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Antiviral Activity of Antilipidemic Compounds on Herpes Simplex Virus Type 1

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Two antilipidemic compounds, clofibrate and procetofene, inhibited the replication of herpes simplex virus type 1 (HSV-1) in African green monkey kidney cells. Clofibrate, at a concentration of 400 μmol/liter caused a 63% reduction ($P < 0.001$) in HSV-1 yield and at 100 μmol/liter caused a 62% reduction ($P < 0.001$) in plaque formation. Two stereoisomeric analogs of clofibric acid, (−)- and (+)-desmethyl clofibric acid, also caused a significant inhibition of HSV-1 replication. Procetofene at 5 μmol/liter caused a 56% reduction ($P < 0.001$) in HSV-1 plaques and at 10 μmol/liter caused a significant reduction ($P < 0.001$) in both viral yield (42 to 54%) and plaque formation (65%). Procetofene also inhibited the development of HSV-1 plaques. A concentration of 5 μmol/liter resulted in a 26% reduction ($P < 0.001$) in plaque diameter. Because of their nonspecific inhibitory effect on the uptake of cellular macromolecular precursors for nucleic acid and protein biosynthesis, these antilipidemic compounds may exert their antiviral activity by affecting one or more key metabolic host cell pathways.

A conceptual basis for using antilipidemic compounds to inhibit virus replication has been suggested by Grossberg and associates (6, 7), who demonstrated that the sodium salt of clofibrate [sodium 2-(*p*-chlorophenoxy)-2-methylpropionate; Fig. 1] decreased both viral titers and circulating lipid levels associated with arbovirus infections. Steinhart et al. (18) further demonstrated the antiviral efficacy of clofibrate [Atromid S; ethyl 2-(*p*-chlorophenoxy)-2-methylpropionate; Fig. 1] by noting its ability to inhibit herpes simplex virus type 1 (HSV-1) replication in vitro at nontoxic concentrations. It has been hypothesized by Grossberg et al. that clofibrate antiviral activity is mediated via lipid biogenesis either in the viral envelope or in the host membranes necessary for viral development (6, 7). Clofibrate is known to alter cellular lipid metabolism at different levels, suggesting that this drug could deprive replicating viruses of essential lipid components (3, 10, 14, 15, 19, 20). In addition, Steinhart and associates (18) have demonstrated that clofibrate antiviral activity is directed at some late step in HSV maturation such as during envelopment of the virion by host membranes.

The purpose of this study was to further investigate clofibrate analog activity against HSV-1 and to examine structural relationships for antiviral activity. For these latter studies, two stereoisomeric analogs of sodium clofibrate [(−)- and (+)-desmethyl clofibric acid; Fig. 1] were used (21, 22). In addition, a *p*-chlorobenzoyl-substituted analog of clofibrate, procetofene [W-13,635; isopropyl (4'-(*p*-chlorobenzoyl)-2-phenoxy-2-methyl)-propionate; Fig. 1], was evaluated for antiviral activity. Procetofene exhibits pronounced hypolipidemic effects in both rats (9) and humans (17, 24, 25), thus prompting its testing for antiviral activity.

MATERIALS AND METHODS

Virus and cell cultures. The virus used was the Tyler strain of HSV-1 and was propagated in either HeLa cells or a continuous African green monkey kidney cell line (BGM-70). Cell cultures were grown in Eagle minimal essential medium containing 10% fetal bovine serum and 1.1 g of sodium bicarbonate per liter. Cell cultures were maintained with Eagle medium containing 5% fetal bovine serum and 1.8 g of sodium bicarbonate per liter. Both growth and maintenance tissue culture media contained the following antibiotics: penicillin, 100 U/ml; streptomycin, 100 μg/ml; polymyxin, 10 μg/ml; and chlortetracycline, 50 μg/ml.

Compounds. Clofibrate (Atromid S; Ayerst Laboratories, Inc., New York) and procetofene, a gift from Alexander M. Moore (Warner-Lambert/Parke, Davis & Co., Ann Arbor, Mich.), were prepared as 0.1 M stock solutions in 0.25% methylcellulose. Each compound was added to 0.25% methylcellulose in Omnimixer cups (Ivan Sorvall Inc., Norwalk, Conn.), placed on ice, and homogenized for 1 min at full speed five separate times with a 1-min rest interval between each blending. As a control, 0.25% methylcellulose received the same homogenization regimen. The methylcellulose (CPS 4000) was a gift from the Dow Chemical Co., Midland, Mich.

