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In vivo efficacy of SDZ 35-682, a new picornavirus capsid-binding agent

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Abstract

SDZ 35-682 is a potent and selective inhibitor of the replication of members of the picornavirus group. It belongs to the group of uncoating inhibitors binding to the hydrophobic pocket in the capsid of the virion. In cell culture it inhibits several rhinovirus serotypes and echovirus 9 at concentrations as low as 0.1 μg/ml. In the echovirus 9 animal model the protective effect of SDZ 35-682 was found to be dependent on both, dose of drug and duration of treatment. Significant protection of newborn mice from paralysis and death could be achieved by either a high dose (126 mg/kg) given only twice, at days 0 and 1 relative to echovirus 9 inoculation, or by a lower dose administered for 4 or 6 days. This finding might be explained by assuming a long half-life for SDZ 35-682. Though clinical usefulness of SDZ 35-682 may be limited by its relatively narrow antiviral spectrum it represents a novel potent and selective inhibitor of rhinovirus and echovirus 9 replication in cell culture and in the organism.

Keywords: SDZ 35-682; Antipicornaviral activity; Echovirus; Rhinovirus

1. Introduction

The picornavirus family includes, among others, the genera enterovirus and rhinovirus. Rhinoviruses are the causative agents of the majority of upper respiratory illnesses, particularly of the 'common cold' (Couch, 1984). The enterovirus group includes poliovirus, coxsackie A and B viruses, echoviruses, and additional other human enteroviruses. It is estimated that 5–15 million enteroviral infections occur each year in

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the US (Kogon et al., 1969), with half of them resulting in symptomatic illness (Spigland et al., 1966). Clinical enteroviral syndromes range from mild upper respiratory disease, gastrointestinal disease, summer exanthems, and acute hemorrhagic conjunctivitis to aseptic meningitis, myocarditis, poliomyelitis, encephalitis, and 'neonatal sepsis'. According to a study of the WHO (Assaad and Cockburn, 1972) about one third of the echoviruses isolated from patients, mostly with aseptic meningitis, was classified as serotype 9. Echovirus type 9, strain Barty, was recovered from the cerebrospinal fluid of a child suffering from aseptic meningitis during the large epidemic of 1957 in Milwaukee, WI (strain A.B., Eggers and Sabin, 1959). Strain Barty produces paralysis in newborn mice with muscle lesions similar to those caused by coxsackie A viruses (Bültmann et al., 1983). Thus, infection of newborn mice with echovirus 9, strain Barty, represents an animal model of enteroviral infection, suitable for in vivo testing of candidate antipicornavirus drugs.

Currently, no drugs are available for the treatment of diseases caused by entero- or rhinoviruses. Antiviral compounds, however, interacting with the capsid of picornaviruses have been known for more than 20 years. These agents were found to bind in a hydrophobic pocket (Smith et al., 1986; Badger et al., 1988; Badger et al., 1989; Chapman et al., 1991) underneath the floor of the 'canyon' (Rossmann et al., 1985) of rhinovirus particles. Depending on the serotype this binding either led to a blockade of uncoating (McSharry et al., 1979; Fox et al., 1986) and/or to inhibition of attachment (Pevear et al., 1989; Kim et al., 1993). The oxazolinyl isoxazol WIN 51711 (Otto et al., 1985) was demonstrated to be protective in echovirus 9-infected newborn mice (Diana et al., 1985) and in poliovirus 2-infected mice (McKinlay and Steinberg, 1986). Nevertheless, a closely related derivative, WIN 54954, failed to exert any antiviral or clinical effect when given prophylactically per os to rhinovirus type 23-infected human volunteers (Turner et al., 1993). The same drug, on the other hand, given by the same route, proved efficacious in preventing experimental human coxsackievirus A21 infection and illness (Schiff et al., 1992). The pyridazinamine derivatives R61837 and R77975 (Andries et al., 1988; Andries et al., 1989; Andries et al., 1990; Andries et al., 1992; Al-Nakib and Tyrrell, 1987) were found to be clinically effective against rhinovirus infection in volunteers (Al-Nakib et al., 1989; Barrow et al., 1990; Hayden et al., 1992).

