incubation at 37 °C in a CO_2 incubator, the cytopathic effects (CPE) of CHIKV and SFV were recorded and the number of wells in which a CPE appeared was evaluated. Data were plotted and analyzed by the isobologram method (Elion et al., 1954). The fractional inhibitory concentration (FIC) for each combination of IFN-α2b and ribavirin was calculated as follows: $FIC_{ribavirin}$ = (concentration of ribavirin in the combination at the end point)/(concentration of ribavirin alone required to achieved the end point) and $FIC_{IFN\text{-}\alpha2b}$ = (concentration of IFN-α2b in the combination at the end point)/(concentration of IFN-α2b alone required to achieved the end point). The combinations resulting in an additive antiviral effect ($FIC_{ribavirin} + FIC_{IFN\text{-}\alpha2b} = 1$) are represented by straight lines (unity line) on the isobolograms. When the combination results in synergy, i.e. stronger antiviral effects than the sum of the individual effects ($FIC_{ribavirin} + FIC_{IFN\text{-}\alpha2b} < 1$), the FIC values fall below the unity line. When the combination results in an antagonistic antiviral effect ($FIC_{ribavirin} + FIC_{IFN\text{-}\alpha2b} > 1$), the FIC values fall above the unity line.

2.8. Statistical analysis

Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA).

3. Results

3.1. Selectivity of compounds as inhibitors of CHIKV and SFV

The cytotoxicity of the compounds for Vero cells was determined both on confluent cells or on exponentially growing cells. Table 1 shows the cytotoxicity values of different tested compounds and their antiviral activity against CHIKV and SFV.

When selectivity indices were calculated as the ratio of the 50% cytotoxic concentration (CC_{50}) for cell viability in monolayer cell cultures to the average EC_{50} (CC_{50}/EC_{50}), all compounds showed selectivity indices ≥3 against CHIKV. The two recombinant IFN-α exhibited the highest selectivity indices (>901), and ribavirin, considered in this study as a positive control, had a selectivity index of 24. The selectivity indices of 6-azauridine (204) and iota-carrageenan (>133) were higher than that of ribavirin, while glycyrrhizin, dextran sulfate, and fucoidan showed selectivity indices of 3, 5, and >12.6, respectively. All compounds showed selectivity indices ≥2 against SFV. The two recombinant IFN-α and iota-carrageenan exhibited the highest selectivity indices (>694). Ribavirin showed a selectivity index of 109. The selectivity indices of 6-azauridine (85) and fucoidan (>62.5) were similar to that of ribavirin and glycyrrhizin and dextran sulfate showed the lowest selectivity indices (2 and 6, respectively).

When selectivity indices were calculated on the basis of the ratio of the 50% inhibitory concentration (IC_{50}) for cell growth to the average EC_{50} (IC_{50}/EC_{50}), ribavirin and 6-azauridine exhibited lower selectivity indices because of their cytostatic effect. These compounds had a marked inhibitory effect on the growth of uninfected cells although none affected normal cell morphology. IFN-α and iota-carrageenan proved to be the most selective inhibitors of the replication of CHIKV and SFV (selectivity index for both CHIKV and SFV >694).

Table 1
Cytotoxicity, antiviral activity and specificity of action of substances as inhibitors of CHIKV and SFV

Compound	CC_{50}[a] ($\mu g\,ml^{-1}$ or $IU\,ml^{-1}$) (A)	IC_{50}[b] ($\mu g\,ml^{-1}$ or $IU\,ml^{-1}$) (B)	EC_{50}[c] ($\mu g\,ml^{-1}$ or $IU\,ml^{-1}$)		Selectivity index[d]			
			CHIKV (C)	SFV (D)	CHIKV		SFV	
					(A/C)	(B/C)	(A/D)	(B/D)
IFN-α2b	>10000	>10000	9.7	11.6	>1031	>1031	>862	>862
IFN-α2a	>10000	>10000	11.1	14.4	>901	>901	>694	>694
Ribavirin	7500	58	83.3	47.0	24	1	109	1
6-Azauridine	51	0.9	0.2	0.4	204	5	85	2
Glycyrrhizin	3125	2418	1025	1462	3	2	2	2
Iota-carrageenan	>500	>500	3.8	0.7	>133	>133	>714	>714
Fucoidan	>1000	>1000	79.5	16.0	>12.6	>12.6	>62.5	>62.5
Dextran sulfate	1630	2000	352.6	280	5	6	6	7