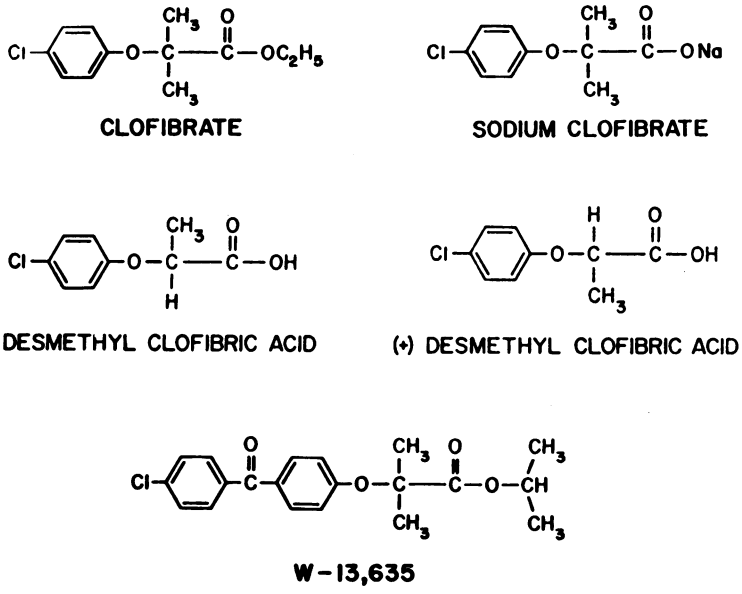

FIG. 1. *Chemical structures of clofibrate, sodium clofibrate, (−)-desmethyl clofibric acid, (+)-desmethyl clofibric acid, and procetofene (W-13,635).*

Clofibric acid, a gift from G. R. Goetchius (Ayerst Laboratories, Inc.), and the isomeric desmethyl clofibric analog (21, 22) were dissolved in 1.0 N NaOH to make a 0.01 M solution. Each compound was further diluted with tissue culture maintenance medium. For each solution, the final pH was 7.8. As a control, similar alkaline solutions without test compounds were used.

Cytotoxicity assays. The cytotoxic effects of clofibrate and procetofene were determined by monitoring the uptake of macromolecular precursors for deoxyribonucleic acid, protein, and lipid synthesis. The effect of clofibrate and procetofene on uptake of precursors for protein and lipid metabolism was determined because clofibrate is reported to directly affect cell lipid metabolism (3, 10, 14, 15, 19, 20, 23) as well as other metabolic pathways (2, 11–13). Inhibition of [^{3}H]thymidine uptake was primarily used as an indicator of cytotoxicity because of similar cytotoxicity studies reported by Ferguson and Prottey (4).

The precursors used for cytotoxicity studies were ^{3}H-labeled protein hydrolysate, [^{3}H]thymidine (Schwarz Mann, Orangeburg, N.Y.), [^{3}H]oleic acid, and [^{3}H]acetate (New England Nuclear Corp., Boston, Mass.). Cells were planted at a density of 40,000 per well in Microtest II tissue culture plates (Falcon 3040) in a volume of 0.2 ml of growth medium and were incubated at 37°C in a 5% CO_2–95% air atmosphere with 95 to 99% humidity for 48 h. When the cells were 80 to 90% confluent, the medium was removed from the wells and 0.2 ml of fresh maintenance medium with or without drug was added. Cells were incubated for 24, 48, or 72 h and were pulsed with 1 μCi of the appropriate ^{3}H-labeled precursors for the last 24 h of any given incubation period. After incubation, cells were trypsinized and collected with a MASH II cell harvester. Exogenous isotope was removed by exhaustive washing with saline. A Beckman LS-7000 liquid scintillation counter was used to determine radioactivity. The percentage of inhibition of ^{3}H-labeled precursor uptake was determined as follows:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{mean cpm of drug-treated cultures}}{\text{mean cpm of non-drug-treated cultures}}\right) \times 100$$

The cytotoxic effects of clofibric and desmethyl clofibric acid analogs were determined as described above or by growth inhibition studies. The inhibition of cell growth was monitored by exposing established BGM-70 cells to various concentrations of compound for 72 h. At 0, 24, 48, and 72 h, mean cell counts from triplet cultures were determined for both drug-treated and nontreated cultures. Results were analyzed by Student's *t*-test for significant inhibition.

