The Antiviral Activity of a Triazinoindole (SK&F 30097)[1] (36163)

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In vitro antiviral activity against rhinoviruses has been described for triazinoindole analogues of methisazione (1). One such compound, [(5-methyl-5*H*-*as*-triazine[5,6,-db] indol-3-yl)amino]-2-butanol, designated SK&F 30097, has shown a broad spectrum of *in vitro* activity extending to all picornaviruses against which it has been tested, as well as to a number of DNA viruses (2, 3). Little or no activity has been observed against a variety of laboratory animal infections (2, 3).

Little information has been provided on the action mechanism of SK&F 30097. In view of the potential importance of this or related compounds with extensive intrinsic *in vitro* activity, its antiviral action was examined more definitively.

Materials and Methods. Viruses. The HGP strain of rhinovirus 2, obtained from D. A. J. Tyrrell, was grown in diploid human embryonic lung cells (WI-38). Poliovirus type 1, Brunhilde strain, was propagated in HeLa cells.

Cell cultures. Tube cultures of WI-38 containing approximately 3×10^5 cells were obtained from Flow Laboratories. HeLa cells were grown in our laboratory; monolayers containing 4×10^6 cells in 60 mm Falcon plastic petri dishes were used for plaque assay, and tube cultures with 2×10^5 cells were used for isotope experiments.

Media. WI-38 cells were maintained in medium consisting of Eagle's minimum essential medium (MEM), Eagle's nonessential amino acids, 2% fetal calf serum (FCS) with 100 units/ml of penicillin G and 100 μg/ml of streptomycin sulfate. Both WI-38 and HeLa cells were grown in MEM with 10% FCS. Medium for rhinovirus plaque assays in HeLa cells was composed of MEM with 2% heated chicken serum, 66 m*M* $MgCl_2$, 200 μg/ml of DEAE-dextran, the above antibiotics, and 1% Noble agar. Medium for poliovirus plaque assays in HeLa cells contained MEM, 2% FCS, the same antibiotics, and 1% Noble agar. The same medium lacking agar was used for maintaining HeLa cells in all radioisotope studies.

Compounds. Stock suspensions of SK&F 30097 at 1000 μg/ml were prepared with sonicating in MEM with 2% FCS. Appropriate dilution in maintenance medium was carried out immediately before use. Actinomycin D was purchased from Calbiochem. Tritiated thymidine (10 Ci/mmole), arginine (10 Ci/mmole), alanine (60 Ci/mmole), leucine (6 Ci/mmole), and uridine (3 Ci/mmole) were obtained from Schwarz Biochemicals.

Virus infection assays. Infectivity of rhinovirus and poliovirus suspensions was determined by plaque assay. Five-tenths milliliter aliquots of 10-fold virus dilutions in MEM with 2% FCS were added to HeLa cell monolayers in petri dishes. After 60 min (37° for poliovirus; 34° for rhinovirus), residual medium was removed and 6 ml of agar-containing maintenance medium were added. Cultures were incubated (37° for poliovirus; 34° for rhinovirus) for 3 and 4 days for

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poliovirus and rhinovirus, respectively. Plaques were counted after staining with 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride for 1 hr at 37°.

Growth curve experiments. Rhinovirus was added to the tube cultures of WI-38 cells at an input multiplicity of 1 unless otherwise stated. Cells were incubated at 34°; unadsorbed virus was removed after 1 hr (0 time), and cultures were washed 3 times with 3 ml of Hanks' balanced salt solution. Nine-tenths milliliter of maintenance medium was then added and the cultures were incubated at 34° on a roller drum operating at 12 rph. One-tenth milliliter of the compound suspension was added at specified times. Total yield of virus was generally assayed: cell-associated virus was released into the medium by 3 alternate cycles of freezing and thawing between —70 and 37°. Cell debris was sedimented by centrifugation prior to assay. When cell-associated virus was quantitated separately, washed cells were disrupted as above in an equivalent volume of fresh medium. Poliovirus growth curves were similarly performed except that input multiplicities of 10 were normally used, and cultures were incubated stationary at 37°.

Isotope experiments. Noninfected or poliovirus-infected HeLa cells were used. Infection was accomplished as in the growth curve experiments using an input multiplicity of 10. After removing virus (0 time), or medium from uninfected cultures, 1 ml of maintenance medium was added. This contained 1 μg/ml of actinomycin D; 1 μCi/ml of radiolabeled precursor, and an appropriate concentration of SK&F 30097, as indicated. For pulse-labeling, 5 μCi/ml of a suitable RNA precursor were added after 5 hr; and cultures were further incubated for 1 hr. The incorporation of radiolabeled precursors into cellular constituents was measured as follows. Two-tenths milliliter of sodium lauryl sulfate was added to each culture. The cells were lysed by 3 alternate cycles of freezing and thawing. One milliliter of 10% trichloroacetic acid (TCA) was added. Cell debris and acid-insoluble precipitate were collected by filtration on a glass fiber disc (Whatman GF/C).

