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SDZ 35-682, a new picornavirus capsid-binding agent with potent antiviral activity

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Abstract

SDZ 35-682 is a potent and selective inhibitor of the replication of members of the picornavirus group. It inhibits several rhinovirus serotypes and echovirus 9 at concentrations as low as 0.1 μg/ml, without exerting any effect on cell proliferation up to 30 μg/ml. As observed with other capsid-binding antipicornavirus compounds, there is a wide variation in sensitivity of the different serotypes within the rhinovirus group. The point of interference of SDZ 35-682 in a single cycle of virus growth is an early event taking place before 2 or 3 h of echo- or rhinovirus replication, respectively. By incorporation of neutral red into the viral capsid and measurement of acquisition of photoresistance it is shown that uncoating of echovirus 9 is inhibited by SDZ 35-682. In addition, efficiency of adsorption of echovirus 9 is reduced by SDZ 35-682. To demonstrate that SDZ 35-682, like other uncoating inhibitors of picornaviruses, binds to the hydrophobic pocket beneath the canyon floor co-crystallization with HRV 14 was performed. Considerable conformational changes occur in VP1 in the HRV 14/SDZ 35-682 complex. SDZ 35-682 is 19 Å long from end to end and thus fills the entire hydrophobic pocket including its innermost end; it is less flexible than other long antiviral agents. It has been suggested that compounds filling the entire hydrophobic pocket will affect the uncoating process of the virion. Thus, inhibition of viral uncoating, as demonstrated with echovirus 9, probably is the predominant mode of action of SDZ 35-682.

Keywords: SDZ 35-682; HRV 14; Antipicornavirus agent; Echovirus; Rhinovirus

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1. Introduction

Picornaviruses are a major cause of virus-associated morbidity in man, and their infection may result in severe disease and death, particularly in immunocompromised patients and in neonates. Currently, no drugs are available for the treatment of diseases caused by entero- or rhinoviruses, the main genera of the picornavirus family. Antiviral compounds, however, interacting with the capsid of picornaviruses have been known for more than 20 years. Some of these molecules, such as rhodanine (Eggers et al., 1970; Eggers, 1977; Rosenwirth and Eggers, 1979), dichloroflavan (Bauer et al., 1981; Tisdale and Selway, 1983), and chalcones, e.g. Ro 09-0410 (Ishitsuka et al., 1982; Ninomiya et al., 1984) are natural products originating from plants. Others, such as arildone (Diana et al., 1977) and the more recent disoxaril (WIN 51711) (Diana et al., 1985), were derived synthetically. Following the finding that arildone is a potent inhibitor of enteroviruses in vitro (McSharry et al., 1979; Kim et al., 1980) and in vivo (McKinlay et al., 1982), a series of analogs, namely oxazolinyl isoxazoles being active against entero- and rhinoviruses, e.g. WIN 51711, have been synthesized and extensively studied by the Sterling-Winthrop research group (Diana et al., 1985; Otto et al., 1985; Fox et al., 1986; McKinlay and Steinberg, 1986). Elucidation of the atomic structures of poliovirus type 1 (Hogle et al., 1985), human rhinovirus types 14 (Rossmann et al., 1985), and 1A (Kim et al., 1989) allowed a more detailed investigation of the molecular basis for the antiviral activity of the WIN compounds. These agents were found to bind in a hydrophobic pocket (Smith et al., 1986; Badger et al., 1988) 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 as was shown for poliovirus type 2 and rhinovirus type 2 (McSharry et al., 1979; Fox et al., 1986) and/or to inhibition of attachment of some rhinoviruses to their cellular receptor (Pevear et al., 1989; Kim et al., 1993).

A different class of compounds has been found at Janssen Research Foundation to inhibit replication of several rhinovirus serotypes (Al-Nakib and Tyrrell, 1987; Andries et al., 1988, 1989, 1990, 1992). One representative of this group of pyridazinamines, R 61837, was demonstrated to bind in the same pocket and to cause similar conformational changes in the rhinovirus 14 capsid as the WIN derivatives (Chapman et al., 1991).

Attempts to improve water-solubility while retaining capsid-binding properties resulted in the synthesis of SCH 38057, a relatively weak inhibitor of entero- and rhinoviruses (Rozhon et al., 1993). However, structural studies of SCH 38057, bound to HRV 14, indicated a larger degree of conformational change than those occurring with either WIN or Janssen compounds (Zhang et al., 1993).

Independently, a class of compounds, sharing the piperazinyl moiety with the Janssen derivative R 61837, and also exhibiting potent and selective antirhinovirus activity, was discovered in our laboratories in 1984. Systematic attempts to broaden the antirhinovirus spectrum of our lead compounds by chemical derivation (to be published elsewhere) led to a derivative with activity not only against a limited spectrum of rhinovirus serotypes, but also against the enterovirus ECHO 9. Here we describe the in vitro characterization and investigation of mode of action of SDZ 35-682 (Fig. 1), a new representative of the piperazine derivative class of antipicornavirus agents.

2. Materials and methods

2.1. Viruses and cell lines

Echovirus type 9, strain Barty, poliovirus type 2, strain P712-ch-2ab and coxsackie virus B3, strain Nancy, were originally supplied by A.B. Sabin (Eggers and Tamm, 1961); all human rhinovirus (HRV) serotypes were purchased from the American Type Culture Collection (ATCC). 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, poliovirus type 2 and coxsackie virus B3 were prepared in GMK cells at 37°C; all rhinoviruses were propagated in HeLa (Ohio) cells at 34°C. Cell lines were grown as monolayers at 37°C in Eagle's minimum essential medium (EMEM), supplemented with 10% fetal bovine serum.

