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SCH 38057: a picornavirus capsid-binding molecule with antiviral activity after the initial stage of viral uncoating

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Summary

The activity of a new water-soluble molecule, SCH 38057, against picornaviruses is described. SCH 38057 inhibited plaque formation of selected entero- and rhinoviruses in a range of 10.2 to 29.1 μM (50% endpoint) and had a therapeutic index of 10 against poliovirus type 2 (polio 2) in HeLa cells. When administered orally or subcutaneously, SCH 38057 protected mice infected with either coxsackievirus B3 (CVB3) or echovirus-9 from mortality. The molecule provided a low level of protection against thermal inactivation of virus, indicating that SCH 38057 interacts with the picornavirus capsid. Binding studies with [^{3}H]SCH 38057 revealed that the molecule binds to CVB3 and human rhinovirus 14 (HRV14) in a ratio of 29 and 19 molecules per viral particle, respectively. The affinity constant for SCH 38057 binding to CVB3 was 7.0×10^{-4} M. When added to cultures of infected cells at 3 h after infection, SCH 38057 markedly inhibited viral RNA synthesis. This finding with lack of inhibition of attachment and loss of infectious virus after attachment were interpreted to indicate that, although SCH 38057 binds to the viral capsid, the molecule exerts its antiviral effect after the initial stage of

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picornavirus uncoating, i.e., after conversion of the 156S infectious viral particle to smaller subviral species.

SCH 38057; Antiviral; Picornaviruses

Introduction

The picornavirus family comprises a large number of viruses which cause a variety of diseases. Two genera of picornaviruses, the rhino- and enteroviruses, are responsible for the highest incidence of infection in humans: for example, the incidence of the common cold, the most common illness caused by picornaviruses is estimated to be 40×10^6 cases per year in the USA (Couch, 1985). Symptomatic infections caused by enteroviruses, which include summer respiratory disease, myocarditis, aseptic meningitis, and pancreatitis, may be as high as 10×10^6 cases per year (Rotbart, 1989).

A variety of molecules having activity against picornaviruses in cell culture or in mice have been described (Alacron et al., 1986; Andrei et al., 1985; Andries et al., 1988; Andries et al., 1992; Diana et al., 1985; Eggers et al., 1985 & 1987; Ishitsuka et al., 1982; Kamano et al., 1988; Kenny et al., 1985 & 1987; McSharry et al., 1979; Ninomiya et al., 1985; Otto et al., 1985; Rombaut et al., 1985; Tyrrell, 1988; Woods et al., 1985). However, only a few of these molecules have been shown to be efficacious in humans in the absence of significant adverse effects: R61837 and pirodavir (R77975) decrease cold symptoms and nasal secretions in volunteers experimentally infected with rhinovirus when these molecules are administered intranasally before infection or prior to onset of symptoms (Al-Nakib et al., 1989; Barrow et al., 1990; Hayden et al., 1992). WIN 54954 is efficacious in experimentally induced coxsackievirus A21 colds when the molecule is given prophylactically by the oral route (Schiff et al., 1992). X-ray diffraction analysis of crystals of human rhinovirus 14 (HRV14) infused with WIN molecules (Smith et al., 1986; Rossmann, 1989) or with R61837 (Chapman et al., 1991) have shown that these molecules bind within the hydrophobic pocket of VP1, one of the four proteins comprising the picornavirus capsid. Studies with poliovirus 2 (polio 2), HRV2, and HRV14 indicate that WIN molecules inhibit early viral processes associated with attachment and uncoating (Fox et al., 1986; Pevear et al., 1989). For example, against HRV14, the WIN molecules act indirectly to inhibit attachment of virus to its cellular receptor by causing a conformational change in the capsid (Pevear et al., 1989).

Antipicornaviral molecules like pirodavir and those of WIN series are hydrophobic and consequently exhibit little solubility in aqueous-based biological systems. Therefore, the goal of this study was to evaluate if H_2O-soluble molecules could be synthesized which would interact with the picornavirus capsid to inhibit early capsid-associated viral processes. This

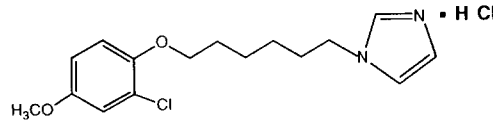


Fig. 1. Chemical Structure of SCH 38057.

resulted in the synthesis of SCH 38057 (Fig. 1). Comparative studies with WIN 51711 indicate that, although SCH 38057 interacts with the capsids of CVB3, polio 2 and HRV14, the major antiviral effect of SCH 38057 is mediated through a mechanism that is exerted later than that of WIN 51711.

Materials and Methods

Antipicornaviral molecules and synthesis of ^{3}H-labelled SCH 38057

SCH 38057 (1-[6-(2-chloro-4-methoxyphenoxy)hexyl] imidazole hydrochloride; Girijavallabhan et al., 1989) and WIN 51711 (Otto et al., 1985) were synthesized at the Schering-Plough Research Institute. The maximum solubility of SCH 38057 in H_2O was >50 mg/ml; at the maximum concentration tested in cell culture, 45 μg/ml (131 μM), the molecule was completely soluble. Studies with WIN 51711 were performed in aqueous solutions containing 1% (v:v) dimethyl sulfoxide (DMSO). In radiolabelled SCH 38057, [^{3}H]atoms were attached to the aliphatic bridge linking the methoxyphenoxy and imidazole rings. This was accomplished by reacting desmethyl SCH 38057 with [^{3}H]methyl iodide (Amersham, 85 Ci/mmol). Desmethyl SCH 38057 (5.63 mg) was dissolved in a mixture of 10 mg tetrabutyl ammonium hydrochloride (40% aqueous) and 1 ml of CH_2Cl_2. [^{3}H]methyl iodide (1.03 mCi) was added followed by carrier methyl iodide (0.002 mmol). After 20 min, the reaction mixture was partitioned between CH_2Cl_2 and 0.1 M ammonium acetate. The organic layer was evaporated in vacuo, the residue dissolved in acetonitrile, and the product was purified using two HPLC systems: (i) Whatman M-9 ODS column with a mobile phase comprising 1:1 acetonitrile and 0.02 M ammonium acetate buffer, and (ii) a Waters Radial Compression 10 μ silica column with a mobile phase consisting of 97.5 parts CH_2Cl_2 and 2.5 parts methanol. The resulting [^{3}H]SCH 38057 had a radiochemical purity of 98% by HPLC (Waters Nova-pak column) and by thin-layer chromatography (Whatman LK6DF silica plate). The specific activity was 329 mCi/mmol.

