The *in Vitro* Antiviral Activity of a Triazinoindole (SK&F 40491) (36801)

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Cyclization of the side-chain of isatin 3-thiosemicarbazone led to the formation of a triazinoindole. Analogues of this triazinoindole had antiviral activity, notably against picornaviruses (1–3). One such compound, 3-(3 hydroxy-3-methylbutylamino)-5-methyl-*as*-triazino (5,6-b) indole (SK&F 30097), was active *in vitro* against all picornaviruses so examined as well as against certain DNA viruses (1–4). A low degree of activity was obtained with SK&F 30097 against virus infections in animals (3, 4); the compound provided low plasma concentrations after oral administration and underwent rapid degradation *in vivo*. The present report describes the biological profile of another, more potent triazinoindole. Its activity against rhinovirus infections in gibbons is reported elsewhere (5).

Materials and Methods. Viruses and cell cultures. The viruses used were standard strains with multiple cell culture passage: Coxsackie A21/Kuykendall, B1/Conn. 5, B2/Ohio 1, B3/Nancy, B4/Benschoten, B5/Faulkner, and B6/Schmitt; ECHO 12/Travis 2-85; equine rhino 1; herpes simplex/HF; influenza A2/Ann Arbor/57; mengo; polio 1/Brunhilde, 2/Lansing, and 3/Leon; parainfluenza 1/Sendai; pneumonia virus of mice (PVM); pseudorabies/Aujeszky; respiratory syncytial/Long; rhino 1A/2060, 2/HGP, 14/1059, 17/33342, 16/11757, 21/47-CV21, 25/5426-CV26, and 45/Baylor 1; and vaccinia/WR.

Cell cultures were propagated in this laboratory by conventional procedures.

Compound. The trizinoindole described in this report is designated SK&F 40491 (Fig. 1). The compound was dissolved in dimethylsulfoxide for addition to cellulose discs in plaque inhibition tests. For other *in vitro* studies suspensions in cell culture maintenance media were prepared. Suspension was facilitated by grinding in a mortar or by sonication. The compound was soluble under these conditions to approximately 20 μg/ml. Aqueous suspensions with Tween 80 were used for animal inoculations. Suspensions were freshly prepared for each experiment.

Plaque inhibition tests. The *in vitro* antiviral spectrum of SK&F 40491 was determined by plaque inhibition tests using 100 μg of compound/disc according to previously described methods (6). Antiviral activity was denoted by a plaque-free zone in the visibly normal cell monolayer surrounding the disc.

Yield reduction tests. In vitro antiviral activity was quantitated as follows. Tube cultures containing 3×10^5 HeLa cells were inoculated with 1 ml of medium (basal medium, Eagle, with Earle's balanced salt solution (BME) + 1% heated chicken serum) containing serial 2-fold dilutions of compound suspension, and virus at a 1:100 input multiplicity. After 1 hr at 23°, unadsorbed virus was removed by washing and the cultures were incubated at 37° (or 34° for human rhinoviruses) in 1 ml of the same compound—containing medium lacking virus. After 18 hr the cultures were frozen and thawed 2 times, and the medium, clarified by centrifugation, was assayed for virus by plaque counting in HeLa cell monolayers (6). The maintenance medium for cultures infected with human and equine rhinoviruses consisted of BME, 1% heated chicken serum, 400 μg/ml DEAE-dextran, 30 m*M* $MgCl_2$, 1% special agar—Noble (Difco), and antibiotics. That for Coxsackie and polio virus infected cultures contained no DEAE-dextran or $MgCl_2$; 5% fetal bovine serum was used instead of chicken serum, and purified agar (Difco) or

Bacto-agar (Difco) was employed for polio and Coxsackie viruses, respectively. Rhinovirus plaques were counted after 4 days incubation (34° for human rhinoviruses and 37° for equine rhinovirus). Coxsackie and polio virus plaques were counted after 3 days at 37°. The difference in virus titer between control and compound-treated cultures was compared.

Virucidal tests. Compound at 250 μg/ml was incubated for 1 hr at 23° with 10^5 plaque forming units (PFU) of rhinovirus 1A in phosphate buffered saline (PBS) at pH 7.5. Residual virus was compared to that in a placebo-treated control by plaque titration using dilutions beyond the limit of compound activity *in vitro*. Alternatively, 50 PFU of the virus were similarly treated, and SK&F 40491 was removed by extraction with chloroform (5%; v/v) prior to virus titration. Extraction was carried out by shaking the solvent with the virus suspension, and then centrifuging the mixture at 1000*g* to separate the aqueous and solvent phases.

Cell-binding of SK&F 40491. The reversibility of inhibition was first examined. HeLa cell monolayers were incubated with SK&F 40491 in PBS for 1 hr at 23°; the medium was then removed, and the monolayers were washed 3 times with PBS. Rhinovirus 1A (50 PFU) was added in PBS. After 1 hr at 23° unabsorbed virus was removed, agar-containing maintenance medium (with or without SK&F 40491) was added, and plaques were counted after incubation.