A class of compounds, sharing the piperazinyl moiety with R61837, and also exhibiting potent and selective antirhinovirus activity, was discovered in our laboratories in 1984. Systematic chemical derivation led to a derivative, being antivirally active not only against a limited spectrum of rhinovirus serotypes, but also against echovirus 9. Here we describe the in vivo evaluation of SDZ 35-682 (Fig. 1) (Rosenwirth et al., 1994), a new representative of the piperazine derivative class of antipicornavirus agents, in the echovirus 9 animal model.

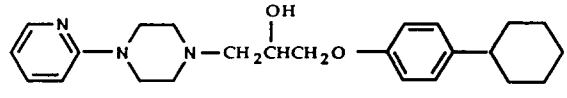

Fig. 1. Chemical structure of SDZ 35-682: 1-[2-hydroxy-3-(4-cyclohexylphenoxy)propyl]-4-(2-pyridyl)piperazine.

2. Materials and methods

2.1. Virus and cell line

Echovirus type 9, strain Barty, was originally supplied by A.B. Sabin (Eggers and Tamm, 1961). The GMK cell line, a continuous line derived from African green monkey kidney cells, was kindly given to us by H. Lennartz (Hamburg, Germany). Stocks of echovirus type 9 were prepared in GMK cells at 37°C. GMK cells were grown as monolayers at 37°C in Eagle's minimum essential medium (E-MEM), supplemented with 10% fetal bovine serum.

2.2. Chemicals and other materials

Most of the chemicals were described before (Rosenwirth and Eggers, 1978a, Rosenwirth and Eggers, 1978b). SDZ 35-682 (1[2-hydroxy-3-(4-cyclohexylphenoxy)-propyl]4-(2-pyridyl)piperazine) (Fig. 1) was synthesized at Sandoz Pharma AG, Basle, in the course of a β-blocker-program in 1977, and passed also the general screening program because of its unique structure. The cyclohexylaryloxy-epoxy intermediate [RN 67006-99-9] and its synthesis have been described in the literature (Jendrichovsky et al., 1978). By refluxing equivalent amounts of epoxide and 2-pyridylpiperazine [supplied by Aldrich] in ethanol for 24 h we obtained SDZ 35-682 in 76% yield as colorless crystals. The melting point of the racemic free base is 128–129°C.

SDZ 35-682 was formulated as an emulsion at a concentration of 10 mg/ml using Tween-80 and Span-80 as emulsifiers; dilutions for injection were made in saline immediately before use.

2.3. In vivo model

Naval Medical Research Institute (NMRI) mice were bred randomly in our own facilities. Newborn mice (< 24 h old) were inoculated subcutaneously in the interscapular region with 0.02 ml of echovirus 9 stock dilution in PBS. The infected animals were checked at least once a day for a period of 15 days, and clinical manifestations as well as deaths were recorded. For growth curve experiments newborn mice were infected subcutaneously with various dilutions of echovirus 9 stock. Groups of five mice were killed on days 0 (= post inoculation), 1, 2, 3, 4, 5 and 6 and were stored at -20°C. Subsequently, a 50% (w/v) suspension in E-MEM of each mouse was prepared (Eggers and Sabin, 1959), and the virus content was determined by plaque assay in GMK cells. Three litters (= 36 animals) were used per virus dilution. Treatment of mice consisted of subcutaneous injection of 0.02 to 0.05 ml per mouse of SDZ 35-682 dilution in saline, once per day for the indicated days, starting 0.5 h before virus inoculation. Three litters were used per variable. Only litters of 11 to 14 mice were taken to minimize differences in body weights.

