Rhinovirus Inhibition by 3-Substituted Triazinoindoles (34642)

JACK M. GWALTNEY, JR.[1]
(Introduced by C. M. Kunin)

Departments of Preventive Medicine and Medicine, University of Virginia, School of Medicine, Charlottesville, Virginia 22901

Fifty-six antigenically distinct rhinoviruses have been reported thus far (1), and the number of types continues to grow. The antigenic diversity of rhinoviruses is a major obstacle to vaccine development. An alternative approach is the use of antiviral compounds with activity against many rhinovirus types. A group of 3-substituted triazinoindoles, which are related to isatin thiosemicarbazone, have recently been reported to show antirhinovirus activity (2). The present report describes the activity of six different triazinoindoles against a large number of rhinovirus strains.

Materials and Methods. Compounds. 3,10-Dimethyl-10*H*-*s*-triazolo[4′,3′:2,3]-*as*-triazino[5,6-b]indole (SK&F 21681), 3-[(5-methyl-5*H*-*as*-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 21687), 5-methyl-5H-*as*-triazino[5,6-b]indole-3-thiol (SK&F 20646), 3-[(5-benzyl-5H-*as*-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 26689), 2,2-dimethyl-3-[(5-methyl-5H-*as*-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 28938), and 2-methyl-4-[(5-methyl-5H-*as*-triazino[5,6-b]indole-3-yl)amino]-2-butanol (SK&F 30097) were obtained from the Research and Development Division of Smith Kline and French Laboratories, Philadelphia, Pennsylvania (Fig. 1). For cell culture tube experiments, the compounds were suspended in equal volumes of minimum essential medium Eagle (MEM) and medium 199 and mixed overnight at room temperature by an electromagnetic stirrer. Two percent fetal calf serum was incorporated into the medium prior to stirring or at the time of testing. Solutions were prepared at weekly or 2-week intervals and stored at room temperature. For gradient plate plaque reduction tests, compounds were dissolved in 2× basal medium Eagle (BME) with 20% agamma calf serum and mixed overnight prior to incorporation into an agar mixture.

Viruses. Rhinovirus strains were isolated during an epidemiological study of colds (3) or were obtained from other investigations.[2]

Cell cultures. Diploid human embryonic lung cell (WI-38) culture tubes[3] were maintained on 49% MEM, 49% medium 199, and 2% fetal calf serum. HeLa (Rhino)[4] cells were grown on BME with 10% agamma calf serum.

Gradient plate plaque reduction technique. The rhinovirus plaquing method of Fiala and Kenny (4) was combined with the gradient plate technique of Kucera and Herrmann (5). HeLa cell monolayers in 60× 15-mm plastic petri dishes[5] were infected with tenfold dilutions of virus, incubated at 37° for 1 hr, washed with Hanks' balanced salt solution and overlaid with agar gradients containing the desired concentrations of compound. In addition to the experimental compound, the final concentration of the ingredients in the overlay were: 0.49% Agarose[6], 30 m*M* $MgCl_2$, 30 μg/ml of DEAE-dextran, 10% agamma calf serum, and 1× BME. Petri dishes were incubated for 3 to 5 days at 35° in an atmosphere of 2–3% CO_2 with high humidity. Cell monolayers were then fixed

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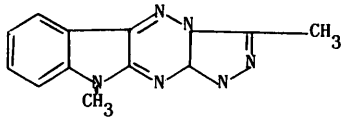
[2] Kindly supplied by Drs. M. A. Mufson, D. Hamre and V. V. Hamparian.

[3] Flow Laboratories, Rockville, Maryland, HEM Research, Rockville, Maryland.

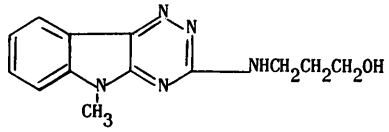
[4] Grand Island Biological Company, Grand Island, New York.

[5] Falcon Plastics, Los Angeles, California.