Viruses, cells, plaquing, radiolabelling and virus purification

CVB3 (Woodruff strain), HRV14 and echovirus 9 (Barty) were gifts from C. Gauntt (University of Texas, San Antonio), R. Rueckert (University of Wisconsin, Madison), and H. Eggers (Universität zu Köln, Germany), respectively. Polio 2 (MEF1) and other entero- and rhinoviruses as well as encephalomyocarditis (EMC) virus were purchased from the American Type

Culture Collection (ATCC). Entero- and rhinoviruses were propagated in HeLa cells (C. Gauntt) and working stocks were prepared as cellular lysates. Similarly, stocks of EMC virus were prepared in buffalo green monkey kidney cells (Whittaker Bioproducts). Standard procedures for plaquing and storing picornaviruses have been described (Trousdale et al., 1977; Rueckert and Pallansch, 1981). Virus was labelled with [^{3}H]uridine and purified by two consecutive rate zonal sedimentations in 20–70% sucrose gradients before pelleting (Rozhon et al., 1982).

Evaluations of SCH 38057 in mice

Studies with mice were performed as recommended in the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 85–23). Forty adult male Balb/c or ICR mice (22 g; Harlan Sprague-Dawley) were inoculated intraperitoneally with 1.75×10^3 PFU of CVB3 or intracranially with 1.87×10^5 PFU of polio 2, respectively. Treatment with SCH 38057 by oral gavage (0.2 ml in H_2O) was initiated immediately after infection (3 times a day on days 1–3 and twice a day on days 4–14). For neonatal mice (Taconic), 64 neonates (8 per mother) were inoculated with either 200 PFU of CVB3 or 10 PFU echo 9 subcutaneously (SC) 24 h after birth. Subcutaneous dosage with SCH 38057 began 4 days after infection and comprised 2 doses per day for 15 days (CVB3) or 5 days (echo). 40 adult or 64 neonatal control animals were infected and administered distilled H_2O using regimens identical to those for the experimental groups. Animals were monitored daily for 21 days to record mortalities. Results were tested for statistical significance using Chi square analysis (Davies and Goldsmith, 1972), and protection was considered to be significant at $P < 0.05$.

Antiviral activity and mechanism of SCH 38057

(i) Plaquing. 150 plaque PFU were mixed with compound (1–45 μg/ml) in Eagles' modified minimal essential medium (EMEM) and added to monolayers of the appropriate cells. After 45 min at 33°, the inoculum was aspirated, the cells washed and plaquing was performed as described above.

(ii) Cell viability and incorporation of [^{3}H] precursors. A colorimetric assay for cell viability, dependent on light absorption by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) formazan has been described (Mossman, 1983; Green et al., 1984). This assay, referred to as the MTT assay, was used to determine the antiviral and cytotoxic IC_{50} levels for SCH 38057 as well as the therapeutic index (TI; cytotoxicity $IC_{50} \div$ antiviral IC_{50}) with polio 2 in HeLa cells. Briefly, SCH 38057 (0.07–131 μM; 0.025–45 μg/ml) and virus were used to infect cells (MOI 1.0) in 96-well microelisa plates, while uninfected monolayers, treated identically with test compound, were used to measure cytotoxicity. Incubation for 18 h was performed prior to addition of MTT (Mossman, 1983). The effect of SCH 38057 on incorporation of [^{3}H]leucine, -thymidine, and -uridine was

determined by incubating uninfected HeLa cell monolayers for 3 h (37°) prior to adding 1 μCi (50 Ci/mM, NEN) of the appropriate radiolabelled precursor and incubating for an additional hour. Acid-insoluble radioactivity was quantitated by liquid scintillation.

(iii) Thermal inactivation. 10^7 PFU of purified virus was incubated with compound at 131 μM (45 μg/ml) for 45 min at 22°, then shifted to 47°. At prescribed times, aliquots were diluted in EMEM to concentrations of compound which were not inhibitory, and plaqued.

(iv) Capsid-associated viral processes. To assess the effect of SCH 38057 on viral attachment, purified, ^{3}H-labelled virus (6×10^8 PFU) was treated with 131 μM of compound (45 min, 22°) prior to infecting HeLa cells. After adsorption (2 h at 4°), the monolayer was washed with phosphate-buffered saline (4°) and lysed by the addition of 100 μl of cell lysis buffer (0.5% NP-40, 150 mM NaCl, and 130 mM Trizma base (pH 7.5). The resulting cytoplasmic extract was assayed for acid-insoluble radioactivity by liquid scintillation.

The ability of antiviral agents to affect viral penetration through the plasma membrane has been described by Eggers (1977). This assay is based on the principle that an agent which prevents virus from entering the cytoplasm will result in virus remaining on the cell surface where it is susceptible to neutralizing antibody. Cells were infected with 200 PFU (30 min, 4°) prior to addition of test compound (131 μM). Following incubation (30 min, 37°), the cells were resuspended and incubated with rabbit anti-CVB3 antibody (22° for 30 min; preparation of antiserum described by Dasmahapatra et al., 1991). Unbound antibody was removed by pelleting cells and washing before cells were overlaid onto a HeLa cell monolayer and plaqued.

An uncoating assay, adapted from Fox et al. (1986), is based on the principle that viruses which are inhibited from the initial stage of uncoating retain the sedimentation constant of extracellular viral particles, which is about 156S for enteroviruses (Rueckert, 1990). Purified, ^{3}H-labelled CVB3 (6.0×10^{11} PFU) or polio 2 (1.1×10^{14} PFU) was treated with 131 μM of compound (45 min, 22°) prior to infecting HeLa cells. After infection (45 min, 33°, MOI 1100), the cells were washed with PBS and incubated for 3 h (37°). Monolayers were then washed with cold EMEM, lysed with cell lysis buffer, and the resulting cytoplasmic extract was subjected to sedimentation in a Beckman SW 50.1 rotor at 110000 $\times$ g for 45 min in a 15–30% (w:v) sucrose gradient (TNE buffer (0.5 M NaCl, 2 mM EDTA, and 20 mM Tris-HCl (pH 7.2)). Acid-insoluble radioactivity in fractions was measured by liquid scintillation.

(v) Viral RNA synthesis. SCH 38057 (1–45 μg/ml; 2.9–131 μM) was added to virus-infected HeLa cells either at the time of infection (t_0) or at 3 h post-infection (t_3): For t_0 virus was pre-incubated with test compound (60 min, 22°) and used to inoculate HeLa cells (MOI 1) which had been treated with actinomycin D (5 μg/ml). After 3 h (37°), 1 μCi/ml [^{3}H]uridine (30.5 Ci/mMol,

New England Nuclear) was added and incubation continued for 1 h. The cells were then washed, lysed with 1% SDS in PBS (w:v), and incorporation of acid-insoluble radioactivity was determined. For the t_3 hour measurement, SCH 38057 and [^{3}H]uridine were added to cells (pretreated with actinomycin D at t_0) at 3 h post-inoculation, incubated for 1 h and processed as described for the t_0 measurement.