3. Results

Echovirus 9, strain Barty, infection of newborn mice results in flaccid paralysis and subsequent death, due to a severe generalized myositis of striated muscles (Eggers and Sabin, 1959; Bültmann et al., 1983) SDZ 35-682 (Fig. 1) was tested for antiviral efficacy in this animal model. The virus dose used for subcutanous infection was adjusted such, that 80 to 100% of the animals developed paralysis, leading to death in the majority of them. The time course of virus production in mice infected with such a virus dose was followed by measuring virus titers in muscle homogenates by plaque assay in GMK cells. Virus replication is noticeable as early as 1 day after inoculation and virus titers reach their highest value (i.e. 1.2×10^8 p.f.u. per ml) at day 4 post infection. Under these experimental conditions onset of paralysis is observed at day 5 and first deaths occur on day 7 post infection (Fig. 2, control). Two weeks after infection the mice have either died or recovered from paralysis. Initially, mice had been treated subcutaneously with SDZ 35-682 twice per day for 8 days with administration of the

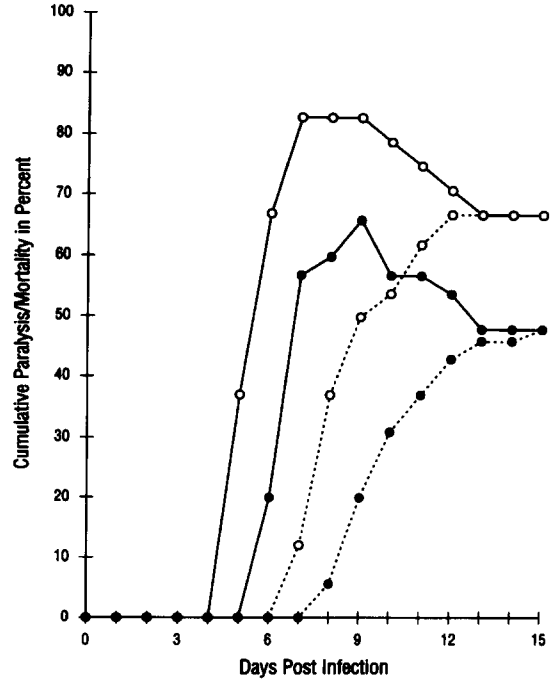

Fig. 2. Time course of ECHO 9-induced paralysis and death of newborn mice: effect of SDZ 35-682 administered subcutaneously. ○———○ Untreated control; cumulative paralysis in percent of total mice per group. ○------○ Untreated control; cumulative mortality in percent of total mice per group. ●———● Treatment with SDZ 35-682 at a dose of 63 (60–66) mg/kg/day, once per day, for 2 days (0 and 1); cumulative paralysis in percent of total mice per group. ●------● Treatment with SDZ 35-682 at a dose of 63 (60–66) mg/kg/day, once per day, for 2 days (0 and 1), cumulative mortality in percent of total mice per group.

Table 1
ECHO 9 infection of newborn mice: statistical significance of the effect of SDZ 35-682 on paralysis and death

Treatment		Cumulative paralysis [b] in percent (*P*-value)	Mean time of paralysis in days (*P*-value)	Cumulative mortality [c] in percent (*P*-value)	Mean survival time in days (*P*-value)
Duration (days)	Dosage group [a]				
2	1	37 (0.0001)	3 (0.002)	16 (0.0001)	18.6 (< 0.001)
(0–1)	2	62 (0.0001)	7 (0.002)	48 (0.0001)	15.7 (< 0.001)
	3	87 (n.s.)	9 (0.003)	58 (0.002)	14.5 (< 0.001)
	4	100 (n.s.)	15 (n.s.)	99 (n.s.)	8.6 (n.s.)
4	2	23 (0.0001)	2 (0.002)	8 (0.0001)	19.4 (< 0.001)
(0–3)	3	59 (0.0001)	6 (0.002)	39 (0.0001)	16.2 (< 0.001)
	4	97 (n.s.)	13 (0.022)	90 (n.s.)	10.5 (n.s.)
6	3	15 (0.0001)	2 (0.001)	9 (0.0001)	19.3 (< 0.001)
(0–5)	4	72 (0.004)	10 (0.006)	72 (0.04)	14.2 (< 0.001)
8 (0–7)	4	55 (0.0001)	6 (0.002)	46 (0.0001)	15.6 (< 0.001)
Control	-	97	14	92	9.7