The retention of SK&F 40491 by monolayers treated and washed as above was determined using the compound with a ^{14}C label on the 3-position (see Fig. 1). Sufficient radiolabeled material was added to each compound concentration to provide 50 dpm by the counting procedure. Total wash fluid and scraped cells were added separately to scintillation medium (PPO-POP + toluene) and counted.

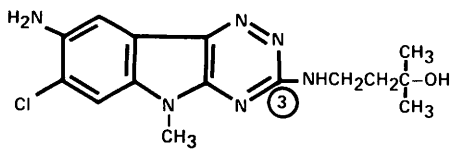

FIG. 1. SK&F 40491: 4-((8-amino-7-chloro-5-methyl-5*H*-*as*-triazino (5,6-b) indol-3-yl)amino)-2-methyl-2-butanol.

TABLE I. Antiviral Spectrum of SK&F 40491 *in Vitro* (Plaque Inhibition Test: 100 μg/Disc).[a]

Virus	Cell	Virus	Cell
Coxsackie A21	HeLa	Polio 3	HeLa
Coxsackie B1	HeLa	Pseudorabies	CE
Coxsackie B2	HeLa	Rhino 1A	HeLa
Coxsackie B3	HeLa	Rhino 2	HeLa
Coxsackie B4	HeLa	Rhino 14	HeLa
Coxsackie B5	HeLa	Rhino 16	HeLa
Coxsackie B6	HeLa	Rhino 17	HeLa
ECHO 12	WI-38	Rhino 21	HeLa
Equine rhino 1	HeLa	Rhino 25	HeLa
Mengo	HeLa	Rhino 45	HeLa
Polio 1	HeLa	Vaccinia	CE
Polio 2	HeLa		

[a] Antiviral activity was denoted by a plaque-free zone in the visibly normal cell monolayer surrounding the disc.

Interferon induction. The compound was inoculated iv at 50 mg/kg into New Zealand white rabbits. Blood was collected after 0, 2, 4, 6, and 24 hr. The serum was assayed for interferon in primary rabbit kidney cells by 50% plaque reduction of vesicular stomatitis virus (7). Rabbit interferon and poly I:C were used to control the sensitivity of the *in vitro* assay.

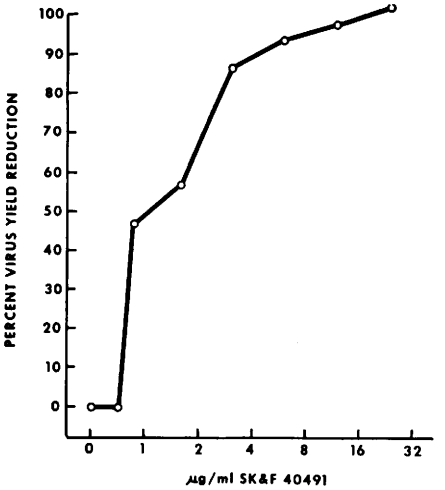

FIG. 2. Effect of SK&F 40491 on the yield of rhinovirus 1A from infected HeLa cells.

TABLE II. Effect of SK&F 40491 on Virus Yield Reduction from Infected HeLa Cells.

Virus	MIC_{50} (μg/ml)
Coxsackie A21	2.0
Coxsackie B1	2.3
Coxsackie B3	1.5
Equine rhino 1	1.4
Polio 1	2.0
Rhino 1A	0.8
Rhino 2	1.3
Rhino 14	3.5
Rhino 21	0.9
Rhino 45	2.0

Results. Antiviral spectrum. As shown in Table I a broad spectrum of picornaviruses, including 8/8 strains of human rhinoviruses, was inhibited by SK&F 40491. No picornavirus tested was insensitive. Activity was also obtained against DNA viruses—pseudorabies and vaccinia. Inhibition was observed in HeLa, WI-38, and primary chick embryo cell cultures. In comparable assays, no activity was obtained against herpes simplex, parainfluenza 1/Sendai, and respiratory syncytial virus.

Dose response. The relationship of SK&F 40491 concentration to antiviral activity was studied in HeLa cells using the yield reduction test. A respresentative dose response curve is shown for rhinovirus 2 (Fig. 2). Inhibition is expressed as percentage reduction in virus titer with respect to that of untreated cultures yielding approximately 1×10^6 PFU. Significant inhibition was obtained at 0.8 μg of SK&F 40491 per ml. Fifty percent inhibition of yield (MIC_{50}), calculated by arithmetic interpolation, was produced by 1.3 μg/ml. SK&F 40491 (100 μg/ml) was well-tolerated for maintenance of HeLa cells in this assay as determined by microscopic observation. Fifty to 100 μg/ml were well-tolerated for 4 to 7 day periods of incubation. The MIC_{50} obtained against other picornaviruses are shown in Table II. All values fell between 0.8 and 3.5 μg/ml.