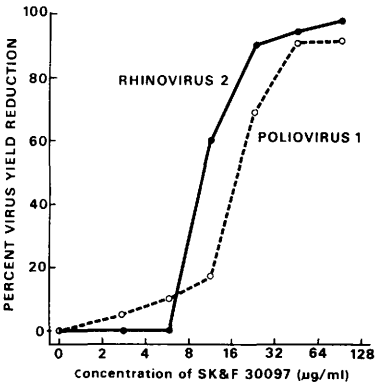

FIG. 1. Inhibition of poliovirus 1 and rhinovirus 2 in WI-38 cells.

The disc was washed with 5 ml of 1% TCA. Ten milliliters of scintillation counting fluid (Toluene containing POP and POPOP) were added to a vial containing the dried disc, and radioactivity was measured with a Packard scintillation counter.

Toxicity. SK&F 30097 was well tolerated for maintenance of WI-38 and HeLa cells at 50 μg/ml, over a 7-day period of incubation as determined by microscopic examination. A 2-fold higher concentration produced slight cytopathic alterations. At least 50 μg/ml permitted optimal growth of WI-38 cells through 5 generations. Similar results were obtained with HeLa cells over 15 generations. The limit of solubility of SK&F 30097 in cell culture media is approximately 50 μg/ml.

Dose response. The relationship of SK&F 30097 concentration to antiviral activity was studied by virus yield reduction in treated cells as described under Growth Curve Experiments. Compound was added at 0 time and total yield of virus was assayed after 6 and 18 hr for poliovirus and rhinovirus, respectively. Figure 1 shows the results obtained with WI-38 cell substrate.

Comparable curves were obtained for rhinovirus using HeLa cells However, poliovirus was more sensitive to inhibition by SK&F 30097 using this substrate; 3 μg/ml provided 50% yield reduction. The degree of viral inhibition obtained with this agent was not obviously dependent on the concentration of challenge virus in that 50 μg/ml provided a 1.3 to 1.8 $\log_{10}$ inhibition of rhinovirus in

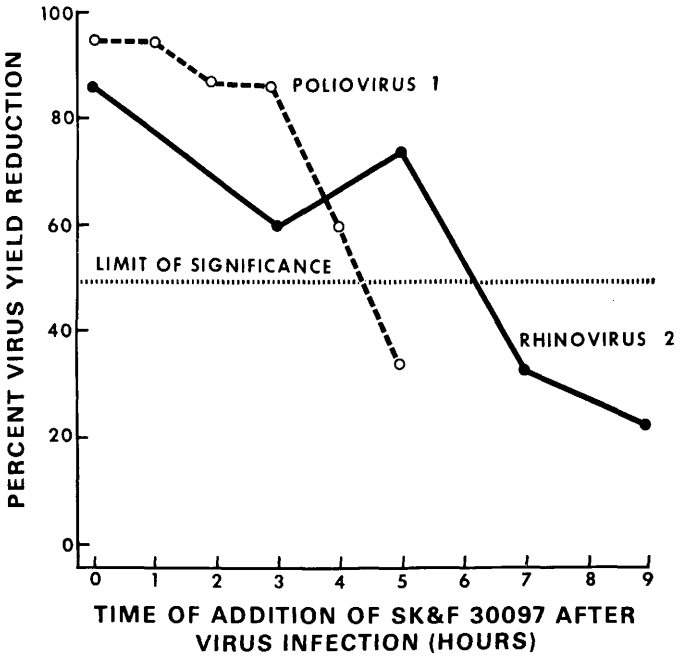

FIG. 2. Degree of viral inhibition in relation to time of addition of SK&F 30097 (50 μg/ml) in WI-38 cells.

WI-38 cells at input multiplicities of virus varying from 0.001 to 10.

Virus inactivation. SK&F 30097 at 250 μg/ml was not virucidal. It did not enhance the rate of rhinovirus inactivation over a 7-hr period at 23° in phosphate buffered saline at pH 6.4, 7.4, and 8.4. Similarly, no virucidal activity was shown against poliovirus over an 18-hr period at 4° and pH 7.4.