2.2. Chemicals and other materials

Most of the chemicals were described before (Rosenwirth and Eggers, 1978a,b). SDZ 35-682 (1-[2-hydroxy-3-(4-cyclohexyl-phenoxy)-propyl]-4-(2-pyridyl)-piperazine) was synthesized at Sandoz Pharma AG, Basle, in the course of a β-blocker program in 1977, and also passed 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 (Scheme 1).

The compound was dissolved in dimethylsulfoxide (DMSO) to 10 mg/ml and diluted at least 1 : 300 in cell culture medium.

2.3. Cytopathic effect (CPE) inhibition assays

Virus-induced cytopathic effects are taken as a measure for virus replication in cell culture, thus, inhibition of virus-induced cytopathic effects by a substance is considered as an indication for interference with virus replication. Serial 3-fold dilutions of test compound in EMEM were prepared in flat-bottomed microtiter plates. Equal parts of virus dilution in EMEM and cell suspension in EMEM with 15% fetal calf serum were added. Virus-infected cultures without compound were included as controls as were uninfected cells treated with compound. The cell input was adjusted such as to give a confluent monolayer after 1–2 days of incubation, and the virus input such as to cause

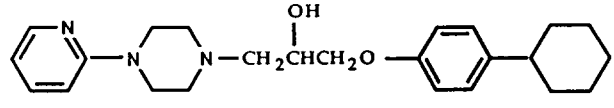
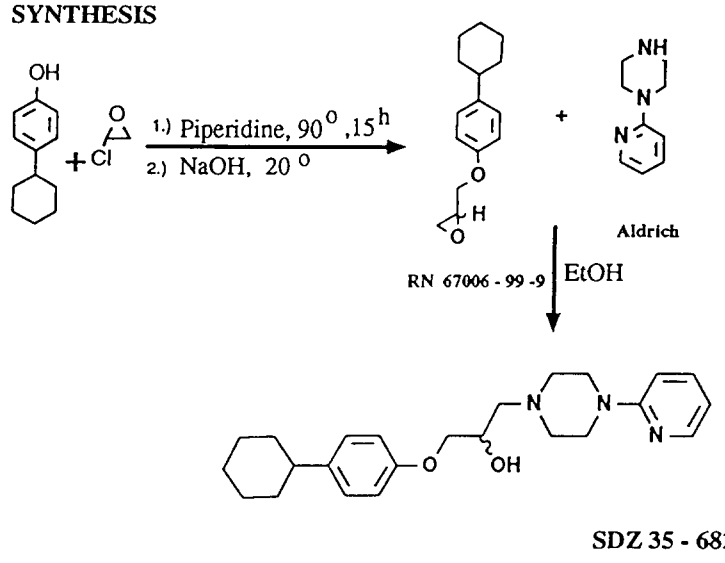


Fig. 1. Chemical structure of SDZ 35-682. 1-[2-hydroxy-3-(4-cyclohexyl-phenoxy)propyl]-4-(2-pyridyl) piperazine.



90–100% CPE in the infected control cell cultures after 3–4 days. At this time, the cells were fixed and stained with crystal violet, and the extent of virus-induced CPE in infected controls and in drug-treated wells was estimated. Alternatively, CPE was monitored for 1–4 days by microscopic observation.

Echovirus 9-, coxsackie B3- and poliovirus-2-induced CPE was observed in GMK cells; all HRV serotypes were tested in HeLa (Ohio) cells.

2.4. Growth curve experiments

All growth curve experiments were carried out at 37°C in a constant-temperature room as described previously (Rosenwirth and Eggers, 1978a).

2.5. Plaque assays

Confluent GMK cell monolayers were infected with a dilution of echovirus 9 stock, suitable to give approximately 30 plaques per tissue culture dish; three plates were used per variable. After adsorption for 1 h at room temperature overlay was added, consisting of equal volumes of 1.8% agar and EMEM at double concentration with 2% fetal bovine serum. The plates were incubated as described previously (Eggers and Tamm, 1961). Plaques were counted after 3 days without staining.

2.6. Virus yield reduction assays

Approximately 80% confluent monolayers of GMK or HeLa (Ohio) cells were infected at room temperature with echovirus 9 or rhinovirus 14, respectively, at a

multiplicity of infection (m.o.i.) of 10 plaque-forming units (PFU) per cell. SDZ 35-682, at a concentration of 1 μg/ml, was either added at the time points indicated and was present until harvest of the infected cultures, or, it was added before the virus and was removed by washing of the cultures at the time points indicated. At various times postinfection aliquots of the plates were frozen at −20°C. Virus yield was determined by plaque assay in the absence of test compound.

2.7. Uncoating assay

Neutral red-sensitized virus was prepared, handled, assayed and irradiated following Kato and Eggers (1969) and Eggers and Waidner (1970).

2.8. HRV 14 crystallization and structure determination complexed with SDZ 35-682

HRV 14 crystals were grown according to procedures described by Arnold et al. (1984) with subsequent modification (Zhang et al., 1993). SDZ 35-682 was dissolved in DMSO to a concentration of 0.5 mg/ml and was then added to 200 μl drops containing 3–4 crystals of sizes varying from 0.1 to 0.2 mm in each dimension, to a final concentration of 5 μg/ml. The drops contained 10 mM Tris buffer, pH 7.2, and 0.25–0.75% (w/v) PEG 8000.