(vi) Viral RNA polymerase and template elongation. The poliovirus RNA-dependent RNA polymerase ($3D^{pol}$) was expressed in *E. coli* using a T7 expression vector similar to that described by Plotch et al. (1989). The $3D^{pol}$ enzyme was isolated as described by Jablonski et al. (1991) and a final purification over poly (U)-Sepharose (Plotch et al., 1989) was performed to give a homogeneous preparation of enzyme, judged to be 95% pure by SDS-polyacrylamide gel electrophoresis and silver-staining. The assay for $3D^{pol}$ activity using a poly A · oligo (U) template/primer has been described (Jablonski et al., 1991). 50 μl reaction volumes containing α-[^{32}P]UTP and compound (0.01–200 mg/ml) were incubated for 30 min (30°). The in vitro-synthesized product was precipitated using 10% trichloroacetic acid, collected on Gelman filters, and the radioactivity determined by liquid scintillation. Assay conditions were adjusted to yield linear incorporation of radioactivity for 30 min and the amount of $3D^{pol}$ used resulted in 75% of maximum incorporation.

Binding studies with SCH 38057 and coxsackie- and rhinoviruses

(i) Measurement of virus-bound SCH 38057. Purified CVB3 (7.3×10^{12}) or HRV14 particles (4.7×10^{11}), determined by optical density (Hsu et al., 1988; Rueckert, 1990), was mixed with 150 μM of [^{3}H]SCH 38057 (final volume 10 μl). It was assumed that SCH 38057 binds to non-infectious viral particles to the same extent as infectious particles (particle:PFU for both viruses was 400:1). The mixture was incubated at 4° for prescribed times to allow SCH 38057 to bind to virus. Virus-bound and free SCH 38057 were separated by submitting the mixture to rate zonal sedimentation in a 20–70% sucrose gradient (in TNE buffer (10 mM Trizma (pH 7.2), 150 mM NaCl, 1 mM EDTA); Beckman SW41 rotor, 150 000 $\times$ *g* for 3 h). Fractions were assayed directly for radioactivity by liquid scintillation. The number of virion-bound SCH 38057 molecules was calculated using total dpm recovered in peak viral fractions and the specific activity of the [^{3}H]SCH 38057 preparation. In parallel sucrose gradients, a 70% recovery of purified ^{3}H-labelled virions and PFU was determined and used to correct for loss of virus attributed to experimental procedures.

(ii) Determination of affinity constant, K_i, for SCH 38057 binding to virus. Microfuge tubes containing 5×10^{11} particles of CVB3, 7.447 pmol [^{3}H]SCH 38057, and various concentrations of unlabelled SCH 38057 were prepared (final volume 20 μl) and incubated for 1 h (22°). 200 μl of TNE buffer

was added, mixed, and 200 μl were loaded onto a Sephadex G-25 (medium grade; Pharmacia, Piscataway, NJ) column (0.5 × 20 cm; 2.5 ml) to separate virion-bound and unbound SCH 38057. Columns had been pre-equilibrated overnight with TNE buffer which also was used as the eluant. Fractions (120 μl) were collected and assayed directly for radioactivity; fractions containing virion-bound SCH 38057 were pooled and SCH 38057 bound to viral particles was determined as described above. Results were plotted as percentage bound radioactivity vs. concentration of unlabelled SCH 38057. The concentration of unlabelled SCH 38057 at which 50% of [^{3}H] molecule is bound to virus represents the affinity constant, K_i (Bennett, 1978).

Results

To assess the potency and validate experimental procedures to define the antiviral mechanism of SCH 38057, WIN 51711 was often tested in parallel with SCH 38057. Since CVB3 was our principal antiviral target, it was the virus of choice for these studies; however WIN 51711 exhibited little activity against the strain of CVB3 which we used. Therefore, polio 2, inhibited by both the SCH and WIN molecules, was used as an alternative to CVB3 in some studies. HRV14 was not evaluated in mechanistic assays, however the number of SCH 38057 molecules binding to HRV14 was determined as a prerequisite for structural analysis of HRV14 crystals infused with SCH 38057 (Zhang et al., 1993).

Antipicornavirus activity and therapeutic index (TI) of SCH 38057

The IC_{50} values for SCH 38057 against CVB3, polio 2, and two other enteroviruses as well as six rhinoviruses, are shown in Table 1A. The compound exhibited an IC_{50} of 21.8 μM against CVB3, but was more active against polio 2 with an IC_{50} of 10.2 μM. In general, SCH 38057 was less active against rhinoviruses and was inactive against encephalomyocarditis (EMC) virus at the maximum concentration tested. WIN 51711 (Otto et al., 1985) exhibited poor activity against CVB3 with an IC_{50} of 130.8 μM but was active against polio 2 (IC_{50} 0.2 μM) (Table 1B). A TI of 10 for SCH 38057 against polio 2 in HeLa cells was determined by the MTT antiviral and MTT cytotoxicity assays (Fig. 2). An additional study, which was performed to determine the effect of SCH 38057 on incorporation of ^{3}H-labelled precursors in uninfected HeLa cells, confirmed the narrow TI of the molecule: IC_{50} values for incorporation of [^{3}H]leucine, -uridine, and -thymidine were 86 μM, 57 μM, and 86 μM, respectively. Thus, because of the narrow TI, it is possible that adverse cellular effects produced by SCH 38057 influenced antiviral measurements. A TI with CVB3 in the MTT assay was not obtained, possibly because SCH 38057 was not inhibitory at multiplicities required to produce a significant cytopathic effect.

TABLE 1

Plaque-neutralizing activities of antiviral molecules against selected picornaviruses

A. Antiviral Activity of SCH 38057

Picornavirus Genus	Virus[1]	IC_{50} (μM)[2]
Enterovirus	CVB3	21.8
	CVA21	14.5
	Polio 2	10.2
	Echo 9 (Barty)	29.1
Rhinovirus	HRV 14	27.6
	HRV 1A	29.1
	HRV 10	29.1
	HRV 28	20.4
	HRV 45	29.1
	HRV 61	29.1
Cardiovirus	EMC	> 130.8

B. Comparison of Antiviral Activities of SCH 38057 and WIN 51711

Virus	IC_{50} (μM)[2]	
	SCH 38057	WIN 51711
CVB3	21.8	130.8
Polio 2	10.2	0.2

[1]CVA21, coxsackievirus A21; Echo 9, enteric cytopathic human orphan virus 9.

[2]The IC_{50} value represents the concentration of compound required to inhibit 50% of the PFU compared to the control, which was not treated with test compound. Each value represents an average of at least two assays.