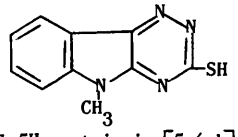
[6] Bausch and Lomb, Rochester, New York.



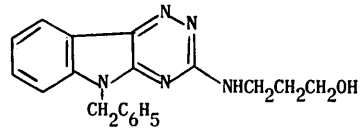
3,10-Dimethyl-10H-s-triazolo[4',3':2,3]-as-triazino[5,6-b]indole (SK&F 21681)



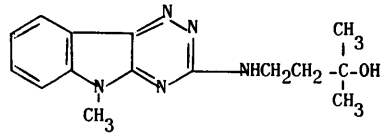
3-[(5-Methyl-5H-as-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 21687)



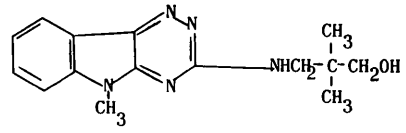
5-Methyl-5H-as-triazino[5,6-b]indole-3-thiol (SK&F 20646)



3-[(5-Benzyl-5H-as-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 26689)



2-Methyl-4-[(5-methyl-5H-as-triazino[5,6-b]indole-3-yl)amino]-2-butanol (SK&F 30097)



2,2-Dimethyl-3-[5-methyl-5H-as-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 28938)

Figure 1.

with 10% formalin and stained with 1% crystal violet in 20% ethanol.

Experimental design. In the WI-38 cell culture tube experiments, compound concentrations ranging between 20 and 100 μg/ml were tested against rhinovirus concentrations ranging between 3 and 100 $TCID_{50}$/0.1 ml. Testing was done in triplicate or quadruplicate cell culture tubes, and virus titrations were maintained simultaneously on medium without compound. Cell control tubes containing test concentrations of compounds and tests of a sensitive rhinovirus were included in each series of experiments. Tubes were incubated at 35 or 37° in a roller drum and read daily for cytopathic effect (CPE). Tests were judged complete when virus control tubes showed 75% or greater destruction of cell sheets. Observed differences in the extent of CPE in experimental and control tubes of 75% or more were graded as +; 75–50% as ±, and less than 50%, — inhibition. Gradient plate experiments employed final compound concentrations of 20 and 50 μg/ml.

Results. SK&F 21681, 21687, 20646, and 26689. The results of testing SK&F 21681, 21687, 20646, and 26689 against 32 rhinovirus strains representative of "H" and "M" sero-

TABLE I Rhinovirus Testing vs. Compounds Related to Isatin Thiosemicarbazone.[a]