Virus adsorption. In the presence of 50 μg/ml of SK&F 30097, the adsorption kinetics of poliovirus to WI-38 cells were unmodified as determined by the number of infectious centers established with time of incubation. Compound and unadsorbed virus was removed from cell monolayers after different intervals of time, the cultures were washed, and the titer of adsorbed virus was determined by plaque assay. Fifty percent of virus adsorption occurred during the initial 20 min of incubation; maximum adsorption was achieved by approximately 1.5 hr. Comparable results have been obtained with rhinovirus (2).

Time of action. The foregoing observations suggest action of SK&F 30097 on an event subsequent to viral adsorption. Activity with respect to time of addition after virus adsorption was therefore examined in growth curve experiments to provide information on the stage of viral replication subject to inhibition. WI-38 cell cultures were infected with rhinovirus or poliovirus. SK&F 30097 (50 μg/ml) was added to the culture medium at different intervals after virus adsorption. After incubation for 6 and 11 hr for poliovirus and rhinovirus, respectively (1 cycle of virus replication), total virus content of cultures was determined. As shown in Fig. 2, inhibition was greatest when compound was added immediately after infection. Activity diminished with delay of compound addition. Activity was still evident when the compound was added 5 hr after infection with rhinovirus and 3 hr after infection with poliovirus. These intervals correspond to the time at which newly synthesized virus is begining to appear. Thus, a late stage(s) in viral synthesis is subject to inhibition.

The effect of discontinuous treatment was studied in an attempt to better define the temporal stages of viral replication which are

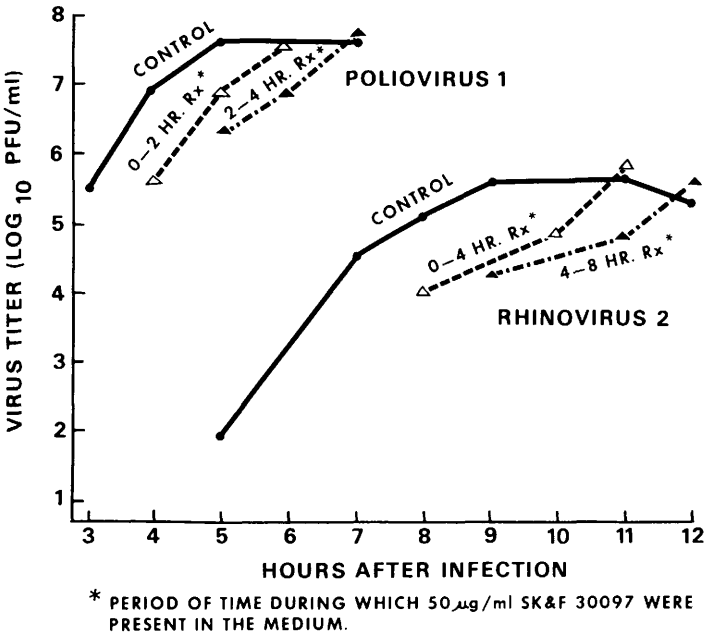

FIG. 3. Effect of discontinuous treatment with 50 μg/ml of SK&F 30097 on virus replication in WI-38 cells.

sensitive to inhibition. Growth curve experiments were carried out with SK&F 30097 present in the medium at 50 μg/ml only during the first or second half of the latent period. Following the interval of treatment, the compound was removed; and total virus yield from cultures was determined at various intervals of time thereafter. The length and time of each treatment period was compared with the delay observed to maximum virus production (Fig. 3). It is evident that with both poliovirus- and rhinovirus-infected cultures, the inhibitory action of SK&F 30097 is reversible. Furthermore, there is approximately a direct relationship between length of the treatment period and delay in time required to achieve maximal virus production after compound removal when cultures were treated during the latter half of the latent period. On the other hand, when cultures were treated with compound for the same length of time, but during the initial half of the latent period, there was a shorter delay to time of maximum virus production.

Virus release. To investigate the possibility that SK&F 30097 inhibits release of virus from treated cultures, growth curve experiments were carried out with poliovirus- and rhinovirus-infected WI-38 cell cultures and compound at 50 μg/ml. Yield of cell-associated virus and virus in culture medium was determined separately after 6 and 11 hr for poliovirus and rhinovirus, respectively. The ratios of virus in the two fractions were unmodified by treatment. At the times of assay, approximately 75 and 95% of rhinovirus and poliovirus, respectively, were cell associated.

Isotope experiments. The nature of the antiviral action of SK&F 30097 was further investigated by tracer studies. The first question to be asked was whether the compound is a specific inhibitor of viral RNA synthesis. When ^{32}P and tritiated uridine were used to measure RNA synthesis in uninfected HeLa cells in the presence of SK&F 30097, the results obtained were not identical (Table I). While the tritiated uridine data indicate an apparent inhibition in cellular RNA synthesis, the ^{32}P data suggest that RNA synthesis is not affected by the presence of SK&F 30097. In experiments where ^{32}P was used to follow RNA synthesis, aliquots of the mixture were treated with RNase; and the re-

TABLE I. Effect of SK&F 30097 on Uptake of Radiolabeled Precursor Into the RNA of Uninfected HeLa Cells (cpm).