[a] SDZ 35-682 was administered subcutaneously once per day; four concentrations of SDZ 35-682 were used: group 1: 10 mg/ml, group 2: 7.5 mg/ml, group 3: 5 mg/ml, group 4: 2.5 mg/ml; the volume administered was increased from 0.02 ml/mouse stepwise to 0.03, 0.04 and 0.05 ml/mouse, according to the increasing body weight, to give an approximately constant dose over time of treatment. The increase in body weight was determined by weighing twelve mice from one litter every day. Their mean body weight increased from 1.52 g on day 0 to 2.47 g on day 4 and to 3.51 g on day 7. The mean dose per kg per day in the four dosage groups was (range given in parentheses): group 1: 126 (120–132) mg, group 2: 102 (90–107) mg, group 3: 71 (60–81) mg, group 4: 36 (30–41) mg. In addition to the variation of the dose four different durations of treatment were evaluated: 2 days (on days 0, 1 relative to virus inoculation), 4 days (on days 0–3), 6 days (on days 0–5), and 8 days (on days 0–7). Three litters were used per dosage group and per duration of treatment.
[b,c] The numbers of paralyzed and dead animals as well as the respective time intervals after infection were recorded daily. The values are expressed in percent of total mice/group, i.e. are based on 33 to 42 animals/group. Statistical significance of the differences as compared to the control group, was assessed by the χ^2 test with Yates correction for small numbers (Sacks, 1984).

first dose 0.5 h before virus inoculation. Then we noticed that treatment once per day was equally effective, a result indicating a long half-life of the drug. Consequently, in the following experiments test compound was administered once per day starting 0.5 h before virus inoculation. The doses used for drug treatment were adjusted according to the fast increasing body weight of the mice. Since it was obvious from the time course of virus replication that inhibition was crucial up to day 4 after inoculation, duration of treatment was also varied. Toxic effects (i.e. deaths during the first days of treatment) were observed only with doses higher than 150 mg/kg/day.

In a series of experiments dose and duration of subcutaneous treatment with SDZ 35-682 were varied in the non-toxic range (Table 1, Figs. 2, 3 and 4). In Fig. 2 an example of the time course of echovirus 9-induced disease in untreated and SDZ 35-682-treated mice is shown. While onset of paralysis in control mice is observed at day 5 and reaches its maximum at day 7, mice treated with 63 mg/kg/day of SDZ