Type	Strain	Cell tropism	SK&F 21681 conc				SK&F 21687 conc				SK&F 20646	SK&F 26689
			285 μM (80 μg/ml)		71 μM (20 μg/ml)		389 μM (100 μg/ml)		78 μM (20 μg/ml)		462 μM 100 μg/ml	300 μM 100 μg/ml
1A	SF 1382	M	—(30)[b]	—(100)	—(30)	—(100)					+(30)	±(30)
1B	SF 704	M	—(30)	—(100)	—(30)	—(100)	+(30)	+(100)	—(30)	—(100)	+(30)	—(30)
2	HGP	M	—(30)		—(30)	—(100)	+(30)	+(100)	—(30)	—(100)	—(30)	—(30)
3	SF 1399	H	+(30)	+(100)	±(30)	±(100)	+(30)	+(100)	—(30)	—(100)		
4	SF 748	H	—(30)	—(100)	—(30)	—(100)	+(3)	+(10)	+(3)	+(10)	—(30)	—(30)
4	SF 1394	H					+(30)	+(100)	+(30)	±(100)		
6	SF 1349	H	—(30)	—(100)	—(30)	—(100)					±(30)	—(30)
11	SF 747	H	—(30)	—(100)	—(30)	—(100)	+(30)		—(30)	—(100)	+(30)	+(30)
13	SF 1384	H	—(30)	—(100)	—(30)	—(100)	+(30)	+(100)	—(30)	—(100)	—(30)	—(30)
14	SF 725	H	+(30)	+(100)	—(30)	—(100)	+(30)	+(100)	+(30)	±(100)		
14	SF 732	H					+(30)	+(100)	+(30)	±(100)		
14	NIH 1059	H									+(30)	+(30)
15	SF 525	H	+(30)	+(100)	—(30)	—(100)						
17	SF 460	H	—(30)	—(100)	—(30)	—(100)	+(30)	+(100)	±(30)	±(100)	—(30)	—(30)
21	SH 51	H	+(30)	+(100)	+(30)	+(100)					±(30)	—(30)
23	SF 1322	H	+(30)	+(100)	—(30)	—(100)	+(30)	+(100)	±(30)	—(100)		
26	127-1	H	+(30)		+(30)		+(30)	+(100)	±(30)	—(100)		
26	Cor 27	H									+(30)	±(30)
27	SF 274	H					+(30)	+(100)	—(30)	—(100)		
29	SF 127	M	—(30)	—(100)	—(30)	—(100)	+(30)	+(100)	±(30)	—(100)	±(30)	—(30)
30	SH 91	M	—(30)	—(100)	—(30)	—(100)					±(30)	—(30)
31	SF 1240	M	+(30)	+(100)	±(30)	—(100)	+(30)	+(100)	—(30)	—(100)	—(30)	—(30)
32	SF 578	H	—(30)	—(100)	—(30)	—(100)	+(30)	+(100)	+(30)	±(100)	±(30)	—(30)
33	SF 692	H	±(30)	+(100)	—(30)	—(100)	+(30)	+(100)	±(30)	—(100)	+(30)	+(30)
34	SF 1540	H	+(30)	+(100)	±(30)	—(100)					±(30)	+(30)
35	SF 795	H	—(30)	—(100)	—(30)	—(100)						
39	SF 299	M	+(30)	+(100)	±(30)	—(100)	+(30)	+(100)	—(30)	—(100)	—(30)	—(30)
41	SF 220	H	+(30)	+(100)	—(30)	—(100)	+(30)	+(100)	—(30)	—(100)	±(30)	—(30)
46	SH 202	H	+(30)	+(100)	—(30)	—(100)					—(30)	—(30)
Cor 20[c]	21	H	—(30)	—(100)	—(30)	—(100)						
Cor 43[c]	SF 682	H	+(30)	+(100)	—(30)	—(100)	+(30)	+(100)	—(30)	—(100)	+(30)	±(30)
SF 1338[d]	SF 1338	M	—(30)	—(100)	—(30)	—(100)						

[a] Grading scale explained in text. [b] Virus $TCID_{50}$/0.1 ml. [c] Provisional type. [d] Untyped vs. 60 antisera.

types are shown in Table I. Each of the four compounds showed inhibitory activity against rhinoviruses. SK&F 21681 inhibited 13 of 27 (45%); SK&F 21687, 20 of 20 (100 %); SK&F 20646, 14 of 21 (67%); and S K&F 26689, 7 of 21 (33%) virus strains at one or more concentrations of compound. The inhibition was reversible. In some experiments cells in the tubes containing compound were re-fed regular medium at the time that the virus titrations were completed. This was followed by the appearance of viral CPE in 1 to 3 days.

Strains of 21 virus types were tested against the three compounds that inhibited some but not all virus strains (Table II). The five viruses that were sensitive to all three compounds were H strains. Two of these viruses had been previously tested against D-(—)-2-(α-hydroxybenzyl)-benzimidazole · HCl (D-HBB · HCl) by the same procedure and had shown maximum sensitivity to this compound. Three of the four viruses that were the most resistant to inhibition by the triazinoidoles were also H strains. Two of these showed good or moderate sensitivity to D-HBB · HCl. The other resistant virus was an M strain which required a maximum concentration of D-HBB·HCl for inhibition. Other M strains (types 1B, 29, and 30) were sensi-

TABLE II. Comparison of Rhinovirus Inhibition by 3-Substituted Triazinoindoles and D-HBB·HCl.