The data represent average values of six experiments.
[a] Cytotoxic concentration required causing 50% inhibition of Trypan blue exclusion in confluent cell cultures.
[b] Inhibitory concentration required causing 50% inhibition of Trypan blue exclusion in exponentially growing cells.
[c] Effective concentration required to reduce virus-induced cytopathogenicity by 50%.
[d] Selectivity index is the ratio CC_{50}/EC_{50} or IC_{50}/EC_{50}.

3.2. Inhibitory effects of IFN-α2b, ribavirin, 6-azauridine, and glycyrrhizin on CHIKV and SFV yield in infected cells

IFN-α2b, ribavirin, 6-azauridine, and glycyrrhizin were further studied for their inhibitory effect on CHIKV and SFV replication in Vero cells. For each compound virus titer reduction was determined at different non-cytotoxic concentrations below the maximum tolerated concentration (MTC) which was the highest concentration that did not cause microscopically detectable cytotoxic effects.

For CHIKV, at the highest studied concentration of these compounds, the virus titer reduction was 5.4 $\log_{10}$ with IFN-α2b, 5.2 $\log_{10}$ with glycyrrhizin, 1.8 $\log_{10}$ with 6-azauridine or ribavirin (Table 2). The concentration of these compounds necessary to decrease virus yield 100-fold (99% effective concentration—EC_{99}) was 230 $IU\,ml^{-1}$ with IFN-α2b and 1250 $\mu g\,ml^{-1}$ with glycyrrhizin. These concentrations were, respectively, 43.5- and 2-fold lower than the MTC. The EC_{99} value with ribavirin and 6-azauridine was close to their MTC value. All these compounds caused a concentration-dependent reduction in the virus yield (one-way ANOVA, $P < 0.05$).

The four compounds (IFN-α2b, 6-azauridine, glycyrrhizin, and ribavirin) were also studied for their inhibitory effect on SFV replication in Vero cells. Results are reported in Table 2. At the MTC of the compounds, the virus titer reduction was 5.0 $\log_{10}$ with glycyrrhizin, 4.8 $\log_{10}$ with IFN-α2b, 2.9 $\log_{10}$ with 6-azauridine, and 2.8 $\log_{10}$ with ribavirin. The EC_{99} was 193 $IU\,ml^{-1}$ with IFN-α2b, 625 $\mu g\,ml^{-1}$ with glycyrrhizin, 229 $\mu g\,ml^{-1}$ with ribavirin, and 2.7 $\mu g\,ml^{-1}$ with 6-azauridine. These concentrations were, respectively, 52-, 4-, 4-, and 2-fold lower than the MTC. These four compounds caused a concentration-dependent reduction in virus yield (one-way ANOVA, $P < 0.05$).

Table 2
Inhibitory effect of selected compounds on CHIKV and SFV replication in Vero cells

Compound	Concentration[a] ($\mu g\,ml^{-1}$ or $IU\,ml^{-1}$)	Virus titer reduction[b] ($\log CCID_{50}\,ml^{-1}$)	
		CHIKV	SFV
IFN-α2b	1.0	0	0.2
	10.0	0.3	0.9
	100.0	0.7	1.5
	1000.0	3.1	3.1
	10000.0	5.4	4.8
Ribavirin	62.5	0.9	1.2
	125.0	1.0	1.4
	250.0	1.2	2.3
	500.0	1.5	2.6
	1000.0	1.8	2.8
6-Azauridine	0.3	0.3	0
	0.6	0.4	0.3
	1.3	0.7	1.1
	2.5	1.5	1.4
	5.0	1.8	2.9
Glycyrrhizin	156.3	0.1	0.9
	322.5	0.5	1.0
	625.0	0.7	2.0
	1250.0	2.0	2.6
	2500.0	5.2	5.0