Cell–virus interaction. The observed activity of SK&F 40491 was not the result of virucidal action. Compound at 250 μg/ml did not influence the titer of either high or low concentrations of rhinovirus 1A on incubation of the virus–compound mixture for 1 hr at pH 7.5, the conditions of maximal contact between free virus and compound in yield reduction tests. Therefore, the compound must affect a step in viral replication.

The reversal of inhibition by SK&F 40491 was examined in HeLa cell monolayers. The cells were treated for 1 hr and then washed prior to infection with rhinovirus 1A. Residual activity was measured by reduction in plaque number and size. No plaques were produced in cells treated with 250 μg/ml of SK&F 40491. There was 85 to 100% reduction in plaque number followng a 50 μg/ml treatment; the observed plaques were reduced in size. No effect was obtained at 10 and 2 μg/ml. When the same concentrations of compound were also added to the maintenance medium no plaques were observed at 10 μg/ml; at 2 μg/ml there was reduction in plaque size, but not number. Thus, cells treated with SK&F 40491 before infection remained refractory to viral replication after removal of all free compound by washing. However, the compound was effective at lower concentrations when present in the medium during the entire incubation period.

The binding of SK&F 40491 to HeLa cell monolayers was studied using ^{14}C-labeled compound. In experiments comparable to those described above, cells were incubated for 1 hr with SK&F 40491, excess compound was removed, and the cells were washed with PBS. Recovery of SK&F 40491 from the cell fraction was quantitated. At treatment concentrations of 250, 50, and 10 μg/ml the cell fractions retained 83, 20, and 2 μg/ml, respectively. The amounts of cell-bound SK&F 40491 following treatment with 250 and 50 μg/ml were sufficient to provide complete inhibition as shown above, whereas that remaining after 10 μg/ml treatment was not.

In contrast with poly I:C and other known inducers of interferon, no such product was observed after iv inoculation of a high concentration of SK&F 40491 in rabbits. Thus, the broad-spectrum antiviral activity of the compound could not be explained on this basis. SK&F 40491 *per se* apparently inhibits a step in viral replication, but the site of action is not now known.

Discussion. It was shown that SK&F 40491 inhibited a broad spectrum of viruses *in vitro*. The activity extended to all picornaviruses so examined. In this respect the compound is similar to several previously described triazinoindoles in this chemical series (1–3). However, SK&F 40491 is the most potent. Activity was observed at 0.8 to 3.5 μg/ml against 10 picornaviruses. In comparable tests SK&F 30097 was as much as 21-fold less active against the same infections.

The action mechanism of SK&F 40491 is not yet defined. It presumably inhibits a step in virus replication selectively. Not all viruses were sensitive in comparable test systems. Also, there was no evidence of cytotoxicity at antiviral concentrations. No virucidal effect was observed at a high compound concentration against a sensitive rhinovirus, and despite its broad antiviral spectrum, interferon induction was not demonstrated by a sensitive method of assay. SK&F 30097, a close analogue of SK&F 40491, was shown to selectively inhibit RNA synthesis of a picornavirus (8). It is likely that SK&F 40491 has a similar action.

We have directed considerable attention to the potential of SK&F 40491 for use against viral infections *in vivo*. Systemic use in multidose prophylactic regimens provided no activity against Coxsackie A21, B1, and B3; herpes simplex, influenza A2; mengo; PVM; pseudorabies; or vaccinia in mice. Also, intranasal application against influenza A2 and PVM had no effect. Rhesus monkey infections with equine rhinovirus were not modified by oral or aerosol prophylactic treatments. Similarly, a human rhinovirus infection in gibbons was insensitive to SK&F 40491 using oral prophylactic dosing (5). In contrast, significant activity was obtained against a human rhinovirus infection in gibbons when SK&F 40491 was applied by intranasal spray prophylactically (5). Since SK&F 40491 was bound tenaciously at antiviral concentrations to cultured cells, a single antiviral dose to the nasal mucosa may show prolonged effect.

The lack of systemic activity with SK&F 40491 can be largely explained on the basis of its metabolic profile (this laboratory, unpublished data). The maximum quantities of a 25 mg/kg oral dose which were recovered in the urine of mice and monkeys were 40 and 9%, respectively. Peak plasma levels were only 5.8 μg/ml for mice and 5.2 μg/ml for monkeys. Its half-life in plasma was exceedingly short, 1.5 hr for both species. Although stable to breakdown in both species, the compound was extensively conjugated, in part to glucuronides, in the monkey.

Summary. SK&F 40491, a triazinoindole, exhibited a broad spectrum of antiviral activity *in vitro*, notably against picornaviruses. Its activity was selective, and was observed at concentrations well below cytotoxic levels.

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