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Antipicornavirus activity of SCH 47802 and analogs: in vitro and in vivo studies

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Abstract

SCH 47802 and its derivatives are potent inhibitors of enteroviruses in vitro. The IC_{50} for SCH 47802 ranges from 0.03 to 10 μg/ml when tested against a spectrum of enteroviruses in plaque reduction assays. The compounds have in vitro therapeutic indices of at least 81 based on viral cytopathic effect (CPE) assays. The in vitro activity of SCH 47802 translates into in vivo activity in the murine model of poliovirus encephalitis. In an oral dosing regimen, SCH 47802 protects mice from mortality at 60 mg/kg per day. Consistent with the in vivo efficacy, pharmacokinetic analyses after oral dosing with SCH 47802 demonstrate serum levels of the compound above the in vitro IC_{50} for poliovirus for at least 4 h. SCH 47802 and its active analogs stabilize poliovirus to thermal inactivation indicating that the compounds bind to the virus capsid. Mechanistic studies with poliovirus indicate that SCH 47802 acts early in viral infection. This series of molecules represents potential candidates for the treatment of human enterovirus infections.

Keywords: Picornaviruses; Enteroviruses; Antiviral agent; SCH 47802

1. Introduction

The family Picornavirus contains three groups of human pathogenic viruses: enteroviruses, rhinoviruses and hepatitis A. These groups share a

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unique structural organization of viral capsid as delineated by the crystallization and resolution of the three-dimensional structure of human rhinovirus 14 (HRV-14) and poliovirus (Rossmann et al., 1985; Hogle et al., 1985; Rueckert, 1990). The capsid, which encapsulates the viral RNA, is composed of 60 protomers each of which contains one copy of the viral proteins VP1, VP2, VP3 and VP4. Among the features on the viral capsid is the 'canyon' formed by the junctions of VP1 and VP3. The canyon serves as the site for binding to the viral cellular receptor, e.g. ICAM-1 for HRV-14 (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989). Underneath the canyon lies a hydrophobic cavity which is accessible through a pore in the canyon floor and serves as the binding site for capsid-binding inhibitors.

A variety of diverse structures have been shown to bind to the viral capsid including rhodanine (Eggers et al., 1970; Eggers, 1985), flavanoids (Ishitsuka et al., 1982), a series of aralkylaminopyridines (Kenny et al., 1985, 1987), a series of oxazolinyl isoxazole compounds (e.g. arildone, WIN 51711, WIN 54954: Diana et al., 1985; McSharry et al., 1979; Otto et al., 1985; Woods et al., 1989) a pyridazinamine series (R61837, R77975: Andries et al., 1988, 1992) and a (phenoxyalkyl) imidazole series (SCH 38057: Rozhon et al., 1993). X-ray crystallographic studies of crystals of HRV-14 infused with WIN molecules (Badger et al., 1988; Rossmann, 1989; Smith et al., 1986), with R61837 (Chapman et al., 1991) or with SCH 38057 (Zhang et al., 1991, 1992) and the analysis of mutant viruses which demonstrate cross-resistance to several of the above compounds (Heinz et al., 1989; Heinz, 1990) confirm that these molecules bind to the hydrophobic cavity underneath the canyon in VP1. A number of compounds active against rhinoviruses and enteroviruses have been investigated in clinical studies (Al-Nakib et al., 1989; Barrow et al., 1990; Hayden et al., 1992, Schiff et al., 1992; Turner et al., 1993). To date no compound has been approved for diseases caused by rhinoviruses or enteroviruses.

This paper describes the identification of a series of molecules which bind to the picornavirus capsid and possess potent biological activity. These molecules were identified using a rapid screening assay which identified structures that prevented thermal inactivation of poliovirus and thus suggested potential interaction with the viral capsid. Molecular projections based on the X-ray crystallography of an early inhibitor, SCH 38057, in the VP1 canyon of HRV-14 also assisted in the design of these inhibitors (Zhang et al., 1991, 1992, Rozhon et al., 1993; O'Connell et al., 1995).

2. Materials and methods

2.1. Compounds

SCH 47802 and its derivatives were synthesized at the Schering-Plough Research Institute (Girijavallabhan et al., 1995).

2.2. Viruses

Poliovirus type 2 (MEF) and enteroviruses were obtained from Dr M. Pallansch, Centers for Disease Control, Atlanta, Georgia or from the American Type Culture Collection (ATCC). Poliovirus and coxsackieviruses were propagated in HeLa cells and echoviruses in BGMK cells, and virus working stocks were prepared as cellular lysates. Standard procedures for quantitation of virus by plaque assay and storing viral stocks have been described (Trousdale et al., 1977; Rueckert and Pallansch, 1981).