Crystals were irradiated 12–17 h after the addition of SDZ 35-682, and data were collected on the monochromatic beam lines A1 and F1 at the Cornell High Energy Synchrotron Source (MacCHESS). Three crystals were exposed to the X-ray beam at the A1 line (wavelength of 1.54 Å) for 3 min and each gave one useful 0.3° oscillation picture. The crystal-to-film distance was 100 mm. The F1 line was set at a shorter wavelength (0.9105 Å) and allowed at least two useful 30- or 60-s exposures from a single crystal. Ten films were processed from 4 crystals exposed to this beam with the film being 168 mm distant from the crystal.

The films were digitized using a Photoscan System P-1000 photo scanner with a 50-mm raster step. The data were processed and scaled as described in Zhang et al., 1993. Data between 10 and 3 Å were used giving a total of 78,617 full unique reflections and 60,785 partial unique reflections with ($F > 3\sigma$), representing 20% of observable data to 3-Å resolution. A complete data set would be 20-fold redundant. Since the electron density maps were averaged 20-fold over the non-crystallographic symmetry in HRV 14 crystals, the current data set would be approximately 20% of 20-fold, i.e. 4-fold redundant. The resultant images in the electron density maps are therefore comparable in quality to a complete data set. Native phases were used to calculated the maps. These phases were obtained from a 70.8% complete native data set and were iteratively averaged and reconstructed over the 20-fold non-crystallographic symmetry in the asymmetric unit (Arnold and Rossmann, 1988).

Since complete 100% occupancy is not expected for the drug in every available site and since the occupancy was not refined as a parameter, a series of adjusted maps were calculated with arbitrary fractions of native data (F_n) subtracted from the observed data set intensities (F_d), as explained by Smith et al. (1986). The equation used was: $kF_d - (1 - k)F_n$, where k is an adjustment factor. The map of choice was that which

showed the most continuous, non-disrupted density for both the compound and the amino acids in the coat proteins. In the case of 100% occupancy a pure difference ($F_d - F_n$) map would be calculated and would have a corresponding k-value of 0.5. A value greater than that describes the decrease in occupancy relative to a fully occupied site (i.e. more native-like). Modeling of the HRV 14/SDZ 35-682 complex was performed using a map calculated with $k = 0.55$ indicating an occupancy close to 100%. This contributes to the strong signal of the new density in the electron density map used.

The starting structure for SDZ 35-682 was modeled with the aid of the Biosym molecular graphics program Insight II, version 2.2.0, using the build mode (Biosym, Inc.) and using the coordinates for 4′-fluoro-4-(4-(2-pyridyl)-1-piperazinyl) butyrophenone (Koch et al., 1977) for the pyridyl-piperazinyl moiety. The nitrogen of the piperazine ring bound to the pyridine ring (N2, Fig. 1) adopts an almost planar configuration in this molecule. This configuration was incorporated into the model for SDZ 35-682. The starting model for the capsid was the coordinates for the refined native structure (Arnold and Rossmann, 1988; entry 4RHV in the Protein Data Bank). Electron density fitting was performed using computer graphics of the FRODO and O programs (Cambilleau and Horjales, 1987; Jones et al., 1991) on a 4D120 GTXB IRIX and an Indigo2 work station.

3. Results

3.1. *In vitro efficacy of SDZ 35-682*

Antiviral spectrum of SDZ 35-682

The activity of SDZ 35-682 against a series of human rhinovirus (HRV) serotypes and some enteroviruses was measured as inhibition of virus-induced CPE, and was expressed as minimum inhibitory concentrations (MIC, Table 1). The spectrum of antiviral activity, namely the capacity to inhibit certain rhinovirus serotypes, is clearly different from that of the previously described antipicornavirus compounds dichlorofla-

Table 1
Spectrum of picornavirus inhibition by SDZ 35-682

MIC [a] (μg/ml)	Virus inhibited
< 0.1	HRV type: 14, 26, 35, 37, 43, 48
0.3–1	ECHO 9
	HRV type: 3, 5, 55, 72
3–10	HRV type: 1A, 1B, 4, 8, 23, 29, 30, 32, 33, 40, 47, 50, 58, 62, 71, 75, 83
> 10	HRV type: 2, 13, 15, 16, 19, 20, 31, 38, 51, 64, 68, 77, 85
	Coxsackie B3, polio 2

[a] Minimum inhibitory concentration (MIC): concentration required to reduce virus-induced cytopathogenicity by at least 50%.
Minimum cytotoxic concentration was > 30 μg/ml.

Table 2
Effect of multiplicity of infection on the activity of SDZ 35-682 against ECHO 9

m.o.i. [a] (PFU/cell) [b]	MIC [c] (μ/ml)		
Day of reading:	1	2	3
1000	3.3	30	> 30
333	0.92	30	> 30
100	0.33	10	> 30
33	0.092	0.92	> 30
10	0.053	0.21	9.2
3	0.023	0.10	0.92
1	– *	0.067	0.33
0.3	– *	0.067	0.33

[a] m.o.i. = multiplicity of infection.
[b] PFU = plaque-forming units.
[c] Minimum inhibitory concentration (MIC): concentration required to reduce virus-induced cytopathogenicity by at least 50%.
* No cytopathic effect detectable yet.

van, Ro 09-0410, WIN 51711, R 61837, R 77975, and SCH 38057 (Bauer et al., 1981; Ninomiya et al., 1985; Otto et al., 1985; Andries et al., 1990, 1992; Rozhon et al., 1993). As is characteristic for this group of capsid-binding compounds, SDZ 35-682 is highly active against certain HRV serotypes with MIC values of 0.1 μg/ml and has no, or low activity against others. Surprisingly, it effectively inhibits echovirus 9 replication, but is inactive against poliovirus type 2 and coxsackie virus B3. Dose–response curves were steep: at concentrations 3-fold higher than MIC values inhibition of CPE was essentially complete (> 90%).