The data represent average values of four experiments.
[a] For each compound, the virus titer reduction was determined at different non-cytotoxic concentrations below the maximum tolerated concentration (MTC) which was the highest dose that did not cause microscopically detectable cytotoxic effects.
[b] The antiviral activity was evaluated by the difference of the virus titer of virus controls (virus-infected non-drug-treated cells) and the virus titer of virus assays (virus-infected drug-treated cells). Mean virus titers for untreated control viruses were 6.9 $\log CCID_{50}\,ml^{-1}$ and 6.3 $\log CCID_{50}\,ml^{-1}$ with CHIKV and SFV, respectively.

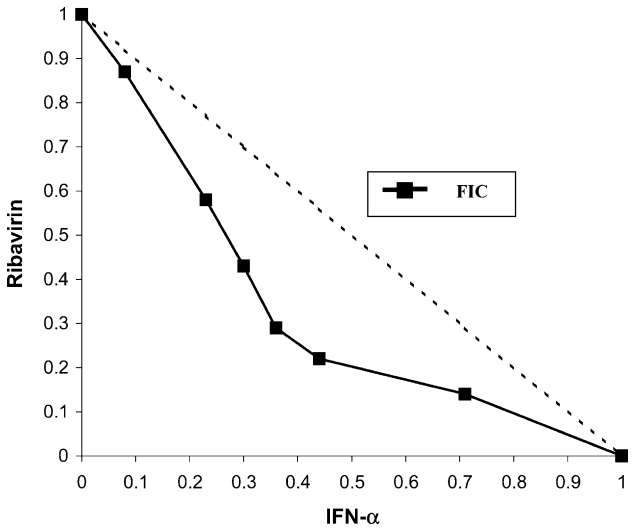

Fig. 1. Isobologram representing synergy of the combinations of IFN-α2b and ribavirin in antiviral activity against the cytopathic effect of CHIKV in Vero cells. $FIC_{ribavirin} + FIC_{IFN\text{-}\alpha2b} = 0.95$, 0.81, 0.73, 0.65, 0.66, 0.85, and <1. These results were observed in three different experiments. FIC: fractional inhibitory concentration.

3.3. Antiviral activity of the combination of IFN-α2b and ribavirin against CHIKV and SFV replication in cell culture

IFN-α2b and ribavirin inhibited CHIKV and SFV replication at concentrations that were significantly lower than the cytotoxic concentration. CHIKV and SFV replication was reduced to 50% of that of the control at an IFN-α concentration of 8.9 IU ml^{-1} (CHIKV) and 13.2 IU ml^{-1} (SFV). Ribavirin reduced CHIKV replication by 50% at a concentration of 86.5 μg ml^{-1} and inhibited SFV replication by 50% at a concentration of 51.0 μg ml^{-1}. When the infected cells were treated with both IFN-α2b and ribavirin, a subsynergistic effect was observed.