Type	Cell tropism	20646	26689	21681	D-HBB · HCl MIC (μM)
14	H	+	+	+	69
26	H	+	±	+	58
33	H	+	+	+	NT
34	H	±	+	+	115
Cor 43[a]	H	+	±	+	NT
11	H	+	+	—	108
1A	M	+	±	—	185
1B	M	+	—	—	>192
29	M	+	—	—	>192
30	M	±	—	—	92
6	H	±	—	—	58
32	H	±	—	—	NT
21	H	±	—	+	92
41	H	±	—	+	NT
31	M	—	—	+	92
39	M	—	—	+	>192
46	H	—	—	+	142
2	M	—	—	—	250
4	H	—	—	—	NT
13	H	—	—	—	81
17	H	—	—	—	123

[a] Prototype candidate.

TABLE III. Inhibition of Rhinovirus CPE in WI-38 Cells by 3-Substituted Triazinoindoles.[a]

Rhinovirus			SK&F 30097	SK&F 28938
Sero-type	Strain	Cell tropism	350 μM (100 μg/ml)	350 μM (100 μg/ml)
14	1059	H	+(100)[b]	+(100)
12	Cor 16	H	+(100)	
23	Cor 24	H	+(100)	+(100)
24	Cor 25	H	+(3)	+(3)
25	Cor 26	H	+(30)	+(30)
14	1059	H	+(100)	+(100)
28	Cor 29	H	+(100)	+(100)
29	Cor 30	M	+(100)	+(100)
30	106-F	M	+(100)	+(100)
32	363	H	+(100)	+(100)
14	1059	H	+(30)	+(30)
36	342-H	H	+(100)	+(100)
37	151-1	H	+(30)	+(30)
39	209	M	+(30)	+(30)
			175 μM (50 μg/ml)	
14	1059	H	±(30)	
40	1794	H	+(100)	
42	56822	H	+(3)	
44	71560	M	±(30)	
45	Ba 1	H	+(30)	
14	1059	H	+(10)	
46	Ba 2	H	±(30)	
48	1505	H	+(100)	
50	A_2 No. 58	H	+(100)	
52	FO1 3772	H	+(30)	
53	FO1 3928	H	±(100)	
14	1059	H	±(100)	
43	58750	H	±(100)	
54	FO1 3774	H	+(100)	
55	WIS 315E	H	±(100)	
8	Cor 12	H	—(100)	
24	Cor 25	H	+(100)	
14	1059	H	+(100)	
18	Cor 17	H	+(100)	
16	11757	H	+(10)	
20	Cor 19	H	+(10)	

[a] Grading scale explained in text.
[b] Virus $TCID_{50}$/0.1 ml.

tive to SK&F 20646 and resistant to the other two compounds, while types 31 and 39 were only inhibited by SK&F 21681.

SK&F 30079 and 28938. Cell culture tube tests were conducted with two compounds SK&F 30097 and 28938, of similar structure to SK&F 21687 but with paired methyl groups in the side chains (Table III). Concentrations of 350 μM (100 μg/ml) of both compounds gave maximum suppression of the CPE of all 12 rhinovirus strains tested. SK&F 30097 inhibited (+ or ±) 17 of 18 strains at a concentration of 175 μM (50 μg/ml). Virus breakthrough appeared after the cells were re-fed regular medium.

SK&F 30097 plaque reduction. Five rhinovirus strains showed complete plaque inhibition in experiments employing a concentration of 175 μM (50 μg/ml) of SK&F 30097 (Table IV). With a compound concentration of 70 μM (20 μg/ml) the minimum inhibitory concentrations for six strains ranged between 21 and 32 μM (6-9 μg/ml). A single agar monolayer containing a concentration of SK&F 30097 (25 μM) that was twice the MIC for the test virus gave complete plaque suppression (Fig. 2). SK&F 30097 at a concentration of 350 μM (100 μg/ml) showed no toxicity for HeLa cells as judged by gross morphologic appearance.