2.3. Cytopathic effect (CPE) assay

This assay was used to determine the antiviral (IC_{50}) and cytotoxic (LC_{50}) levels of the compounds as well as their therapeutic indices (cytotoxicity LC_{50}/antiviral IC_{50}). Test compound and virus were pre-mixed for 45 min at 22°C and used to infect cells (multiplicity of infection (MOI) = 1.0) in 96-well microelisa plates. Following a 45-min incubation (37°C), the culture fluid was aspirated and the monolayers washed. Eagle's modified minimal essential medium (EMEM) with 1% fetal calf serum (FCS) containing the compound at the same concentration as used for the pre-incubation step was added, and incubation continued for 18–24 h prior to measuring cellular viability with MTT (3-(4,5-dimethylthiazol-2-yl)-

2,5-diphenyl tetrazolium bromide; Sigma) formazan (Mossmann, 1983).

2.4. Pre-mix plaque assay

One hundred and fifty plaque forming units (PFU; infectious particles) were mixed with test compound (0.0001–50 μg/ml in 1% dimethyl sulfoxide, DMSO) in EMEM with 1% FCS for 45 min and added to monolayers of appropriate cells. After 45 min at 33°C, the inoculum and compound mixture were aspirated, the cells washed, and incubated with a methylcellulose overlay for 2–3 days without further addition of compound (Rozhon et al., 1993). The number of PFU was determined at each concentration and plotted as a percentage of control.

2.5. In vivo evaluations

Studies with mice were performed as recommended in the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23). Groups of 15–25 male mice (16–20 g; Harlan Sprague Dawley) were infected intracranially with 5.2×10^5 PFU of poliovirus type 2 (polio-2). Treatment with compounds was by oral gavage in 0.3 ml of corn oil beginning 24 h before infection. Survival was monitored daily for 21 days. Results were evaluated for statistical significance using χ^2 analysis at $P < 0.05$ (Davies and Goldsmith, 1972).

An additional analysis of efficacy called AUC activity (area under the curve activity) is calculated from the following formula:

$$\text{AUC Activity} = \frac{\text{exp. AUC} - \text{placebo AUC}}{\text{max. AUC} - \text{placebo AUC}} \times 100.$$

This calculation represents the area under the survival versus days curve (AUC) and normalizes it for the placebo group, and provides a distinct advantage in that it allows for direct comparison of efficacy obtained in different regimens while accounting for changes in the mortality of the placebo group.

2.6. Pharmacokinetics

Mice were administered SCH 47802 in corn oil by oral gavage and bled by cardiac puncture at various times after dosing. Levels of SCH 47802 were determined by high pressure liquid chromatography (HPLC).

2.7. Thermal inactivation

10^7 PFU of purified polio-2 were incubated with compound for 45 min at 22°C, then shifted to 50°C for 20 min. Aliquots were then diluted 7-fold in EMEM to concentrations of compound which were not inhibitory and recovered virus was measured by plaque analysis or by CPE assay.

2.8. Extraction with chloroform

Polio-2 was mixed with SCH 47802 at 1 μg/ml for 45 min at 22°C in a final volume of 1 ml. For extraction, an equal volume of 100% $CHCl_3$ was added, the sample vortexed, and centrifuged at $5000 \times g$ for 1 min. The aqueous phase, which contained virus, was collected and the titer was determined by plaque assay.

2.9. Yield assay

SCH 47802 (dose range 10–0.001 μg/ml) was pre-incubated with polio-2 (MOI = 10) for 45 min at 22°C. Monolayers of HeLa cells were inoculated with the virus–SCH 47802 mixture for 45 min, after which the monolayers were washed and fresh medium without compound was added. Alternatively, virus was added to cells for 45 min, monolayers were washed and compound was added at different times after infection. Following a 6-h incubation at 37°C, the monolayer was washed, the cells removed by scraping, and virus was recovered by lysing cells using three cycles of freeze–thawing (methanol–dry ice/room temperature cycles). Finally, cellular lysates were clarified by centrifugation ($500 \times g$ for 10 min) and virus in the supernatant was quantitated by plaque assay.