Inhibitory potency of SDZ 35-682

It is well known that experimental conditions like m.o.i. do greatly influence the determination of MIC of drugs. The dependence of MIC values obtained for SDZ 35-682 on m.o.i. and on the day postinfection, when virus-induced cytopathic effect was measured, is demonstrated in Table 2. Obviously, MICs are lowest with low m.o.i. and early reading: with an m.o.i. $\leqslant 1$ MIC values of 0.067–0.33 were measured against echovirus type 9 on days 2 and 3 postinfection, respectively.

Inhibition of single-cycle growth of echovirus 9 by SDZ 35-682

GMK cell monolayers were infected with a multiplicity of infection (10 PFU/cell) sufficient to ensure synchronous replication of the virus. Under these conditions, typical single-cycle growth curves are obtained (Fig. 2). In the untreated control culture the inoculum virus titer slightly decreased until 3–4 h postinfection (h.p.i.); then, from 5 h.p.i. on, virus replication was evident: a steep increase of virus titers was observed. In the presence of 10 μg/ml of SDZ 35-682 the inoculum virus titer decreased, too, but only a minor increase was detectable at 6–7 h.p.i. indicating some breakthrough of virus replication. Thus, SDZ 35-682 inhibits echovirus 9 replication by more than two orders of magnitude also under conditions of high inoculum and single-cycle growth.

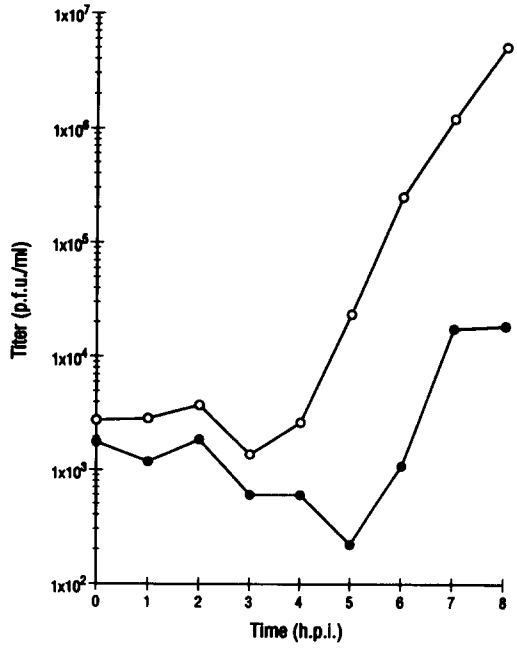


Fig. 2. Time course of ECHO 9 replication in the absence and presence of SDZ 35-682. Confluent GMK cell monolayers were inoculated with virus at a multiplicity of infection of 10 PFU/cell in the absence or presence of 10 μg/ml SDZ 35-682. The drug was present from 0.5 h before infection on throughout the experiment. After adsorption (4°C, 1 h) the inoculum was removed and cell culture medium with or without SDZ 35-682 was added. Aliquots of the infected cultures were harvested at different times postinfection. Lysates were prepared and quantitated by plaque assay in the absence of drug. ○, without SDZ 35-682; ●, with 10 μg/ml SDZ 35-682.

Inhibition of multiple-cycle growth of echovirus 9 by SDZ 35-682

To form a plaque in a GMK cell monolayer, multiple cycles of echovirus 9 replication have to occur. The effect of 1 μg/ml of SDZ 35-682 on echovirus 9 plaque formation in GMK cells, when being present for different time periods, was determined. At this concentration SDZ 35-682 reduced plaque formation by more than 1000-fold when being present from 10 min before infection until the reading of plaque numbers. Pretreatment of cells prior to virus adsorption reduced plaque numbers by only a factor of two, which points to a viral and not to a cellular target for this compound. This minor effect may indicate that SDZ 35-682 could not be completely removed from the cells by washing. When SDZ 35-682 was present only during the 1-h adsorption period, significant inhibition (80.6%) was observed, which suggests an effect of the drug on early processes of virus replication. When SDZ 35-682 was added to the agar overlay, but was not present during the adsorption period, 85.0% inhibition was measured as well. This apparent discrepancy is easily explained by the multi-cycle nature of plaque formation. Inhibition of events in the first hour of the first cycle of virus replication clearly is effective in blocking plaque formation. However, inhibition of early events in the second and further cycles of viral growth will also reduce plaque formation.

Obviously, maximal inhibitory effects (> 99.9% inhibition) are obtained when the compound is added early in the replication cycle and remains present throughout the multiple cycles of virus infection leading to plaque formation.

Effect of SDZ 35-682 on cell proliferation

The in vitro cytotoxic/cytostatic potential of SDZ 35-682 was assessed by measuring the effects of the drug on cell growth. GMK or HeLa (Ohio) cell numbers increased approximately 10-fold over a period of 4 days. Up to 30 μg/ml of SDZ 35-682 did not exert a significant inhibition of cell proliferation (data not shown).