Discussion. Compounds with a common 3-substituted triazinoindole structure inhibited rhinoviruses in WI-38 and HeLa cell cultures. Three of the compounds SK&F 21687, 30097, 28938, inhibited all virus strains tested; the latter two appeared to be the most potent. Paired methyl groups in the side chains may account for the enhanced rhinovirus inhibitory activities of SK&F 30097 and 28938.

TABLE IV. Minimum Inhibitory Concentrations of SK&F 30097 for Selected Rhinovirus Strains in Gradient Plate Plaque Reduction Tests in HeLa Cells.

Virus			Virus			
			Dilution log 10	PFU/0.5 ml of innoc.		
Type	Strain	30097 conc (μM)	$TCID_{50}$/0.1 ml	Control	Exp.	Min. inhib. conc (μM)
5	Sal Norm	70	2.5	510[a]	69	32
27	Cor 28	70	1.0	160[a]	25	25
39	209	70	4.0	65	12	21
44	FO1 3774	70	2.0	80[a]	17	21
		70	3.0	8	1	—
50	A_2 No. 58	70	3.0	inn[b]	inn	21
		70	4.0	inn	inn	25
55	WIS 315E	70	2.0	500[a]	39	21
		70	3.0	50	5	—
14	1059	175	3.0	49	0	<42
		175	4.0	5	0	<42
1A	JH	175	2.0	250[a]	0	<42
		175	3.0	25	0	<42
8	Cor 12	175	4.0	250[a]	0	<42
		175	5.0	25	0	<42
52	FO1 13772	175	3.0	130[a]	0	<42
		175	4.0	13	0	<42
53	FO1 13928	175	2.0	28	0	<42
		175	3.0	0	0	<42

[a] Estimated.

[b] Innumerable.