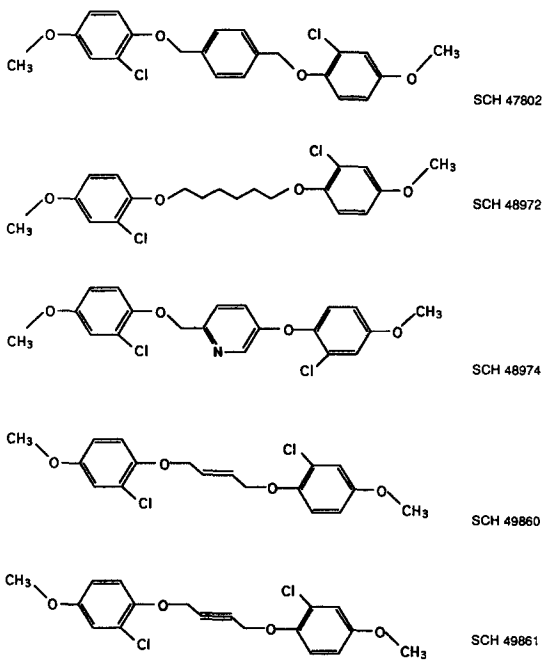

Fig. 1. SCH 47802 and analogs.

3. Results

3.1. Structures of antiviral molecules

The structures of SCH 47802, SCH 48972, SCH 48974, SCH 49860, SCH 49861 are shown in Fig. 1.

3.2. In vitro antiviral activity

The antiviral activity of the compounds as determined by an MTT-based CPE assay using poliovirus demonstrates that the molecules are potent antiviral agents with substantial therapeutic indices of at least 81 (Table 1).

To test for the broadness of antiviral activity, plaque reduction assays were performed using a panel of enteroviruses including 12 representatives of the 15 common enterovirus strains (Table 2). The molecules demonstrated potent activity in the range 0.02–10 μg/ml. Cytotoxicity assays performed on both HeLa and RD cells in a similar manner as the plaque assays (virus/compound washed away after 2 h) indicated that the LC_{50} for the compounds was greater than 50 μg/ml (data not shown). Thus, similar to the MTT-CPE results, the molecules possess anti-enterovirus activity in the absence of significant cytotoxicity.

3.3. In vivo evaluations

To assess whether in vitro antiviral activity translated to in vivo activity the compounds were screened in a murine model of poliovirus-induced encephalitis (Jubelt et al., 1980). This model is a stringent test of antiviral activity since direct inoculation of virus leads to high mortality and high virus loads in the target tissues, brain and spinal cord. The compounds SCH 49860, SCH 48961, and SCH 48972 failed to demonstrate significant oral activity in mice at 40 mg/kg per day (data not shown). SCH 48974 had a marginal effect on survival when administered at 90 mg/kg per day (Table 3). Prophylactic administration of SCH 47802 protected mice from lethality when administered orally in dosages of 60, 90 and 120 mg/kg per day (Fig. 2). At these dosages the survival on day 21 was 57%, 47% and 66%, at 60, 90 and 120

Table 1
Antiviral activity and therapeutic indices (T.I.) for SCH 47802 and its derivatives against polio-2 as determined in an MTT-based CPE assay[a]

Compound	Antiviral IC_{50} (μg/ml)	Cytotoxicity LC_{50} (μg/ml)	T.I.
SCH 47802	0.01 ± 0.001	23.0 ± 3.3	2300
SCH 48974	0.08 ± 0.03	$>50 \pm 0$	>625
SCH 49860	0.15 ± 0.05	$>50 \pm 0$	>333
SCH 48961	0.26 ± 0.08	$>50 \pm 0$	>192
SCH 48972	0.62 ± 0.28	$>50 \pm 0$	>81

[a] IC_{50} values represent the mean and standard error of three experiments.

Table 2
Antiviral spectrum of SCH 47802 and derivatives as determined by plaque assay[a,b]

Virus	SCH 47802	SCH 48974	SCH 49860	SCH 49861	SCH 48972
Polio	0.04	0.03	0.10	0.04	0.3
Echo 3	8.00	10.00	–	–	–
Echo 4	0.04	0.25	0.20	0.10	1.00
Echo 5	7.00	4.5	–	–	–
Echo 6	0.06	0.12	0.20	0.20	0.4
Echo 7	0.05	0.38	0.30	0.12	1.7
Echo 11	5.5	3.6	–	–	–
Echo 30	1.6	0.30	–	–	–
CVA9	0.02	0.14	0.10	0.02	0.20
CVB1	>10	4.00	–	–	22
CVB2	0.06	0.1	–	–	–
CVB3	>10	>10	–	–	–
CVB5	<10	>10	–	–	–

[a] Values represent mean IC_{50} in μg/ml as determined by plaque assay (1–5 experiments).
[b] All viruses except polio-2 are representative of the 15 most common enterovirus strains.

mg/kg per day, respectively. Survival in SCH 47802-treated mice was significantly different from controls for 12–15 days and the activity based on AUC was 54–76% (Table 3). At the lower dosages of 20 mg/kg per day the activity was not significant.