3.2. Mode of antiviral action of SDZ 35-682

Effects of time of addition or removal of SDZ 35-682 on yield of echovirus 9 or HRV 14

The results of the plaque reduction experiment suggested that SDZ 35-682 inhibits an early event in viral replication. To confirm this, GMK or HeLa (Ohio) cells were infected with echovirus 9 or HRV 14, respectively, and compound was added either at time −1 (i.e. immediately before virus), time 0 (i.e. post 1 h adsorption at 20°C) or 2, respectively 3, h after the end of the adsorption period. The drug remained present until virus was harvested at the times indicated. Or, compound was added at time −1 and was removed by washing with phosphate-buffered solution (PBS) at time 0 or 2, respectively 3. Virus yields obtained at the indicated hours postinfection were quantitated by plaque assay in the absence of test compound.

As in the plaque reduction assay, the inhibitory effect on echovirus 9 and HRV 14 replication was maximal when SDZ 35-682 was added immediately before the virus; thus, was already present during adsorption (Table 3). It was slightly less pronounced when the addition of compound was postadsorption, and inhibition was significantly reduced, but not completely abolished, when SDZ 35-692 was added after 2 or 3 h of

Table 3
Effect of time of addition of SDZ 35-682 on inhibition of ECHO 9 and HRV 14 replication

Virus	Time of addition (h)	Yield (PFU, % control) [a] at h postinfection				
		0	2	4	6	8
ECHO 9	−1	0.035	< 0.01	< 0.01	0.67	3.5
	0		0.053	0.017	2.1	6.3
	2			< 0.01	6.3	24
	Untreated control	0.16	0.14	0.36	50	100
		0	3	6	9	12
HRV 14	−1	1.97	0.16	0.29	2.5	4.7
	0		1.0	1.6	2.0	7.9
	3			1.1	13.2	17.9
	Untreated control	0.47	2.0	3.3	100	62

[a] Virus yields obtained at the indicated times in the presence or absence of 1 μg/ml of SDZ 35-682 were determined by plaque assay in the absence of compound; PFU = plaque forming units; control = highest yield obtained in the untreated culture; time −1 = immediately before virus; time 0 = post 1 h adsorption at 20°C.

Table 4
Effect of time of removal of SDZ 35-682 on inhibition of ECHO 9 and HRV 14 replication

Virus	Time of removal (h)	Yield (PFU, % control) [a] at h postinfection				
		0	2	4	6	8
ECHO 9	0		0.067	0.038	11	21.9
	2			< 0.01	2.8	4.9
	Untreated control	0.094	0.19	0.36	74	100
		0	3	6	9	12
HRV 14	0		0.67	0.43	74	17
	3			0.17	3.2	12
	Untreated control	0.7	1.7	2.8	100	70

[a] Virus yields obtained at the indicated times in the presence or absence of 1 μg/ml of SDZ 35-682 were determined by plaque assay in the absence of compound; PFU = plaque forming units; control = highest yield obtained in the untreated culture; time 0 = post 1 h adsorption at 20°C.

virus replication at 37 or 34°C, respectively. These results indicate, that the event in the viral replication cycle being susceptible to SDZ 35-682 inhibition takes place during, but also shortly after, the adsorption period.

The time-of-removal experiment (Table 4) yielded a similar result: removal of compound immediately after adsorption (time 0) reduced significantly, but not completely, the antiviral efficacy of SDZ 35-682; though it has to be kept in mind, that SDZ 35-682, possibly, was not completely removed by washing. Removal of test compound after 2, respectively 3, h of virus replication, obviously, was too late to impair the inhibitory effect.

The conclusion from the results of the time of addition and the time of removal experiment is, that SDZ 35-682 interferes with events at and shortly after adsorption of the virus.

Effect of SDZ 35-682 on echovirus 9 infectivity

SDZ 35-682 did not significantly reduce infectivity of echovirus 9. After a 1-h incubation of virus stock at room temperature in the presence or absence of 40 μg/ml of SDZ 35-682, a concentration more than 100 times the MIC in cell culture, virus titers as measured by plaque assay were indistinguishable from those of the untreated controls (data not shown).

Effect of SDZ 35-682 on echovirus 9 adsorption

Echovirus 9, radioactively labeled with [^{3}H]uridine and purified as published previously (Rosenwirth and Eggers, 1978a), adsorbed to GMK cells at the same rate in the presence or absence of 30 μg/ml SDZ 35-682, when high m.o.i. ($\geqslant$ 10 PFU/cell) was used (Rosenwirth and Eggers, 1982). Under these conditions, the drug had no detectable inhibitory effect on adsorption (data not shown).

However, under conditions of low m.o.i., i.e. when echovirus 9 was diluted to give countable plaque numbers in GMK cell monolayers, a different result was obtained. The virus was adsorbed at 20°C in the presence of 10 μg/ml of drug to GMK cells already

Table 5
Effect of SDZ 35-682 on uncoating of neutral red-sensitized ECHO 9

Time at 37°C (h)	Irradiation	Infective centers per plate [a]	
		Untreated	SDZ 35-682
0	−	6.0×10^3	4.2×10^3
	+	$<1\times10^1$	$<1\times10^1$
3	−	1.5×10^4	1.8×10^3
	+	2.1×10^4	1.1×10^2
5	−	4.7×10^4	2.0×10^3
	+	3.6×10^4	1.7×10^2

[a] Neutral red-sensitized echovirus 9 was adsorbed to GMK cells at 4°C for 1 h in the dark in the absence and presence of 20 μg/ml SDZ 35-682. Some cultures were transferred to 37°C in the dark for 3 or 5 h; some cultures were exposed to light. Then, cells were trypsinized, the cell suspensions were diluted and plated on fresh monolayers for infectious center assay.

pretreated for 1 h, then compound was removed by washing, and plaque formation was allowed in the absence of SDZ 35-682. In this experiment plaque numbers obtained were 10-fold lower for drug-treated samples than for controls (data not shown). This result indicates an inhibitory effect to SDZ 35-682 on adsorption.