SK&F 30097 (μg/ml)	Tritriated uridine	^{32}P	
		No treatment	Treated with RNase
50	12,000	25,500	6400
12.5	30,000	30,000	5300
3.125	41,000	30,100	5600
0.8	48,000	NT	NT
0	50,000	15,000	7400

sults were compared with untreated samples. Since uridine uptake *per se* was inhibited by SK&F 30097, whenever this precurser was used to measure RNA synthesis, SK&F 30097 was removed before its addition. Pulse-labeling with tritiated uridine for 1 hr was found to be adequate for most experiments. In contrast to its effect on uridine uptake, SK&F 30097 has no effect on arginine or lysine incorporation into the protein of uninfected HeLa cells over a 48-hr period.

These data substantiate the biological observations that SK&F 30097 is nontoxic at 50 μg/ml for cultured cells.

Pulse-labeling with ^{3}H-uridine was used to determine the sensitivity of viral RNA to inhibition by SK&F 30097. As shown in Figure 4, the addition of 50 μg/ml of SK&F 30097 to poliovirus-infected cultures of HeLa cells at time 0, provided approximately 80% inhibition of viral RNA synthesis. A similar level of inhibition was obtained when SK&F 30097 addition was delayed for 2 hr. At 3 hr, inhibition was significantly less, though still considerable. Later addition was not measured. When the effect of delayed treatment was measured by virus yield reduction (Fig. 2), a significant loss of activity was first observed at 4 hr. This time difference may not be significant, since the results were obtained in different experiments. Both illustrate an inhibitory action when compound is added at any time during the first half of the latent period.

Discussion. These data support previous conclusions that antipicornavirus activity is obtained with SK&F 30097 in cell culture at nontoxic concentrations (2, 3). The site of this inhibition is intracellular and involves an inhibition of a stage of viral synthesis. Since viral RNA synthesis is inhibited, it is postulated that this effect is responsible for the antiviral action of the compound. The action of the compound is reversible. When present during the latter half of the latent period and then removed, the time lag to maximal virus synthesis corresponds to the duration of time that the compound was in the medium. When present only during the initial half of the latent period, the delay in time to maximum virus synthesis after compound removal is shorter, suggesting that incomplete inhibition is obtained during this treatment period, or that a primary event, such as viral uncoating, is refractory. However, the significance of this difference remains in question until more data are available. The correspondence between treatment period late in infection and lag time to maximum virus yield may be fortuitous. If viral precursors or enzymes are broken down during inhibition,

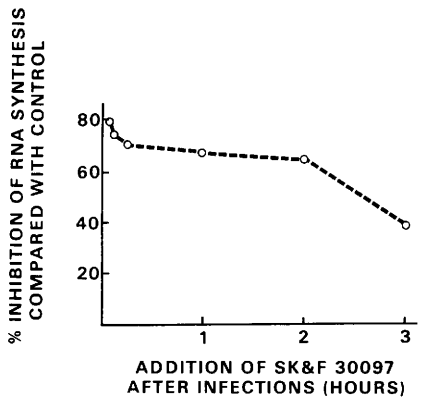

FIG. 4. Degree of inhibition of polio RNA synthesis in relation to time of addition of SK&F 30097 at 50 μg/ml in HeLa cells.

they would have to be resynthesized after compound removal. This process could account for the longer apparent inhibition.

The selective inhibition of uridine uptake by uninfected cells in the presence of SK&F 30097 was an unexpected event, apparently not pertinent to the antiviral action of the compound. Other compounds of different structure have been shown to have a similar effect (4, 5).

It has been reported that isatin-3-thiosemicarbazone, an analogue of SK&F 30097, inhibits synthesis of vaccinia virus by blocking late in RNA translation (6). The synthesis of the viral DNA genome (6, 7) and RNA (7) is not inhibited. Methisazone, a closely related compound, presumably inhibits poxvirus synthesis by a similar means; its action mechanism against selected RNA viruses is not reported (8). The foregoing results with SK&F 30097 raise questions as to whether methisazone inhibits sensitive RNA and DNA viruses by a common mechanism.

Summary. SK&F 30097, a triazinoindole, exerts selective antiviral activity against picornaviruses *in vitro*. Its activity apparently relates to specific inhibition of viral RNA synthesis.

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