Viral quantitation. Ten-fold dilutions of HSV-1 were plaque assayed in tissue culture dishes (160 by 15 mm; Falcon 3030). A 0.75% methylcellulose overlay was used to prevent the formation of secondary foci. A microplaque assay, similar to the one described by Gentry and Aswell (5), was also used to titrate HSV-1. For the microplaque assay, titrations were carried out without an overlay in 24-well cell culture trays (Flow Laboratories, Inc., Linbro Div., Hamden, Conn.). Plaques were counted with the aid of an inverted microscope at 48 h after staining of monolayers with 0.5% crystal violet in 10% formaldehyde.

Yield and plaque reduction assays. Yield reduction assays were performed in tube cultures and in 24-well cell culture trays to mimic the conditions used in the cytotoxicity studies. Cultures were grown to 80 to 90% confluency before virus was allowed to adsorb for 1 h at 37°C. After adsorption, maintenance medium

containing drug was added and the cultures were reincubated for 34 to 48 h. The infected cultures were then subjected to three cycles of freeze-thawing, and the resulting supernatants were clarified at 800 × *g* for 20 min. Supernatants were then titrated for infectious virus.

The plaque reduction assay was performed by adding 75 to 150 plaque-forming units (PFU) of HSV-1 in 50 μl to monolayers of BGM-70 cells. After adsorption, 1 ml of maintenance medium containing various concentrations of drug was added to respective wells. After 48 h, plaques were enumerated and measured for size with the aid of an inverted microscope.

RESULTS

Cytotoxicity studies. Inhibition of [^{3}H]thymidine uptake was found to be a reliable indicator of toxicity. Figure 2 illustrates the effect of different concentrations of clofibrate and procetofene on [^{3}H]thymidine uptake and demonstrates the relative toxicity of the two compounds. Procetofene was toxic at greater than 5 μmol/liter, whereas clofibrate was tolerated up to 600 μmol/liter. This dramatic difference in toxicity was also seen in the ability of these same two compounds to inhibit [^{3}H]acetate and ^{3}H-labeled amino acid uptake. Table 1 illustrates their effects at 48 h. Similar results were seen at 24 and 72 h. Clofibrate with respect to ^{3}H-labeled amino acid uptake was nontoxic at 600 μmol/liter at 24, 48, and 72 h, whereas procetofene was nontoxic at <10 μmol/liter at 24 h and at <5 μmol/liter at 48 and 72 h. In addition, clofibrate had no inhibitory affect on [^{3}H]acetate or oleic acid uptake at 1,000 μmol/liter. Clofibrate toxicity appeared to decrease with time (Fig. 2), and at 1,000 μmol/liter caused a 55 ± 3, 24 ± 11, and 2 ± 10% inhibition of [^{3}H]thymidine uptake at 24, 48, and 72 h, respectively.

In vitro effect of clofibrate on HSV-1. A series of experiments was performed to determine whether clofibrate could inactivate HSV-1 in vitro. Initial experiments were carried out using clofibrate concentrations several times higher than those found to inhibit HSV-1 in cell culture. When HSV-1 was incubated for 2 h at 4°C in medium containing 10 mmol of clofibrate per liter, no significant reduction in viral titers occurred (no drug, 265 ± 17 PFU/ml × 10^5; with drug, 270 ± 10 PFU/ml × 10^5, n = 4). Further investigation demonstrated that clofibrate at 500 μmol/liter, a nontoxic concentration (Fig. 2), did not inactivate HSV-1 directly when incubated at 37°C (no drug, 24 ± 2 PFU/ml × 10^5; with drug, 21 ± 2 PFU/ml × 10^5, n = 4). However, when HSV-1 was incubated at 37°C in the presence of 10 mmol of clofibrate per liter, a reduction in viral titers occurred (no drug, 132 ± 4 PFU/ml × 10^5; with drug, 90 ± 12 PFU/ml × 10^5, n = 4). Noteworthy, however, is the observation that 10 mmol/liter is a concentration 20 times greater than any nontoxic concentration used to inhibit replication of HSV-1 in cell culture.