Effect of SDZ 35-682 on echovirus 9 uncoating

Echovirus 9 grown in the presence of neutral red is photosensitive as long as the dye remains within the viral capsid (Kato and Eggers, 1969; Eggers and Waidner, 1970), i.e. as long as uncoating has not taken place. Neutral red-sensitized echovirus 9 was adsorbed to GMK cells in the dark at 4°C in the absence or presence of SDZ 35-682. Aliquots of the cultures were irradiated after adsorption or at the indicated times after transfer to 37°C. The capacity of irradiated cells and of those kept in the dark to form infectious centers was assayed. The results show (Table 5), that at the end of the adsorption period, in the absence or presence of drug, all of the virus was photosensitive, indicating that no uncoating took place at 4°C. Already after 3 h at 37°C the capacity of the untreated, infected cells to cause infectious centers had become completely photoresistant. On the other hand, cells infected and incubated in the presence of SDZ 35-682 up to 5 h.p.i. were still up to 90% sensitive to irradiation. This result clearly demonstrates inhibition of echovirus 9 uncoating by SDZ 35-682. Notably, the number of infectious centers caused by drug-treated cells after 3 or 5 h at 37°C in the dark was about 10-fold lower than that of the control, indicating again, in addition, an influence of SDZ 35-682 on adsorption.

X-ray structural analysis of SDZ 35-682 bound to HRV 14

The electron density in the antiviral agent binding pocket that does not correspond to protein atoms was strong and continuous (Fig. 3A). The density was long (19 Å), filling the pocket entirely. It had a bend at the center forming an angle of about 140° and a two-lobed density on either end, suitable for two adjacent rings. SDZ 35-682, which has two adjacent rings on either end, can be fit into these lobes in either orientation. From

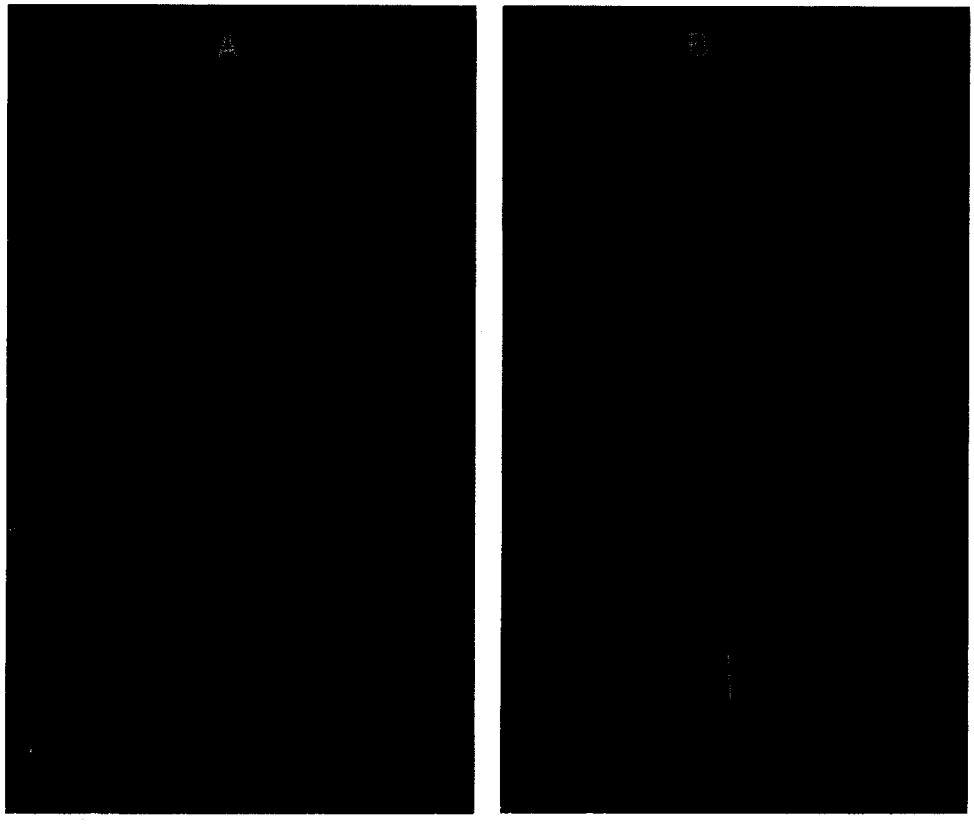


Fig. 3. X-ray results of SDZ 35-682 binding to HRV 14. Both A and B are viewed in the same orientation. (A) The electron density for SDZ 35-682, contoured at 2δ, as seen in the difference map calculated with a coefficient of 0.55. The density entirely fills the pocket. It has a 140° bend in the center. The aromatic rings in the deeper end of the binding pocket are shown (Tyr^{128}, Tyr^{152} and Phe^{186}). (B) Two possible conformations of SDZ 35-682, color coded by atom, as fit in the electron density map in A. The two best fitting models of SDZ 35-682 in the density were of opposite orientation, one (yellow) having the cyclohexane ring deepest in the pocket (down) and the other having the pyridine ring at the innermost end (green).