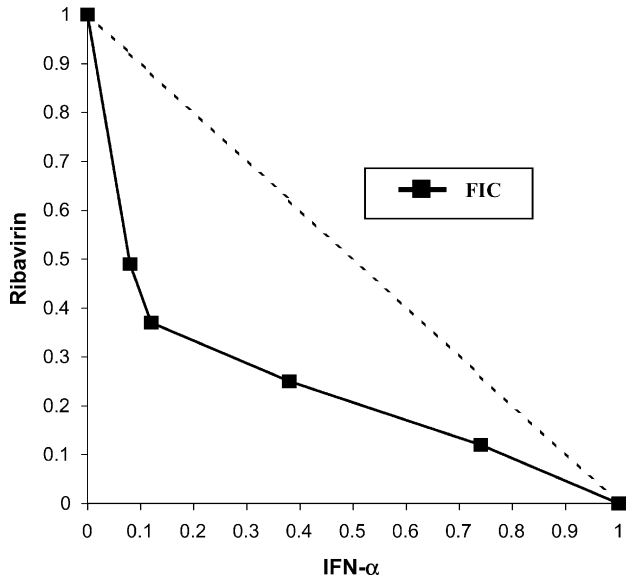

Fig. 2. Isobologram representing synergy of the combinations of IFN-α2b and ribavirin in antiviral activity against the cytopathic effect of SFV in Vero cells. $FIC_{ribavirin} + FIC_{IFN\text{-}\alpha2b} = 0.57$, 0.49, 0.63, 0.86, and <1. These results were observed in three different experiments. FIC: fractional inhibitory concentration.

A combination of IFN-α2b, at a concentration of 3.9 IU ml^{-1} (1/2 EC_{50}), and ribavirin at a concentration of 18.75 μg ml^{-1} (1/5 EC_{50}), inhibited CHIKV replication by 50% (Fig. 1). A combination of IFN-α2b, at a concentration of 3.2 IU ml^{-1} (1/3 EC_{50}), and ribavirin at a concentration of 25 μg ml^{-1} (1/3 EC_{50}), inhibited CHIKV replication by 50% (Fig. 1).

A combination of IFN-α2b, at a concentration of 1.1 IU ml^{-1} (1/12.5 EC_{50}), and ribavirin at a concentration of 25 μg ml^{-1} (1/2 EC_{50}), reduced SFV replication to 50% (Fig. 2). A combination of IFN-α2b, at a concentration of 5 IU ml^{-1} (1/3 EC_{50}), and ribavirin at a concentration of 12.5 μg ml^{-1} (1/4 EC_{50}), reduced SFV replication to 50% (Fig. 2).

The different combinations of IFN-α with ribavirin used in this study showed no significant cytotoxic effect ($P > 0.05$; *t* test) for the 48-h cell exposure experiments.

4. Discussion

In our study, IFN-α2a and IFN-α2b, ribavirin, 6-azauridine, glycyrrhizin, and three sulfated polysaccharides (iota-carrageenan, fucoidan, and dextran sulfate) were evaluated for their inhibitory effects on CHIKV and SFV replication in Vero cell cultures. These compounds inhibited CHIKV and SFV replication at concentrations which were significantly lower than the cytotoxic concentrations in quiescent cell cultures.

Sulfated polysaccharides were not selected for virus yield reduction determinations and synergy study because their antiviral activity has not been demonstrated in vivo (Witvrouw et al., 1994).

IFN-α, ribavirin, 6-azauridine, and glycyrrhizin which have already been used in patients in the treatment of other diseases were further studied for their effects on CHIKV and SFV yield in infected cells. These four compounds reduced the virus titer in a concentration-dependent manner.

Glycyrrhizin only showed antiviral activity at high subtoxic concentrations, but it proved significantly active against CHIKV and SFV replication. This drug has been used in Japan for the treatment of chronic viral hepatitis C (Arase et al., 1997; van Rossum et al., 1999; Miyake et al., 2002). Its use for the treatment of alphavirus infections should be further evaluated in animals at lower concentrations than that required for its antiviral activity in vitro in order to obtain synergistic effect with other drugs.

6-Azauridine is a broad-spectrum anti-metabolite that inhibits both DNA and RNA virus multiplication (Rada and Dragun, 1977). In our study, 6-azauridine showed a significant inhibitory effect on CHIKV and SFV replication at very low concentrations (0.8 and 1.6 μM, respectively),

but this compound was not chosen for synergy studies because it has not been approved for human use as an antiviral drug. 6-Azauridine triacetate has already been used in a wide range of diseases including severe forms of psoriasis (Deneau and Farber, 1975), without notable adverse effects. This drug, which can be used at high doses (up to 200 mg kg^{-1}; Crutcher and Moschella, 1975), could exhibit plasma concentrations higher than those enabling inhibition of alphavirus replication in vitro. Therefore, 6-azauridine should be evaluated in infected mice before its eventual use for the treatment of alphavirus infections in man.