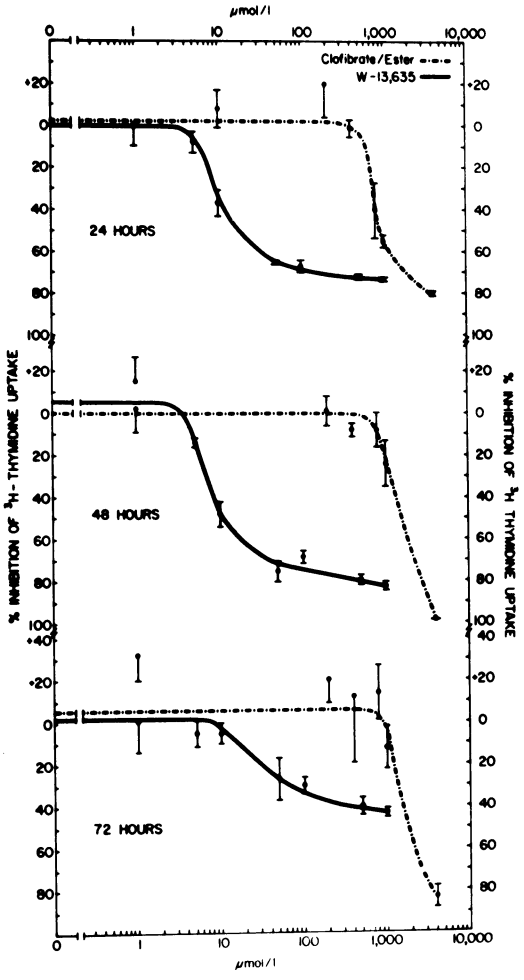

FIG. 2. *Effect of clofibrate and procetofene (W-13,635) on uptake of [^{3}H]thymidine by BGM-70 cells after exposure to the compounds for 24, 48, and 72 h.*

Effect of clofibrate and procetofene on HSV-1 replication. Before determining the antiviral potential of clofibrate and procetofene, a comparison for quantitating virus was made between a standard macro-plaque assay and a scaled-down micro-plaque assay. A 0.75% methylcellulose overlay was used for the macro-plaque assay; none was used with the micro-plaque assay. There were no statistical differences in sensitivity between the macro and micro methods (70 ± 12 versus 69 ± 8 PFU/ml × 10^5, n = 4). The micro-plaque assay, however, was less expensive and less cumbersome than the standard macro method. By not adding 0.75%

TABLE 1. *Effects of clofibrate and procetofene on uptake of macromolecular ^{3}H-labeled precursors for protein and lipid synthesis*[a]

Compound	Concn (μmol/liter)	Amino acids		Acetate		Oleic acid	
		cpm ± SD[b]	% Control	cpm ± SD	% Control	cpm ± SD	% Control
Clofibrate		12,950 ± 526		11,303 ± 1,185		30,306 ± 8,393	
	100	10,909 ± 2,222	84	12,289 ± 2,718	109	55,431 ± 13,852	183[c]
	400	10,330 ± 478	80[c]	13,673 ± 1,127	121[c]	42,292 ± 5,711	139
	600	14,108 ± 2,059	109	12,846 ± 3,472	114	34,994 ± 8,344	115
	800	11,606 ± 1,815	90	14,888 ± 2,580	132[c]	34,776 ± 5,647	115
	1,000	10,399 ± 610	80[c]	13,771 ± 505	122[c]	29,564 ± 2,279	97
	4,000	1,464 ± 645	11[c]	759 ± 91	7[c]	7,641 ± 759	25[c]
Procetofene		7,592 ± 379		25,878 ± 1,594		25,816 ± 4,921	
	1	8,367 ± 908	110	22,681 ± 5,249	87	32,350 ± 3,337	125
	5	7,079 ± 515	93	18,352 ± 1,181	71[c]	27,334 ± 5,400	105
	10	5,693 ± 403	75[c]	19,140 ± 1,504	74[c]	25,135 ± 5,778	97
	50	4,208 ± 420	55[c]	17,596 ± 1,016	68[c]	25,175 ± 6,688	97
	100	4,656 ± 227	61[c]	17,223 ± 1,912	66[c]	27,884 ± 7,321	108
	500	4,522 ± 111	59[c]	16,197 ± 925	62[c]	25,946 ± 2,945	100
	1,000	3,791 ± 303	50[c]	16,891 ± 1,679	65[c]	29,422 ± 6,893	114

[a] BGM-70 cells were incubated in maintenance medium with or without drug for 48 h at 37°C. Cells were pulsed with the ^{3}H-labeled precursor for the last 24 h of the incubation period. Mean counts per minute was determined from four samples.

[b] SD, Standard deviation.