the shape of these lobes it is not clear which correspond to aromatic, flat rings. Fitting the compound in either orientation allows for aromatic interactions with tyrosine residues in the pocket. The central portion of the density is slightly bent and has a small bulge that could correspond to the hydroxyl oxygen. Since there is a racemic mixture of two enantiomers (C12 asymmetric carbon), both enantiomers were tested for their fit to the density. Only the *S*-enantiomer fit well into this bulge and gave possible hydrogen bonding geometry with the hydroxyl group of Tyr^{128} of VP1. At 3 Å resolution, this is consistent with the density around Tyr^{128} being connected to the density for the compound. Possibly, the compound appears in the pocket in two orientations (Fig. 3B), one with the pyridine ring deep in the interior and one with it at the entrance. The two orientations would be slightly shifted relative to each other as a result of a requirement for hydrogen bond with Tyr 128. Therefore, if the density in the pocket is average of

both orientations, then the sum would be longer than each individual orientation. Thus, SDZ 35-682 could not unambiguously be placed in the electron density. Despite this ambiguity, a number of conclusions can be inferred from the appearance of the electron density and the approximate fitting of the two orientations. Residues in contact with this density (within 4 Å from atoms of both conformations) are Ile^{104}, Leu^{106}, Ser^{107}, Leu^{116}, Phe^{124}, Ser^{126}, Tyr^{128}, Tyr^{152}, Pro^{174}, Val^{176}, Phe^{186}, Val^{188}, Val^{191}, Tyr^{197}, Ile^{215}, Met^{224} and His^{245} of VP1 and Ala^{24} of VP3.

A significant movement of protein residues is observed in all structures of HRV 14 complexed to antiviral agents (for a review see Zhang et al., 1992). This movement was observed in the electron density and the protein coordinates were modified to fit the new density. Side chain torsion angles of residues in the pocket had to be adjusted as well. Although the protein conformation may depend on the orientation of the antiviral compound, we see no evidence for multiple protein conformations which might be signaled by weak or broad electron density. As a result, shifts of up to 8 Å were observed for some side chain atoms, even for residues that do not directly contact (within 4 Å) the antiviral agent. The residues with largest conformational shifts were 215–222 of VP1 which are part of the FMDV loop implicated as being part of the receptor binding site, in the floor of the canyon. This is expected to alter the binding affinities of virions to cells. Other residues adjusted were 103–104 and 151–155. The displacement of α carbons relative to the native HRV 14 structure are of a similar magnitude as those observed in the structures of HRV 14–WIN complexes (Smith et al., 1986; Badger et al., 1988; Chapman et al., 1991) and less than those in the HRV 14–SCH 38057 complex (Zhang et al., 1993).

The side chain density of Tyr^{128} of VP1 is merged with the density of the compound. This most likely indicates the formation of a hydrogen bond. In either orientation, atoms O_1 and N_3 of the compound are within the distance range expected for forming a hydrogen bond with the phenoxyl group of Tyr^{128}. There do not seem to be any other hydrophilic interactions between the protein and the antiviral agent.

As observed by Zhang et al. (1993), there is a consensus in the binding positions of aromatic groups belonging to a diverse set of antiviral agents. The authors suggested that aromatic interactions between the compounds and the protein may determine their positioning in the pocket. Here, too, the aromatic rings play a critical role. The two aromatic rings of the compound (phenoxy and pyridine) lie in the same vicinities that are occupied by aromatic groups of other antiviral agents complexed with HRV 14. Another portion of density for SDZ 35-682 makes a continuous connection with the electron density for the aromatic side chain of Phe^{186} of VP1. The arrangement and shape of these densities are consistent with an edge-to-face interaction between the pyridine ring of SDZ 35-682 and the phenyl ring of Phe^{186}. This interaction would be possible only in the case where the compound is oriented with the pyridine end being deepest in the pocket.

4. Discussion

SDZ 35-682 is demonstrated to be a potent and selective inhibitor of the replication of members of the picornavirus group. It inhibits several rhinovirus serotypes and