3.4. Pharmacokinetics of SCH 47802

Single oral administration of SCH 47802 at a dose of 45 mg/kg yielded a C_{max} of 1.2–1.3 μg/ml (Fig. 3). There is a plateau in plasma drug level between 1 and 2 h after dosing. This is likely due to the slow absorption of compound in corn oil suspension. The serum level of SCH 47802 at 4 h (0.62 μg/ml) remains well above the IC_{50} for poliovirus (0.04 μg/ml).

3.5. Thermal stability and virucidal assays

SCH 47802 and its derivatives stabilize virus against thermal inactivation. The concentrations necessary to reduce the loss of virus by heat by 50% were in the range 0.004–0.1 μg/ml (Table 4). Structure/activity comparisons indicate that the two molecules, SCH 47802 and SCH 48974, having a middle aromatic ring system were more potent inhibitors of thermal stability (IC_{50} of 0.004 μg/ml). Consistent with the ability to recover of SCH 47802-treated virus after thermal exposure, the binding of SCH 47802 to virus is not virucidal. After incubation of the poliovirus with the compound at 1 μg/ml and extraction with chloroform, the virus titer was not significantly different from solvent-treated virus (data not shown).

3.6. SCH 47802 acts early in virus life-cycle to inhibit viral uncoating

The time-course addition of SCH 47802 indicates that the molecule acts early in the virus replicative cycle (Fig. 4). Pre-incubation of SCH 47802 with poliovirus reduced viral yield, quantitated 6 h after infection, by 1.2 logs, while addition of compound at 1 h after infection had no effect on viral titers.

4. Discussion

SCH 47802 and its related derivatives possess potent in vitro anti-enterovirus activity as demonstrated in MTT-based CPE and plaque reduction assays. A compound for the treatment of enterovirus disease must have broad activity against a number of serotypes (Rotbart, 1989). Fifteen enterovirus serotypes account for the majority

Table 3
Oral efficacy of SCH 47802 and derivatives in the murine poliovirus encephalitis model

Compound	Dosage[a] (mg/kg)	Days of significance[b]	Activity (based on AUC)[c] (%)
SCH 47802	120	14	74
	90	12	54
	60	15	76
	20	1	20
SCH 48974	90	6	17
	20	0	6
SCH 49860	40	0	0
SCH 48961	40	0	0
SCH 48972	40	0	0

[a] Compounds were administered prophylactically beginning 24 h before infection and continued for 15–21 days. The total mg/kg dose was administered in two equal dosages 8 h apart.
[b] The number of days of significance was determined by χ^2 analysis ($P<0.05$) on each day of the study compared to placebo.
[c] The degree of activity was determined by comparing the area under the survival curve (AUC) for the experimental group with that of the placebo-treated group as described in Section 2.

(65–89%) of the enterovirus isolates for a given year in the US (Strikas et al., 1986). While SCH 47802 and its derivatives demonstrate activity against a number of different isolates representative of the common 15 serotypes, a potential weakness in the spectrum was the lower, or lack, of activity of SCH 47802 and SCH 48974 against coxsackieviruses. For example the IC_{50} of SCH 47802 against CVB1, CVB3, CVB5 is >10, >10 and 10 μg/ml, respectively.

The activity of capsid-binding molecules to prevent thermal inactivation does not absolutely correlate with antiviral potency within all chemical series (Andries et al., 1989). All the inhibitors in the SCH 47802 series with potent anti-viral activity ($IC_{50} < 1$ μg/ml) also act to stabilize the virus against heat inactivation (data not shown). The discrepancies in the degree of thermal stabilization and the antiviral potency among compounds suggests that the two activities are independent, but related, measures of the capsid binding. The ability of the SCH 47802 series to stabilize virus against thermal inactivation and to inhibit viral replication early in the infectious cycle is consistent with the binding of the molecule within the VP1 cavity. Additional data provided by X-ray diffraction of crystals of HRV-14 infused with

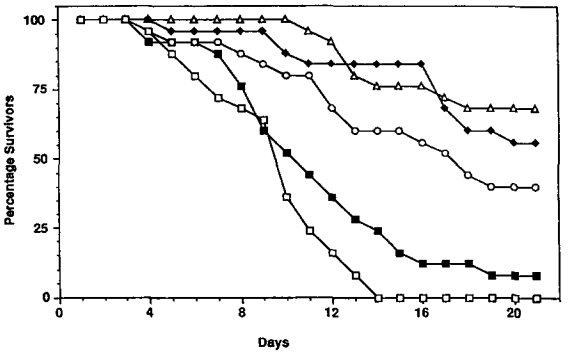

Fig. 2. Efficacy of SCH 47802 after oral dosing of mice infected with poliovirus. Mice were treated with SCH 47802 at 120 (△), 90 (○), 60 (◆) and 20 (■) mg/kg per day, or with corn oil vehicle (□).