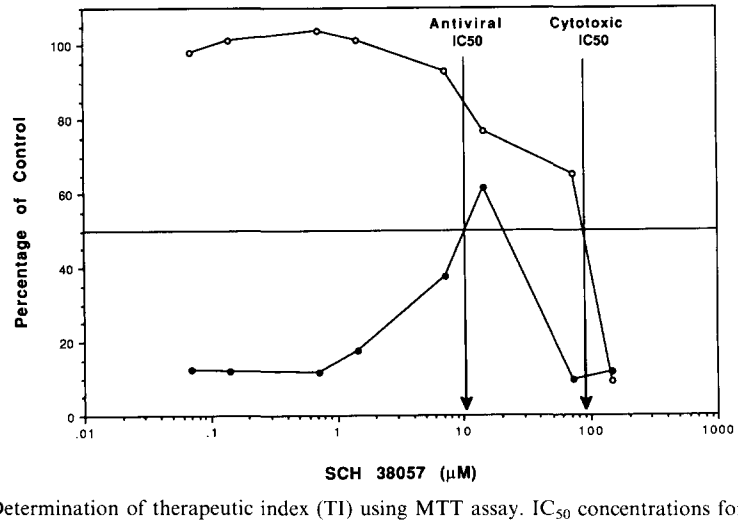


Fig. 2. Determination of therapeutic index (TI) using MTT assay. IC_{50} concentrations for SCH 38057 in polio 2-infected HeLa cells (●) and uninfected, SCH 38057 cytotoxicity control (○) are indicated by verticle arrows. Horizontal line (———) indicates 50% inhibition.

Evaluation of SCH 38057 in mice infected with CVB3, polio 2, or echo 9

Protection ($P < 0.05$) of adult mice infected with CVB3 was observed when treatment of SCH 38057 was initiated immediately after infection at high oral dosages (120–180 mg/kg body weight daily) (Fig. 3A). Significant efficacy ($P < 0.05$) was also obtained in neonatal mice infected with either CVB3 (Fig. 3B) or echo 9 (Fig. 3C) and treated with SCH 38057 SC. These results indicate that SCH 38057 can protect infected mice, however, the extent of efficacy achieved varied among individual studies. Overall, the studies shown in Fig. 3 are representative of 50% of the efficacy evaluations performed with adult and neonatal mice using these dosage regimens. Such variability may be attributable to natural variation of viral titers in identically treated mice: CVB3 titers in sera of non-treated adult mice at 3 days after infection ranged from 10^4 to 10^8 PFU/ml; titers in livers at 6 days ranged from 7.8×10^5 to 6.9×10^9 PFU/gram, and these ranges were typical of CVB3 titers in the brain, pancreas and heart. Thus, SCH 38057 probably has its greatest protective effect in CVB3-infected animals in which the total viral tissue burden is comparatively low. Since WIN 51711 was inactive against CVB3 in vitro, it was not tested in CVB3-infected mice. SCH 38057, given orally or SC in daily dosages as high as 180 mg/kg body weight, failed to protect adult mice infected intracerebrally with polio 2, while WIN, given orally, was consistently efficacious (not shown). The difference between the SCH and WIN molecules in the polio 2 model is not due to WIN having pharmacokinetic advantages since both molecules exhibit similar plasma half-lives and tissue levels (data not shown). The likely explanation is that SCH 38057 is 50-fold less active than the WIN 51711 against polio 2 (Table 1B).

Studies on the antiviral mechanism of SCH 38057

(i) Virucidal test and thermal inactivation. SCH 38057 did not act in a direct virucidal manner against CVB3 and polio 2 when SCH 38057 (131 μM) was incubated with virus (22°, 45 min), diluted beyond the inhibitory concentration of SCH 38057, and plaqued (data not shown). Since some capsid-binding molecules protect picornaviruses from thermal inactivation (Fox et al., 1986; Andries et al., 1989), the ability of SCH 38057 to do the same for CVB3 and polio 2 was tested. SCH 38057 afforded marginal, but consistent protection, to both viruses for about 20 min of heat treatment as measured by diluting samples beyond the inhibitory concentration and plaguing; however, by 40 min, this protective effect was lost (Fig. 4A and B). WIN 51711 protected 100% of polio 2 from inactivation for as long as 40 min (Fig. 4B). To discriminate between lack of protection and carry-over of virion-bound molecules which might inhibit PFU, it was necessary to show that reduction of PFU did not occur when the virus-SCH 38057 mixture is not subjected to heat treatment, i.e., a virucidal assay (see above). Thus, these data are consistent with SCH 38057 interacting with the viral capsid.

(ii) Capsid-associated viral processes. Attachment of radiolabelled CVB3

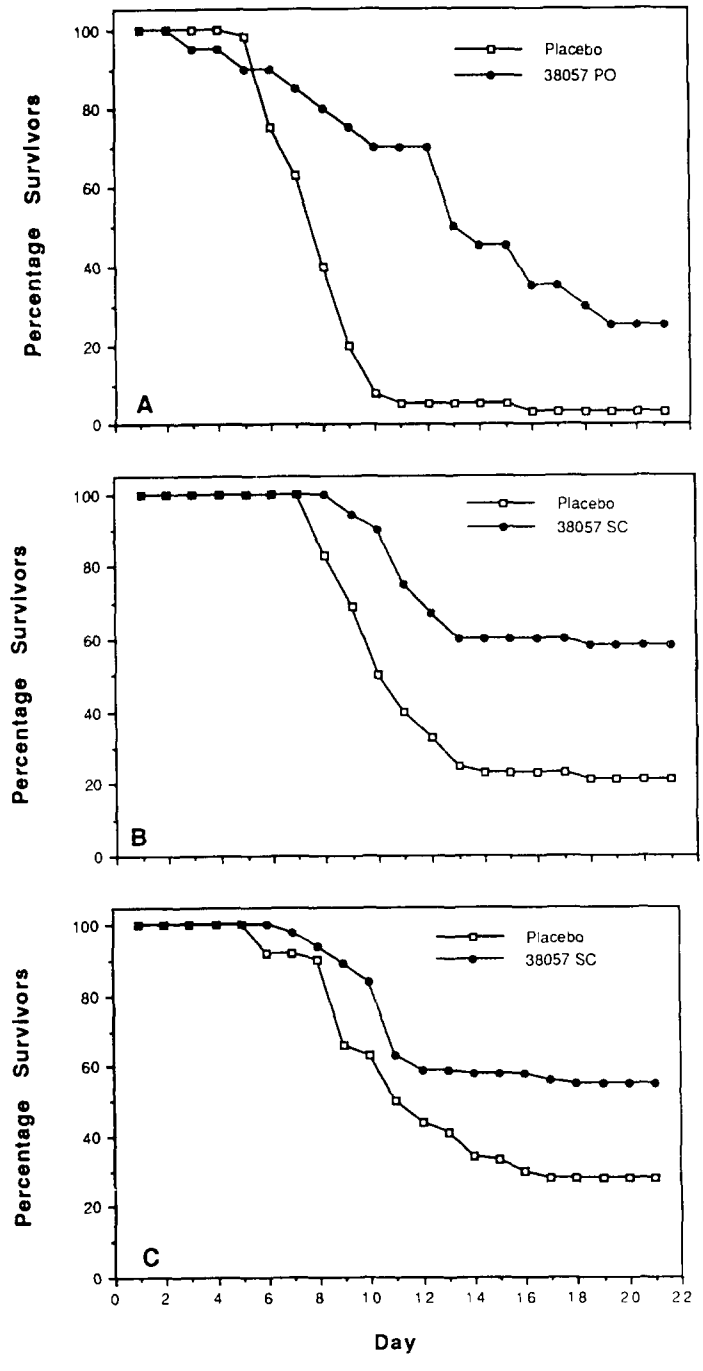


Fig. 3. Protective effect of SCH 38057 in infected mice. (A) SCH 38057 was administered orally (60 mg/kg 3 times per day) to CVB3-infected adult mice as described in the Materials and Methods. Neonatal mice were infected with either CVB3 (B) or echo 9 (C) within 24 h of birth and SC treatment with SCH 38057 (20 mg/kg two times per day) was initiated 4 days later.