echovirus 9 at concentrations as low as 0.1 μg/ml, without exerting any effect on cell proliferation up to 30 μg/ml. Its spectrum of activity seems to be limited to rhinoviruses and the enterovirus ECHO 9; no inhibition of herpes simplex virus types 1 and 2, influenza virus type A and respiratory syncytial virus was detectable (data not shown). As observed with other capsid-binding antipicornavirus compounds, there is a wide variation in sensitivity of the different serotypes within the rhinovirus group, with some showing complete insusceptibility. The spectrum of activity of SDZ 35-682 clearly differs from that of previously described inhibitors. Dichloroflavan, e.g., potently inhibits HRV 2 and does not impair replication of HRV 35 (Ninomya et al., 1985), for SDZ 35-682 the reverse is true. The chalcone Ro 09-0410, too, is a good inhibitor of HRV 2, HRV 15 and HRV 16 (Ninomya et al., 1990), which are refractory to inhibition by SDZ 35-682. WIN 51711 resembles SDZ 35-682 in being a good inhibitor of HRV 3, HRV 5 and HRV 14, but again HRV 2 and HRV 15 behave differently: they are inhibited by WIN 51711 but not by SDZ 35-682 (Otto et al., 1985). The comparison of the antirhinovirus spectrum of SDZ 35-682 with R 61837 and WIN 51711 with regard to subdivision of rhinovirus serotypes in two groups (Andries et al., 1990), reveals, that the serotypes potently inhibited by SDZ 35-682, with the exception of HRV 55, all belong to group A, while R 61837 inhibits preferentially members of group B; WIN 51711 in this comparison is, with the exception of HRV 42, more active against members of group A like SDZ 35-682. Pirodavir (R 77975), a more recent derivative of the group of pyridazineamines (Andries et al., 1992) has a very broad antirhinovirus spectrum; however, HRV 48, which is very sensitive to inhibition by SDZ 35-682, is relatively refractory to inhibition by Pirodavir. Thus, as jugded from the limited number of rhinovirus serotypes tested, SDZ 35-682 differs in antiviral spectrum from all previously described antipicornavirus compounds. The large variation in sensitivity to SDZ 35-682 of different rhinovirus serotypes indicates that the compound possesses specific antiviral activity.

The inhibitory potency of SDZ 35-682 is largely dependent on the multiplicity of infection (m.o.i.): the lower the m.o.i., the lower was the minimum inhibitory concentration. The inhibitory effect of SDZ 35-682 was demonstrated under conditions of high m.o.i. in a single-cycle growth experiment, and under conditions of low m.o.i., namely in a plaque assay. SDZ 35-682 reduced virus yield by more than two orders of magnitude in the single-cycle growth experiment. In the plaque assay, the test compound proved most effective when added before virus and present throughout the experiment. When present only during the adsorption period SDZ 35-682 caused significant inhibition of plaque formation, too. In time of addition and time of removal experiments, SDZ 35-682 showed maximal inhibitory efficacy when added before the virus; it was slightly less effective when added after adsorption. When the compound was added after 2 (for echovirus 9) or 3 h (for HRV 14) of virus replication, its antiviral effect was significantly reduced. If SDZ 35-682 was added before the virus and was removed after adsorption, inhibition was reduced, but still evident. Removal of drug after 2, respectively 3, h of virus replication was too late to reverse the inhibitory effect of the compound. These results clearly indicate that the point of interference of SDZ 35-682 with viral multiplication is an early event taking place before 2, respectively 3, h of virus replication; at least partly, it may be the adsorption process itself.

No direct and irreversible effect of SDZ 35-682 on the infectivity of virus particles could be detected. Adsorption of radiolabeled echovirus 9 particles to cells was not inhibited by SDZ 35-682. However, when test compound was present only before and during adsorption under plaque assay conditions, significant reduction ($\geqslant 80\%$) of plaque formation was achieved. Incorporation of neutral red into the viral capsid renders the particles photosensitive. Uncoating of the infecting virus leads to release of the neutral red from the capsid, and from this moment on, infection is photoresistant. Inhibition of uncoating by SDZ 35-682 was clearly demonstrated in this experimental setting: while after 3 h at 37°C, the capacity of untreated, infected cells to cause infectious centers was completely photoresistant, cells infected in the presence of SDZ 35-682 still contained virus being up to 90% sensitive to irradiation. Thus, SDZ 35-682, like the members of other classes of antipicornavirus agents mentioned above, is an inhibitor of the uncoating process of echovirus 9. It may, in addition, interfere, to some extent, with adsorption.

It was expected that SDZ 35-682, like the other uncoating inhibitors of picornaviruses, would bind to the hydrophobic pocket beneath the canyon floor (Zhang et al., 1992). Since echovirus 9 has not been crystallized so far, cocrystallization of SDZ 35-682 with HRV 14, which is highly sensitive to inhibition by this compound, was performed. As suggested by Chapman et al. (1991), it may be beneficial for the compounds to fill the innermost end of the pocket and that the space should be occupied efficiently. SDZ 35-682 is an example of just such a compound. It is 19 Å long from end to end and thus can fill the entire hydrophobic pocket. It is less flexible than other long antiviral agents such as WIN 51711, since it has more rings and consequently less torsional freedom. This reduction in flexibility has not prevented the compound from entering the pocket. Actually, the rigidity may favor binding to the sterically restricted pocket, since there would not be as great a change in the entropy upon binding as in the case of a more flexible compound.

As seen in the X-ray structure, SDZ 35-682 imparts a similar pattern of conformational changes to the receptor binding site-of HRV 14 as do other antiviral agents likely interfering with receptor attachment (Pevear et al., 1989). Thus, it is expected that part of the antiviral effect of SDZ 35-682 against HRV 14 is due to disruption of attachment during adsorption. These changes do not correlate directly to magnitude of inhibition. The reverse has actually been noted. In the case of SCH 38057 (Zhang et al., 1993), the compound imparts larger than average conformational changes to the protein, yet it is one of the weaker antiviral agents crystallographically studied. It has been suggested in a review by Zhang et al. (1992) that compounds not filling the entire hydrophobic pocket may allow the pocket sufficient degrees of flexibility to only partially affect the uncoating process of the virion. In the case of SDZ 35-682, there is very little volume in the pocket that is unoccupied. This may contribute to the inhibition of viral uncoating, as demonstrated with echovirus 9.

In conclusion, SDZ 35-682 belongs to the chemically very divergent 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 compounds with broadspectrum antipicornaviral activity.

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