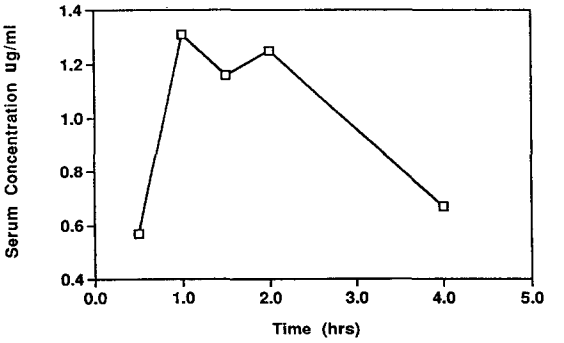

Fig. 3. Serum levels of SCH 47802 after oral dosing in mice. SCH 47802 was administered orally at a dose of 45 mg/kg. The serum concentration of SCH 47802 (□) was determined by HPLC.

Table 4
Thermal stability of SCH 47802 and derivatives

Compound	IC_{50} (μg/ml)[a,b]
SCH 47802	0.004
SCH 48974	0.004
SCH 49860	0.01
SCH 48961	0.05
SCH 48972	0.1

[a] The data represents the mean of 2–4 experiments.
[b] The IC_{50} represents the concentration of compound which resulted in 50% CPE compared to non-heated controls (see Section 2).

representative SCH compounds, including SCH 47802, confirms the binding of compounds within the hydrophobic pocket (personal communication, Dr Edward Arnold, Center for Advanced Biotechnology and Medicine, Rutger University). Other inhibitors which prevent thermal inactivation of virus have been demonstrated to bind within the VP1 cavity (Smith et al., 1986, Alacron et al., 1986; Andries et al., 1990, 1992; Fox et al., 1986; McSharry et al., 1979; Zeichardt et al., 1987).

SCH 47802 was the only molecule with significant in vivo efficacy. The lack of a dose response over the dose range studied (60–120 mg/kg per day) is likely due to the plateau absorption of the compound which is observed with other molecules in this series (data not shown). The inability of the other molecules to demonstrate in vivo efficacy cannot be attributed to differences in antiviral potency since several of the molecules possess similar activity against poliovirus as SCH 47802 in plaque reduction assays (e.g. SCH 48974, SCH 48961). Based on the pharmacokinetics of other compounds in this series it seems likely that the lack of in vivo activity is due to poor oral bioavailability. The molecules in this series are highly hydrophobic, thus allowing interaction with the amino acids in the largely hydrophobic VP1 cavity. The oral delivery of these hydrophobic molecules has been challenging, and a critical factor for the therapeutic development of this series is the discovery of compounds with suitable pharmacokinetic profiles.

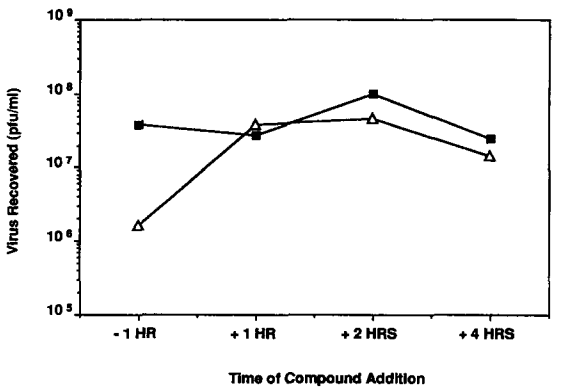

Fig. 4. Effect of time of SCH 47802 addition on poliovirus yield. Virus was treated with SCH 47802 (Δ) or DMSO (■) before infection (−1 h), or at 1, 2 or 4 h post-infection.

To date there is no approved antiviral agent for the treatment of enterovirus infections. While respiratory illness has been the focus of the clinical efforts in developing anti-picornavirus molecules, other illnesses are potential targets for therapy, including aseptic meningitis (Modlin, 1990), febrile illness (Dagan et al., 1989), persistent infections in agammaglobulinemic patients (McKinney et al., 1987), and acute hemorrhagic conjunctivitis (Higgins et al., 1974; Yin-Murphy, 1984). It remains unknown whether anti-enterovirus agents such as SCH 47802 can impact on the morbidity and mortality associated with these diseases.

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