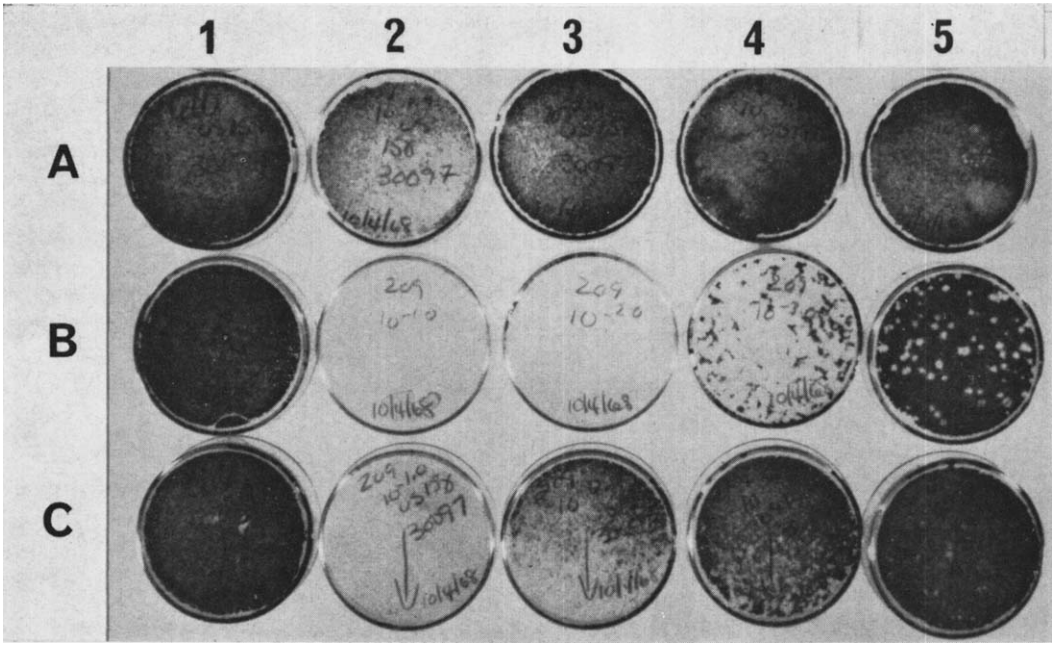


FIG. 2. Inhibition of rhinovirus type 39 by incorporation of SK&F 30097 in agar: columns: (1) cell control, (2) rhinovirus type 39 log 10^{-1} dilution, (3) 10^{-2} dilution, (4) 10^{-3} dilution, (5) 10^{-4} dilution. Lines: (B) virus titration without compound; (C) gradient plate with 15 μg/ml of SK&F 30097; (A) 15 μg/ml of compound in a monolayer.

An attempt was made to place rhinovirus strains in subgroups on the basis of the following characteristics: (i) inhibition by one or more triazinoindoles, (ii) cell tropism, (iii) degree of D-HBB·HCl sensitivity. There may have been some tendency for viruses to group by these characteristics. For example, four M strains (1A, 1B, 29, 30) showed almost identical patterns of triazinoindole susceptibility and three of the four were relatively insensitive to D-HBB·HCl. If such subgroups exist, however, they were not clearly discernible from these data, and many inconsistencies in grouping were observed.

The plaque reduction experiments suggest that SK&F 30097 is more effective against rhinoviruses than D-HBB·HCl. Minimum rhinovirus inhibitory concentrations of the latter have been found to range from $\leq$ 77 μM (20 μg/ml) to $\geqq$ 192 μM (50 μg/ml) (6). SK&F 30097 suppressed rhinovirus plaque production at a concentration of 25 μM (7 μg/ml) which is well below the gross cytotoxic concentration. D-HBB·HCl has a virus specific inhibitory action which occurs at an early stage of intracellular virus replication, and is thought to represent interference with the initation of viral RNA chains (7). The site of the inhibitory action of the 3-substituted triazinoindoles is currently unknown. The reversibility of the antiviral effect by delayed removal of compound suggests that the action takes place during intracellular virus synthesis. Evidence is lacking on whether it is virus specific. Of interest, is the fact that all of the 3-substituted triazinoindoles reported contain a $=N-\overset{|}{C}=N-$ sequence at the site of side chain attachment which is similar to the $\begin{matrix}\diagdown \\ \\ \diagup\end{matrix}\!\!N-\overset{|}{C}=N-$ sequence found in the picornavirus inhibitory compounds, HBB and guanidine. It has been proposed that one (or both) of these nitrogen atoms may be essential for binding HBB to its target (7).

The favorable results of these *in vitro* studies, particularly the broad antirhinovirus action of several of the compounds, should

encourage further study of the 3-substituted triazinoindoles. The wide spectrum of activity makes them suitable candidates for development for clinical trials.

Summary. Six compounds in a series of 3-substituted triazinoindoles were tested for inhibitory activity against a large number of rhinovirus strains. All of the compounds produced some inhibition of rhinovirus growth as measured by the suppression of viral CPE in WI-38 cell culture tubes. Two of the compounds, 2,2-dimethyl-3-[(5-methyl-5H-*as*-triazino[5,6-b]indole-3-yl)amino]-1-propanol (SK&F 28938) and 2-methyl-4-[(5-methyl-5H-*as*-triazino[5,6-b]indole-3-yl)amino]-2-butanol (SK&F 30097), with paired methyl groups in the side chains were the most active. In gradient plate plaque reduction experiments using HeLa cells, SK&F 30097 had a minimum inhibitory capacity for rhinoviruses ranging from 21 to 32 μM (6–9 μg/ml). HeLa cell morphology was not altered by SK&F 30097 at a concentration of 350 μM (100 μg/ml). The mechanism of the antiviral action of the 3-substituted triazinoindoles is currently unknown.

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