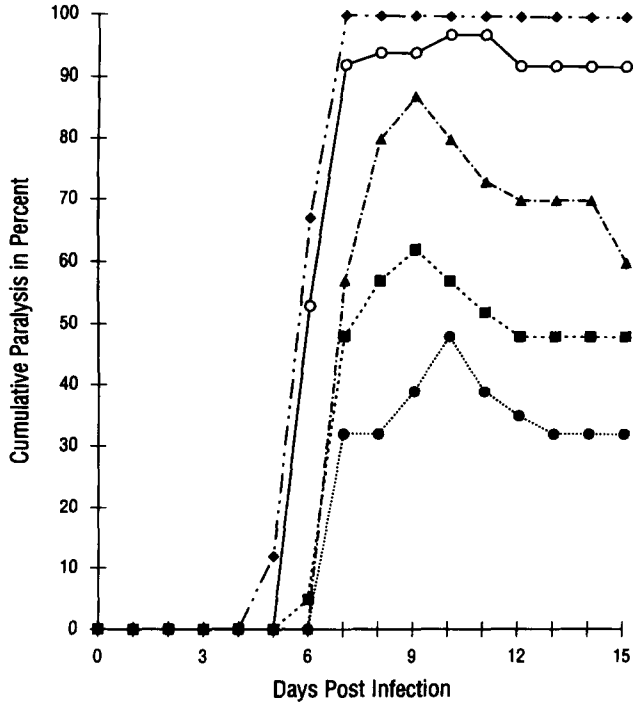

Fig. 3. Time course of ECHO 9-induced paralysis: dose-dependence of the protective effect of SDZ 35-682. Cumulative paralysis in percent of total mice per group: ○———○ Untreated control. ●· · · ·● Treatment with SDZ 35-682 at a dose of 126 (120–132) mg/kg/day, once per day, for 2 days (0 and 1). ■----■ Treatment with SDZ 35-682 at a dose of 95 (90–99) mg/kg/day, once per day, for 2 days (0 and 1). ▲-·-·-·-▲ Treatment with SDZ 35-682 at a dose of 63 (60–66) mg/kg/day, once per day, for 2 days (0 and 1). ◆-··-◆ Treatment with SDZ 35-682 at a dose of 32 (30–33) mg/kg/day, once per day, for 2 days (0 and 1).

35-682 at days 0 and 1 start to develop paralysis on day 6 and reach a lower maximum percentage of paralyzed animals than the untreated control at day 9. Thus, the paralytogenic effect of echovirus 9 is delayed and reduced by SDZ 35-682. The same is true, if virus-induced death is used as the endpoint. Figs. 3 and 4 illustrate, that the protective effect of SDZ 35-682 against echovirus 9-caused paralysis is dependent on both, dose of drug and duration of treatment. Approximately 85% to 90% protection from echovirus 9-induced death could be achieved by either low dose (71 mg/kg, dosage group 3) given for 6 days, or high dose (126 mg/kg, dosage group 1) administered only at days 0 and 1. In addition to the reduction of cumulative paralysis and mortality, mean time of paralysis was shortened and mean survival time was prolonged by treatment with SDZ 35-682. These protective effects were highly significant (Table 1). Delay of start of treatment for 24 h significantly reduced the antiviral efficacy of SDZ 35-682 (data not

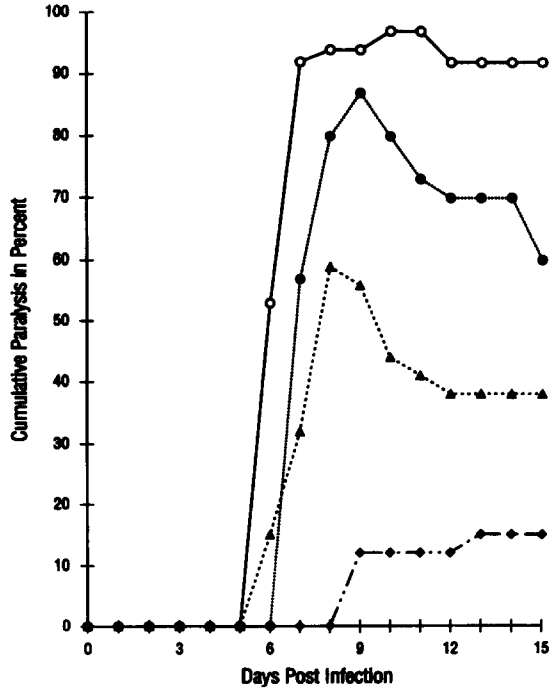

Fig. 4. Time course of ECHO 9-induced paralysis: dependence of the protective effect of SDZ 35-682 on duration of treatment. Cumulative paralysis in percent of total mice per group. O——O Untreated control. ●····● Treatment with SDZ 35-682 at a dose of 63 (60–66) mg/kg/day, once per day, for 2 days (0 and 1). ▲---▲ Treatment with SDZ 35-682 at a dose of 68 (60–74) mg/kg/day, once per day, for 4 days (0 through 3). ◆-..-◆ Treatment with SDZ 35-682 at a dose of 71 (60–81) mg/kg/day, once per day, for 6 days (0 through 5).

shown). In conclusion SDZ 35-682 proved active in vivo, when administered subcutaneously, in protecting mice from echovirus 9-induced paralysis and death.