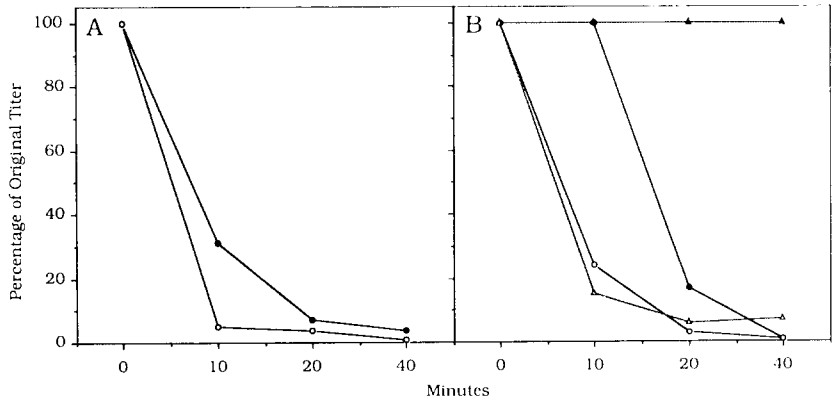


Fig. 4. Thermal inactivation of CVB3 (Panel A) and polio 2 (Panel B) at 47° in presence of SCH 38057 (●). WIN 51711 (▲) was used as a positive control with polio 2. Media controls without test compound were EMEM (○) for SCH 38057 and EMEM with 1% DMSO (△) for WIN 51711.

(Fig. 5A) and polio 2 (not shown) to HeLa cells in the presence of SCH 38057 was similar to that observed with the control (Fig. 5A). As reported (Fox et al., 1986), WIN 51711 did not prevent attachment of polio 2 to HeLa cells (not shown). Results from the penetration study (Fig. 5B) indicated that the number of PFU obtained from cells treated with SCH 38057 and the non-treated

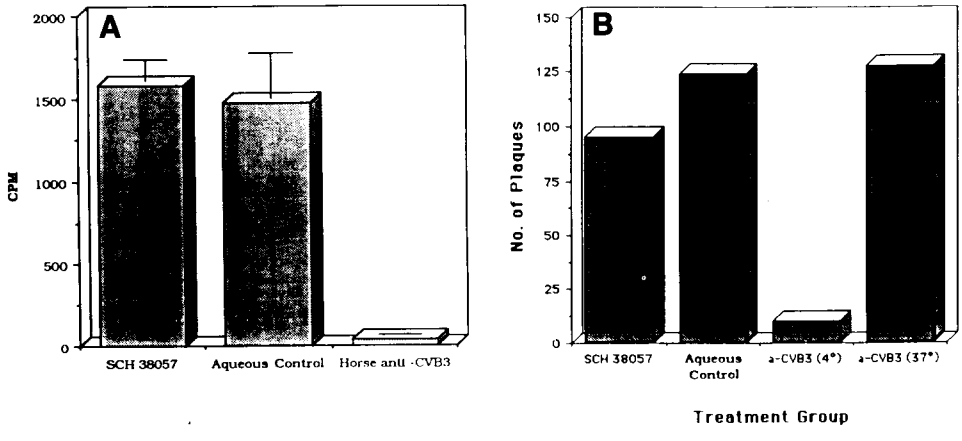


Fig. 5. Effect of SCH 38057 on attachment of CVB3 to HeLa cells (Panel A) and effect on penetration of CVB3 through the plasma membrane (Panel B). Attachment of radiolabelled CVB3 was measured by the cellular association of radioactivity, and horse anti-CVB3 serum (ATCC) was used as a positive control (antiserum coincubated with virus prior to adsorption). For penetration studies shown in columns 1 and 2 of Panel B, rabbit anti-CVB3 serum (a-CVB3) was added to cells immediately after the 30 min, 37° incubation period. Anti-CVB3 serum was also used as both a positive and a negative control depending on its time of addition to infected cells. When a-CVB3 is added to infected cells at 4°, the antibody inhibits penetration when the incubation temperature of infected monolayers is raised to 37° (column 3). When a-CVB3 is added to infected cells after the 37° shift, the antibody acts as a negative control (column 4). The number of plaques produced by the positive and negative control groups was significantly different ($P < 0.05$).

control were similar (Student's t test).

Sucrose sedimentation of polio 2-infected cell extracts after treatment with SCH 38057 resulted in a small peak of radioactivity (fractions 4–8, Fig. 6A) whose sedimentation was similar to that of 156S purified polio 2 (arrow in Fig. 6A). After dilution beyond inhibitory concentrations of SCH 38057, PFU were recovered from these peak fractions to confirm infectious particles. Thus, it was concluded that this small peak comprised intact poliovirions which had not undergone the initial stage of uncoating that leads to loss of the 156S intact virus particle. Although the minor peak seen in the SCH 38057-treated group (fraction 16, Fig. 6A) might have represented partially uncoated virus, we chose