Ribavirin exhibited the expected antiviral activity. This broad-spectrum antiviral drug was previously shown to be inhibitory to togaviruses in vitro (Huggins et al., 1984a) and, particularly, CHIKV (Andrei and De Clercq, 1993) and SFV (Van Tiel et al., 1985). Ribavirin also showed inhibitory effect in vitro, alone or in a synergistic combination with selenazofurin, a related compound, against VEEV (Huggins et al., 1984b). Ribavirin which is an antiviral drug that proved to be effective in patients at the beginning of the hemorrhagic symptomatology (Fisher-Hoch et al., 1995; Huggins, 1989; Huggins et al., 1991; McCormick et al., 1986) could be used in alphavirus infections except for encephalitis. However, several ribavirin-related molecules could be used for the treatment of alphavirus infections which cause encephalitis. Indeed, ribavirin 5′-sulfamate, a close analog of ribavirin proved to be effective to protect mice infected by SFV against encephalitis (Smee et al., 1988), although this compound induced severe toxicity. This compound could also be used in the treatment of alphavirus infections to prevent encephalitis.

Human recombinant IFN-α proved to be much more selective and effective than ribavirin in its inhibitory effect on CHIKV and SFV replication. Its selectivity index (CC_{50}/EC_{50}) was >800 in growing cells. Moreover, among all the compounds tested, IFN-α showed the highest potency against CHIKV and SFV. These results complement previous studies, which demonstrated antiviral activity of interferon against alphaviruses in infected mice. In these studies, recombinant IFN-α was shown to protect mice against experimental infection with SFV (Grieder and Vogel, 1999; Pinto et al., 1990) and with the neurovirulent strain of Sindbis virus (Ryman et al., 2000). On the other hand, pegylated IFN-α proved to be effective for the treatment of virulent VEEV-infected BALB/c mice resulting in a greatly enhanced survival from either a subcutaneous or an aerosol infection (Lukaszewski and Brooks, 2000). IFN-α is now used for the treatment of chronic hepatitis C (Lau et al., 1998). Its therapeutic activities on HCV infection in patient, the encouraging results in alphavirus-infected animal models and the results obtained in the present study as to its inhibitory effect on CHIKV and SFV in vitro suggest that IFN-α should also be further evaluated for its efficacy in the treatment of alphavirus infections in man.

The study of antiviral activity of the combination of IFN-α2b and ribavirin on CHIKV and SFV replication in Vero cells showed that this combination is subsynergistic against the two viruses. Ribavirin proved to be active at concentrations much lower than its cytotoxic concentration in exponentially growing cells in increasing the antiviral activity of IFN-α. The combination of IFN-α2b and ribavirin has been approved for the treatment of both acute and relapsing hepatitis C virus (McHutchison et al., 1998; Poynard et al., 1998). Because IFN-α in combination with ribavirin was found to significantly improve the sustained biochemical and virological response rates compared to interferon alone in this disease (Reichard et al., 1997), ribavirin could also increase the efficacy of IFN-α on alphavirus infections and a combination of IFN-α and ribavirin should also be considered for the treatment of this type of infection.

In conclusion, our present results indicate that several compounds including ribavirin, human recombinant IFN-α, 6-azauridine, and glycyrrhizin can be considered as selective and potent anti-CHIKV and anti-SFV agents. These compounds have already been used in patients for their antiviral or anti-tumor activities. Each of these drugs and the combination of IFN-α and ribavirin should be further evaluated for the treatment of alphavirus infections in animals.

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