4. Discussion

SDZ 35-682 is a potent and selective inhibitor of the replication of several rhinovirus serotypes and of echovirus 9 (Rosenwirth et al., 1994). In cell culture it was inhibitory to echovirus 9 replication at a concentration of 0.067 μg/ml. Like the members of other classes of capsid-binding antipicornavirus agents SDZ 35-682 is an inhibitor of the uncoating process of echovirus 9; it may, in addition, interfere to some extent with adsorption. Like the other uncoating inhibitors of picornaviruses (Zhang et al., 1992), SDZ 35-682 binds to the hydrophobic pocket beneath the canyon floor of virus particles. Since echovirus 9 has not been crystallized so far, this has been demonstrated by co-crystallization of SDZ 35-682 with HRV 14 (Rosenwirth et al., 1994).

Echovirus 9, besides being an important pathogen for humans, has the advantage of being pathogenic for newborn mice; thus, an animal model suitable for in vivo testing of candidate anti-picornavirus drugs is available. Subcutaneous inoculation of echovirus 9 into newborn mice resulted in extensive polymyositis, progressive limb paralysis and eventual death. Single daily subcutaneous doses of SDZ 35-682 beginning 0.5 h prior to infection prevented development of paralysis in a dose dependent manner. It has been shown previously (Eggers and Sabin, 1959; Bültmann et al., 1983) that virus replication to a definable threshold in the muscle tissues of echovirus 9 infected mice can be correlated with the onset of paralysis. This threshold level must be reached within the first days of life since five to six day old mice, afterwards, though exhibiting a similar rate of virus multiplication, lose their susceptibility to paralysis. Under our experimental conditions maximum virus titers in muscle tissue were observed 3 to 4 days after inoculation, i.e. 4 to 5 days after birth. The protective effect of SDZ 35-682 was found to be dependent on both, dose of drug and duration of treatment. Significant protection from paralysis and death could be achieved by either a high dose given only twice, at days 0 and 1 relative to virus inoculation, or by a lower dose administered for 4 or 6 days. This finding could be explained by assuming a long half-life for compound SDZ 35-682. The high dose given at days 0 and 1 would lead to high enough levels for a sufficiently long period, while lower doses would have to be administered more frequently in order to reach a certain level of compound in vivo.

Delay of start of treatment for 24 h significantly reduced the antiviral efficacy of SDZ 35-682. One day after inoculation virus titers have increased already by a factor of 50 as compared to day 0, and it appears that inhibition by SDZ 35-682 is already too ineffective at this time point. In experimentally induced picornaviral infections in humans R61837 was efficacious when administered as late as 24 h after viral infection, but not when administered after the appearance of cold symptoms (Al-Nakib et al., 1989). Thus, antiviral potency as well as virus load at time of start of treatment appear to be decisive factors for in vivo efficacy. In this context it should be pointed out that the pathogenesis of enterovirus illness in humans is different from that of our animal model, where virus is injected straight into the disease-producing target organ, namely muscle tissue.

Clinical usefulness of SDZ 35-682 appears limited by its relatively narrow antiviral spectrum. On the other hand, echovirus 9 is the etiologic agent of a large proportion of recorded enterovirus illnesses (Assaad and Cockburn, 1972). SDZ 35-682 represents a potent and selective inhibitor of rhinovirus and echovirus 9 replication in cell culture and in vivo. It belongs to the group of uncoating inhibitors binding in the hydrophobic pocket in the capsid of picornaviruses, and thus contributes to our understanding of the requirements/restrictions for binding within this pocket. This information will be helpful in designing other compounds with broad-spectrum antipicornaviral activity for in vivo use.

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