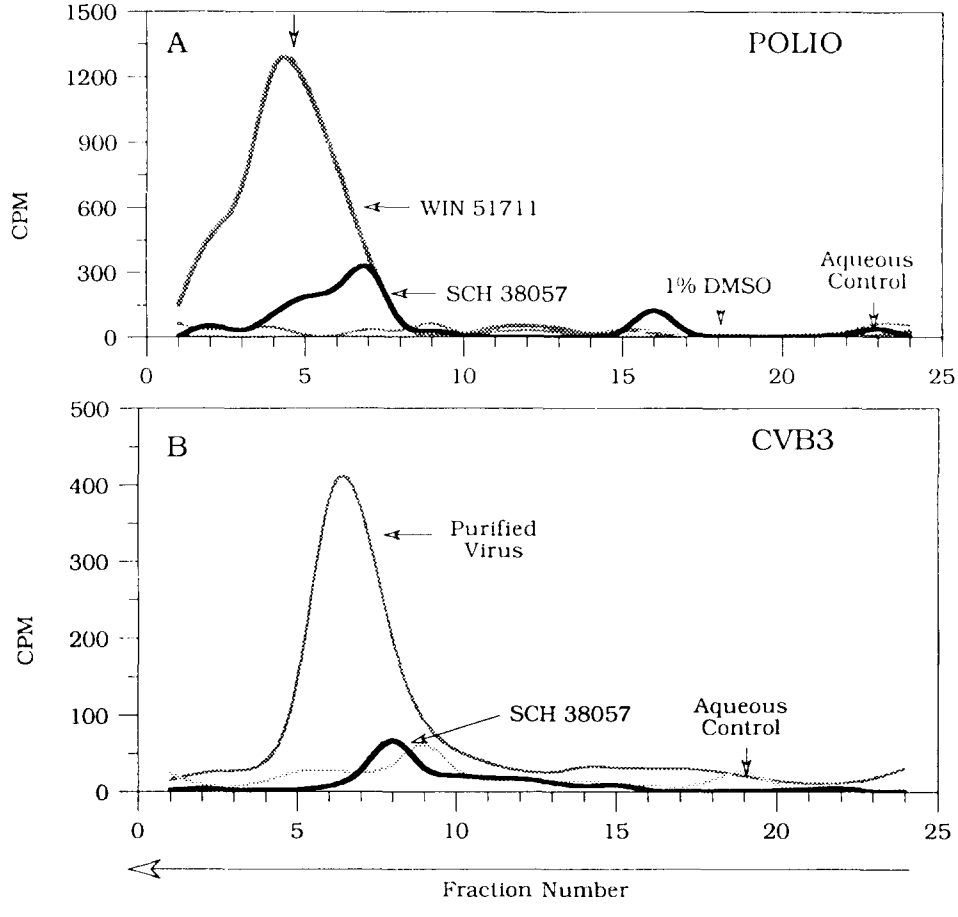


Fig. 6. Sedimentation assay for detecting effect of antiviral compounds on retention or loss of the 156S viral particle. Cells infected with polio 2 (A) and CVB3 (B) and treated with SCH 38057 were lysed and cytoplasmic extracts were sedimented in sucrose density gradients. WIN 51711 against polio 2 was used as a positive control. Media controls without test compound were EMEM for SCH 38057 and EMEM with 1% DMSO for WIN 51711. The position of ^{3}H-labelled, purified polio 2 marker virus is indicated by arrow in panel A, and in panel B the distribution of [^{3}H]CVB3 marker virus is shown.

not to examine the content of this peak since it was not always present in repeated experiments. Compared to SCH 38057, WIN 51711 was more effective in preventing loss of the 156S polio 2 particle: five times more PFU were recovered with WIN-treated virus (Fig. 6A, fractions 3–9) than with SCH-treated polio 2. Sedimentation of polio 2-infected extracts from non-treated controls did not yield a 156S peak, indicating that virus had undergone the initial stage of uncoating. When the effect of SCH 38057 on retention of intact CVB3 particles was examined, a small peak was detected in fractions 7–9 (Fig. 6B), but since a similarly sedimenting peak was detected with the non-treated control, it was concluded that SCH 38057 did not prevent loss of the CVB3 156S particle and that this small peak may have comprised defective particles. That SCH 38057 prevented the loss of at least some 156S polio 2 virions provides additional data supporting SCH 38057 interaction with the polio-2 capsid.

(iii) Viral RNA synthesis and the poliovirus polymerase ($3D^{pol}$). Since the small inhibitory effect that SCH 38057 had on the initial stage of polio 2 uncoating did not seem to account for the antiviral activity observed in the plaque assay and since no inhibition of CVB3 capsid-associated events was observed, the effect of SCH 38057 on viral RNA synthesis was evaluated. SCH 38057 produced a similar inhibitory effect on viral RNA synthesis whether added during adsorption (t_0) or during viral RNA synthesis (t_3) (Table 2). In contrast, WIN 51711 inhibited polio RNA synthesis when added during the early, capsid-associated viral events (t_0), but not at 3 h, when the capsid-associated events presumably were completed. Therefore, it appeared that SCH 38057 inhibited virus after the capsid-associated processes occur with a possible target being synthesis of viral RNA. To evaluate a direct effect on the polio 2 polymerase ($3D^{pol}$), SCH 38057 was tested for inhibition of elongation of a poly A · oligo (U) template/primer catalyzed by $3D^{pol}$. The amount of

TABLE 2

Effect of time of addition of SCH 38057 on RNA synthesis of poliovirus type 2 and coxsackievirus B3

Time of Addition[1]	Virus	Inhibition IC_{50} μg/ml (μM)	
		SCH 38057	WIN 51711
t_0 h	polio	6.9 ± 1.1 (20.1)	0.01 (0.03)
t_3 h	polio	7.2 ± 2.2 (21.0)	>50
t_0 h	CVB3	10.0 (29.1)	–[2]
t_3 h	CVB3	12.2 (35.5)	–[2]

[1]Cells were inoculated with test compound and virus concomitantly at 0 (t_0) h or test compound was added 3 (t_3) h after infection. For both samples, incorporation of [^{3}H]uridine into polio 2 RNA was measured for 1 h beginning 3 h after infection (see Materials and Methods for details).

[2]WIN 51711 was not tested since it is active against this strain of CVB3 only at high concentrations of SCH 38057 (refer to Table 1).

incorporation of radiolabel in the presence of either SCH 38057 or WIN 51711 at 1 mg/ml (280 times the IC_{50} concentration for SCH 38057 against polio 2) was within 5–10% of the control value (data not shown). Spicamycin, an inhibitor of eucaryotic RNA synthesis which was used as a positive control (Acton et al., 1977; Hayakawa et al., 1985), inhibited incorporation by 51% at 200 μg/ml. Thus it was concluded that SCH 38057 does not directly inhibit the activity of the polio 2 $3D^{pol}$.