[c] $P < 0.05$.

methylcellulose into the overlay and scoring the plaques by 48 h, it was possible to reduce the assay time from 6 or 7 to 2 days. Unrestricted spread of HSV-1, due to the lack of methylcellulose in the overlay, did not appear to lead to problems in the accuracy of the micro-plaque assay. Secondary plaque formation, which might affect determination of viral titers, did not readily occur if plaques were counted within 48 h of initial viral infection. Therefore, the micro-plaque assay was chosen for all subsequent titrations of HSV-1 since it had the advantages of low cost, ease of performance, quickness, and reliability. Furthermore, not incorporating methylcellulose into micro-plaque overlays made yield and plaque reduction assays more comparable and simplified the drug cytotoxicity studies.

Clofibrate at a concentration of 400 μmol/liter caused a 63% reduction ($P < 0.001$) in HSV-1 yield. An even greater reduction in viral yield was seen at 800 μmol/liter (94%, $P < 0.001$) (Table 2). In a second experiment, where the viral replication time was 48 rather than 34 h, clofibrate at 400 μmol/liter caused an 88% reduction in viral yield (control, 17,050 ± 323 PFU/ml versus experimental, 2,100 ± 1,131 PFU/ml, $P < 0.001$). In addition to these yield reduction experiments, clofibrate at 100 μmol/liter caused a 62% reduction ($P < 0.001$) in HSV-1 plaque formation. Clofibric acid and the (+)- and (−)- desmethyl analogs, although not as active as clofibrate, also caused a significant inhibition of HSV-1 plaque formation (Fig. 3). At 500 μmol/liter, all three clofibric acids had antiviral activity and were found to be nontoxic by our cytotoxicity or growth inhibition assays (data not shown).

TABLE 2. *Effect of clofibrate on HSV-1 yield reduction assays*[a]

Assay	Clofibrate (μmol/liter)	PFU/ml × 10^5 ± SD[b]
1		950 ± 56
2	400	350 ± 78[c] (63)[d]
3	800	55 ± 7[c] (94)

[a] Virus was adsorbed to African green monkey kidney cells 1 h before incubation with or without clofibrate for 34 h.

[b] Mean PFU was determined from two platings of one to two separate cultures. The multiplicity of infection was 0.40. SD, Standard deviation.

[c] $P < 0.001$.

[d] Percentage of inhibition is given in parentheses.

Procetofene was also found to inhibit the replication of HSV-1 (Table 3). A concentration of 5 μmol/liter gave a 56% reduction ($P < 0.001$) in plaque formation. At 10 μmol/liter, a significant reduction ($P < 0.001$) in both viral yield (42 to 54%) and plaque formation (65%) was observed. Procetofene inhibited not only plaque formation, but also plaque size (Table 3). In a series of

experiments, HSV-1 was used to infect confluent monolayers of BGM-70 cells 2 h before addition of medium either containing or not containing procetofene. After 48 h of incubation, monolayers were fixed and stained with crystal violet. The plaques were scored without knowledge of which were drug treated or controls. A concentration of 5 μmol/liter caused a 26% reduction ($P < 0.001$) in plaque size. Procetofene at 10 and 50 μmol/liter caused, respectively, 44% ($P < 0.001$) and 68% ($P < 0.001$) reduction in plaque diameter.

DISCUSSION

The results of this study confirm the antiherpetic activity of clofibrate first noted by Steinhart et al. (18). Using a different host system, BGM-70 cells (Table 2), clofibrate, at concentrations used in vitro to inhibit viral replication, was shown to have no direct inactivating effect on the virus. Thus, clofibrate appears to inhibit HSV-1 replication by affecting either the host cell or virus-induced events associated with infection.

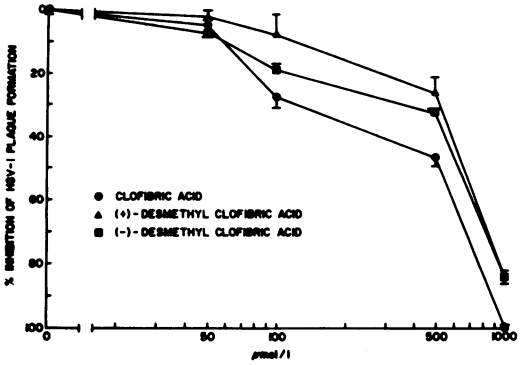

FIG. 3. *Percentage of inhibition of HSV-1 plaque formation by clofibric acid and (+)- and (−)-desmethyl clofibric acid.*

Two stereoisomers of desmethyl clofibric acid caused a significant reduction in HSV-1 plaque formation, but little or no stereoselective effects on activity were observed. These data suggest that the antiviral mechanism can accommodate replacement of methyl with hydrogen in either configuration. Further work with alkyl groups larger than methyl would be required to further define the importance of asymmetric influences on antiviral activity.