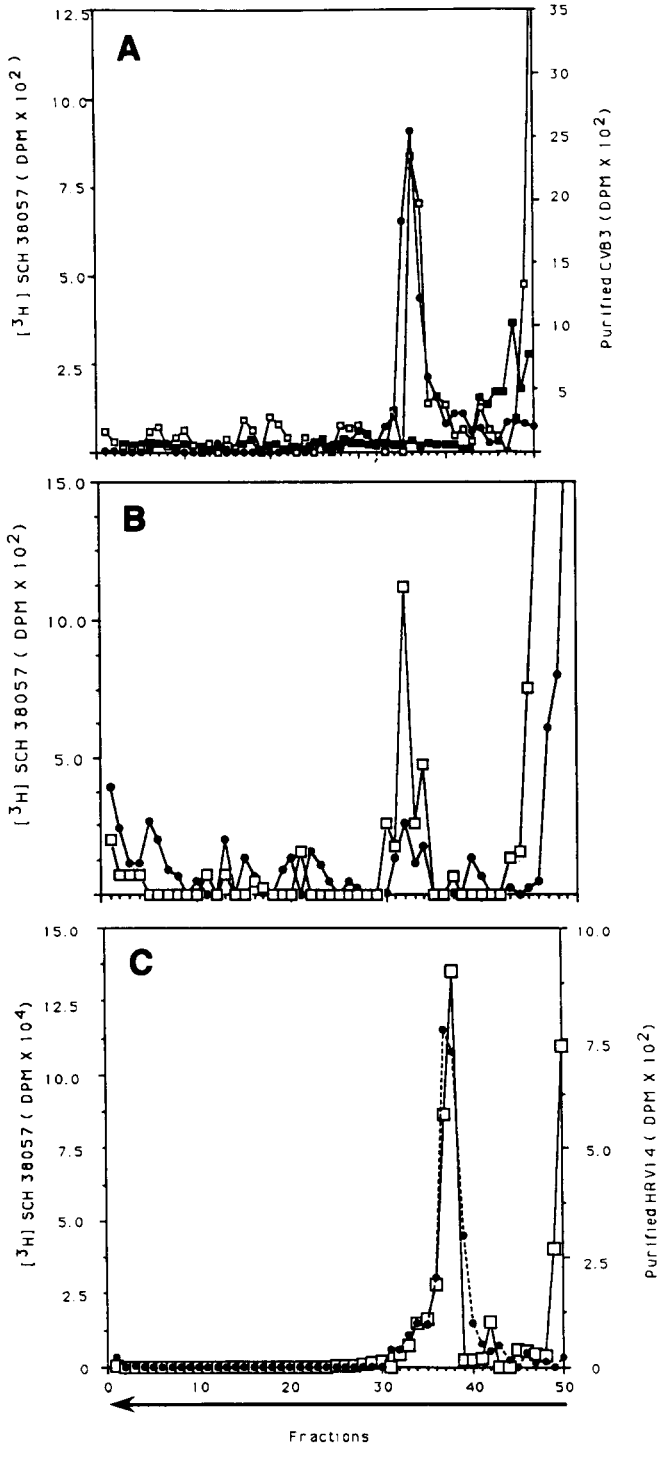
Binding studies with SCH 38057 and picornavirus

(i) Optimization of binding conditions and determination of number of virus-bound molecules of SCH 38057. [^{3}H]SCH 38057 was used in binding studies initially with CVB3 and later with HRV14 to obtain direct evidence that SCH 38057 interacts with the picornavirus capsid. Experimental conditions for [^{3}H]SCH 38057 and CVB3 binding were optimized in regard to time and temperature. In short, maximum binding of SCH 38057 was reached by 24 h (1,866 dpm bound) at 4°; however as early as 1 h, 80% binding had occurred (1,520 dpm bound). Identical samples of CVB3 without SCH 38057 indicated virus could be incubated for as long as 48 h before significant loss of infectivity. Parallel sedimentation of virus-bound SCH 38057 complexes and radiolabelled CVB3 particles demonstrated that binding of SCH 38057 to the viral capsid did not measurably alter sedimentation of the virus (Fig. 7A). Sedimentation of [^{3}H]SCH 38057 alone resulted in 100% of the radioactivity remaining at the top of the sucrose gradient (Fig. 7A), implying that molecules of SCH 38057 which co-sediment with virus bind to viral particles. Since it was possible that the binding capabilities of [^{3}H]SCH 38057 was different than that of the unlabelled molecule, equal molar amounts of the two forms were simultaneously mixed with CVB3 to determine if the two forms would compete for binding. As shown in Fig. 7B, unlabelled SCH 38057 reduced the binding of the isotopic form by approximately half as evidenced by 2288 dpm bound with labelled molecule alone compared to 1176 dpm bound when equimolar quantities were present. The number of SCH 38057 molecules which bound per CVB3 particle was calculated to range from 18–42 with a mean value of 29 $\pm$ 9.3 (S.E.) (6 separate experiments). A similar analysis with HRV14 demonstrated that 19 molecules of SCH 38057 bind per particle (Fig. 7C).

To determine if SCH 38057 binds to CVB3 particles reversibly, virus was pre-incubated (24 h) with [^{3}H]SCH 38057 (150 μM) and then incubated with an equimolar quantity of unradiolabelled SCH 38057 for 24 h. Pooled viral

→

Fig. 7. Analysis of SCH 38057 binding to purified virus using rate zonal centrifugation in sucrose density gradients. Refer to Materials and Methods for experimental details of each reaction. (A) [^{3}H]SCH 38057 and CVB3 (□); purified [^{3}H]CVB3 in parallel gradient as marker (●); and [^{3}H]SCH 38057 alone (■) subjected to centrifugation using conditions identical to that used for [^{3}H]SCH 38057 and CVB3. (B) Equimolar amounts of SCH 38057 and [^{3}H]SCH 38057 reacted with purified CVB3 (●); same concentration of [^{3}H]SCH 38057 alone reacted with CVB3 (□); (C) [^{3}H]SCH 38057 and purified HRV14 (□); purified [^{3}H]HRV14 alone in a parallel gradient (●).


A
B
C
[3H] SCH 38057 (DPM X 10^2)
Purified CVB3 (DPM X 10^2)
[3H] SCH 38057 (DPM X 10^4)
Purified HRV14 (DPM X 10^2)
Fractions

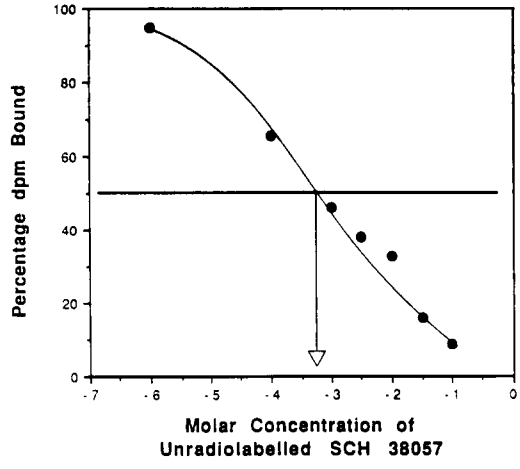


Fig. 8. Competition between SCH 38057 and [^{3}H]SCH 38057 for binding to CVB3. Vertical arrow, extrapolated to abscissa from 50% inhibition of binding of the isotopic form, approximates the affinity constant, K_i.

fractions from the sucrose gradient yielded 2300 dpm which compared to 2288 dpm recovered from incubating CVB3 with 150 μM of the [^{3}H]molecule for 48 h (data not shown). The reverse experiment in which unlabelled molecule was incubated with CVB3 prior to adding the ^{3}H form resulted in little or no radioactivity appearing in the viral peak (data not shown). Therefore, it was concluded that SCH 38057 binds irreversibly to CVB3 under the experimental conditions used.