The antilipidemic drug procetofene was evaluated for antiviral activity since it is more potent than clofibrate as an antilipidemic agent and may have greater pharmacological specificity (9). Although procetofene was relatively more toxic than clofibrate (Fig. 2), it did significantly inhibit (56%) HSV-1 plaque formation (Table 3) and reduce plaque sizes (Table 3), suggesting possible inhibition of spread of virus. Such inhibition would be advantageous for treatment of HSV-1 infections since spread can occur directly between contiguous cells. Alternatively, however, procetofene may merely be slowing down viral replication, and this is reflected morphologically as smaller plaques. It should be noted that concentrations of clofibrate and procetofene that

TABLE 3. *Effect of procetofene on HSV-1 yield and plaque reduction assays*

Procetofene concn (μmol/liter)	Yield reduction[a] (PFU/ml × 10^4 ± SD)		Plaque reduction[b] (PFU ± SD)	Plaque diam[c] (μm ± SD)
	Test 1	Test 2		
	1,320 ± 196	5,200 ± 848	162 ± 3	357 ± 68
1	1,095 ± 75 (18)[d]	4,300 ± 476 (17)		
5			72 ± 5[e] (56)	264 ± 67[e] (26)
10	610 ± 129[e] (54)	3,000 ± 200[e] (42)	57 ± 14[e] (65)	200 ± 38[e] (44)
50			4 ± 2[e] (98)	149 ± 30[e] (68)
100		1,950 ± 550[e] (62)		

[a] Virus was adsorbed to African green monkey kidney cells for 1 h before incubation with or without procetofene for 48 h. The multiplicity of infection for test 1 was 0.40 and for test 2 was 0.15. Mean PFU was determined from two to three platings of two separate cultures, except for the 100-μmol/liter concentration, which is from one culture. SD, Standard deviation.

[b] Mean PFU determined for three platings except for control, which is an average of six platings.

[c] Determined from 50 random plaques which were measured blindly at 40× with a micrometer.

[d] Percentage of inhibition is given in parentheses.

[e] $P < 0.001$.

inhibit viral replication in vitro are well below those attainable in the serum of patients treated with these drugs. Peak serum levels of patients being treated with clofibrate can attain 200 μg/ml (8); with procetofene peak levels can reach 20 to 30 μg/ml (1).

Both clofibrate and procetofene were selected for antiviral evaluation because of their ability to alter cell lipid metabolism. The original hypothesis by Grossberg et al. (6, 7) suggested that clofibrate exerted its antiviral activity by depriving replicating arboviruses of essential lipids. Alternatively, clofibrate may alter host cell energetics. Clofibrate has been shown to inhibit respiration and oxidative phosphorylation in liver mitochondria from different species (2, 11 to 13), suggesting that this drug may regulate energy available to replicating viruses. Further information, suggesting that energy pathways may be involved, comes from Cederbaum and Rubin (2), who demonstrated that clofibrate is more effective than its corresponding sodium salt in inhibiting respiration and decreasing adenosine triphosphate levels in mitochondria. Data (not shown) from our study indicate that clofibrate is more toxic than its free acid in tissue culture, and the ester inhibits HSV-1 at lower concentrations.

Nonspecific effects on macromolecular uptake observed in our toxicity studies also indicate that clofibrate may inhibit a common pathway, as suggested by Pan and Chou (16) in their studies with *Tetrahymena pyriformis*. Inhibition of uptake of labeled biosynthetic precursors for deoxyribonucleic acid, protein, and lipid biosynthesis may be a secondary effect to inhibition of a more common or key pathway involving cell energetics. Furthermore, it is less likely that the antiviral activities attributable to procetofene and clofibrate are specific because these compounds would lack a high variety of independent and highly specific ligand-enzyme interactions (25).

The ability of antilipidemic agents to operate on host metabolic pathways common to viral replication suggests that some of these compounds may have potential use against viruses that require essential lipids. Our studies provide evidence that clofibrate or procetofene can produce a protective effect to the advantage of the virus-infected host. Viral strains resistant to these drugs might not result from therapy, because it is unlikely that a viral genome would undergo mutation in response to drug action directed against a normal cell.

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