(ii) Determination of binding affinity for SCH 38057 and CVB3. The affinity constant, K_i, for SCH 38057 binding to CVB3 was determined in competition binding studies with [^{3}H]SCH 38057 (Bennett, 1978). To separate bound from free SCH 38057 in these studies, Sephadex gel filtration was substituted for sucrose sedimentation since gel filtration was less labor-intensive and resulted in viral recoveries exceeding 99%. Binding data representing two experiments are combined and presented in Fig. 8. The IC_{50} value for SCH 38057 binding was calculated to be 7×10^{-4} M. Non-specific binding of SCH 38057 to CVB3 particles was estimated from binding of [^{3}H]SCH 38057 in the presence of unlabelled SCH 38057 at a concentration 200 times the K_i value: under these conditions, the binding of the [^{3}H]SCH 38057 was 9% of that observed when no unlabelled molecule was added.

Discussion

A summary of our findings with SCH 38057 is shown in Table 3. Based on these findings, we succeeded in synthesizing a water-soluble, antipicornavirus

TABLE 3

Summary of findings with SCH 38057[1]

Virus	Antiviral[2] Activity	TI[3]	Thermal[4] Stability	Mechanism of Action					SCH 38057-Virus Binding	
				Attach.	Penet.	156S[5]	RNA[6] Synthes.	$3D^{pol}$	Molecules per Viral Particle	K_i
CVB3	21.8		+/−	−	−	−	+		29 ± 9.3	7.0×10^{-4} M
Polio 2	10.2	10	+/−	−		+/−	+	−		
HRV14	27.6			+/−[7]					19	

[1]A plus (+) indicates activity, a minus (−) no activity, and a plus/minus (+/−) marginal activity.
[2]Antiviral activity determined in plaque neutralization assay and expressed as IC_{50} in μM.
[3]Therapeutic index (TI) was determined by MTT assay in HeLa cells.
[4]Activity in thermal stability assay is indicative of ability of SCH 38057 to prevent thermal inactivation of virus.
[5]Retention of 156S viral particle in sedimentation of cytoplasmic extracts from infected cells.
[6]Inhibition of viral RNA synthesis when SCH 38057 added to infected cells during viral RNA synthesis.
[7]Results from Zhang et al., 1993.

molecule that binds to the capsids of CVB3, polio 3, and HRV14. Since the WIN and Janssen molecules (e.g., R 61837), when bound in the hydrophobic pocket of the VP1 capsid protein, are associated with protecting virus from inactivation by heat (Fox et al., 1986; Smith et al., 1986; Andries et al., 1989; Chapman et al., 1991; Moeremans et al., 1992; Andries et al., 1992), the ability of SCH 38057 to afford limited thermal protection to CVB3 and polio 2 suggests that the molecule also binds within the VP1 pocket. Recently, using X-ray diffraction analysis of crystals of HRV14 which had been infused with SCH 38057, Zhang et al. (1993) demonstrated that SCH 38057 does indeed bind within the VP1 hydrophobic pocket.

In binding studies between SCH 38057 and CVB3 and HRV14, the number of SCH molecules binding to viral particles ranged from 18–42. This result is consistent with the findings of several groups who have infused crystals of HRV 14 with antiviral molecules and analyzed the complexes by X-ray diffraction: Zhang et al. (1993) estimated that 10 molecules of SCH 38057 were bound per viral particle, while Badger et al. (1988), who studied 8 WIN antiviral molecules, found that the average occupancy rate in the VP1 binding pocket to be 36 molecules per particle. Therefore, the number of molecules bound per virion we report as well as those reported by other groups agree closely with the theoretical maximum number of 60 binding sites per virion (Rueckert, 1990).

Despite binding at the same site as the WIN molecules (Smith et al., 1986; Badger et al., 1988), SCH 38057 manifests its antiviral effect at a later stage of viral replication compared to the WIN molecules, which inhibit early viral processes such as attachment and uncoating (Fox et al., 1986; Pevear et al., 1989). Uncoating of poliovirus involves a stepwise degradation of the infectious 156S particle to several smaller subviral species including 135S, 110S, and 80S

particles (Everaert et al., 1989; Kronenberger et al., 1992). Arildone, a capsid-binding molecule that inhibits picornavirus uncoating (McSharry et al., 1979; Caliguiri et al., 1980), was shown to inhibit conversion of the 156S particle to the 135S particle when added at the time of infection, but if added 30 min later, conversion of the 135S particle to the 110S particle was not inhibited (Everaert et al., 1989). This result implies that arildone and possibly other capsid-binding antiviral molecules like arildone (i.e., WIN), which inhibit a capsid-associated event, act before conversion of the 156S particle to smaller particles. Thus, since SCH 38057 inhibits as late as 3 h after infection, it is certain that SCH 38057 does not have the same antiviral mechanism of the WIN molecules.

When the binding affinities of the SCH and WIN molecules are compared, it is not surprising that SCH 38057 exerts little or no activity at the level of the 156S viral particle. Within a structural series of closely related, capsid-binding WIN molecules, Fox et al. (1991) demonstrated a positive correlation between binding affinity and in vitro antiviral activity against HRV14. For example, the binding affinities, K_ds, reported for WIN 51711 and WIN 52084 were 8.0×10^{-8} M and 2×10^{-8} M, respectively, while their corresponding antiviral IC_{50} values were 0.88 μM and 0.05 μM, respectively. These values compare with a binding affinity of 7×10^{-4} M for the SCH molecule and CVB3. Finally, the conclusion that SCH 38057 binds to the capsid but exerts its antiviral effect on a viral process after conversion of the 156S particle is not without precedent: 3-methylquercetin (3MQ), a flavone class molecule isolated from a plant, has been reported to interact with the capsid of poliovirus (Rombaut et al., 1985), but the antiviral activity of 3MQ is directed against viral RNA synthesis (Castrillo et al., 1986; Vrijsen et al., 1987; Pila et al., 1989).

Regarding the marginal activity SCH 38057 exhibited against polio 2 in the sedimentation assay (Fig. 6A), we concluded that this activity is incidental to the later, more significant antiviral effect observed at 3 h (Table 2). If SCH 38057 were able to exhibit significant early, capsid-associated as well as later (3 h) inhibitory effects against polio 2, it would be expected that the two inhibitory effects would be at least additive, and possibly synergistic. However, the level of inhibition found when the molecule was added at 3 h after infection was similar to that when it was added at the time of infection.

The lack of SCH 38057 activity against the purified polio 2 polymerase does not discount the possibility that the molecule inhibits the picornavirus replication machinery. Poliovirus replication involves the complex interaction of cellular and viral proteins within specialized structures present only in infected cells (Bienz et al., 1987), and whether SCH 38057 interferes with the function of any of these replicative subunits is not known.

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