Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3263–3267, June 1984
Applied Biology

DNA and RNA virus species are inhibited by xanthates, a class of antiviral compounds with unique properties

(inhibitors/viral replication/transformation/reversion)

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Communicated by Peter Medawar, February 3, 1984

ABSTRACT **Various DNA and RNA virus species are inhibited by xanthate compounds at concentrations that leave the mitotic activity of uninfected cells unimpaired. The concentration of tricyclodecan-9-yl-xanthogenate that reduces the yield of herpes simplex virus types 1 and 2 by 50% is between 4.5 and 33 μM. The replication of DNA viruses such as simian virus 40 can be blocked at the DNA and RNA level both early and late after infection. The xanthates are not incorporated into nucleic acids. Episomal bovine papilloma virus DNA replication and transcription are also inhibited in transformed cells. The treated cells revert to the normal phenotype by acquisition of contact inhibition and a flat morphology.**

The development of antiviral compounds has been hampered by the nature of viral replication. Since cellular functions and enzymes are required for the synthesis of viral progeny, it has always been felt that antiviral drugs would exert deleterious effects also on the host cell. This applies in particular to the class of halogenated nucleotide analogs, of which the phosphorylated derivatives become incorporated in both newly synthesized viral DNA and cellular DNA.

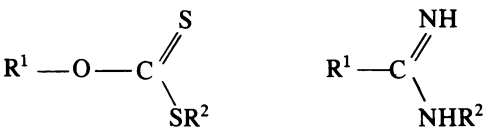
Efforts have therefore been devoted in recent years to the synthesis of compounds that interact with particular virus-encoded enzymes but that leave the host cell enzymes unaffected. Amongst these compounds, phosphonoacetic acid and the structurally related phosphonoformic acid proved to be of interest. They interact with the herpes-virus-encoded DNA polymerase (1). Their retention in the bones of treated animals, however, led to their rejection as therapeutic agents despite their promising specific antiviral effects (2).

The same rationale has guided the development of acyclovir [9-(2-hydroxyethoxymethyl)guanine] as one of the most efficient anti-herpesvirus drugs. Unlike the cellular thymidine kinase, the viral thymidine kinase is capable of phosphorylating acyclovir to the monophosphate. The latter turned out to be a more potent inhibitor of the herpes simplex virus (HSV)-encoded DNA polymerase than of the cellular DNA polymerase (3), hence its excellent antiviral activity in the absence of harmful effects on the host cell.

Compelling as the rationale of administering inhibitors that are virus specific may be, such drugs tend to lead to unwanted drawbacks, such as the accumulation of drug-resistant mutants at high frequencies both *in vitro* and *in vivo* (4).

Another rather different class of pharmacologically active substances, the amidines (5), has been shown to possess antiviral properties. Acting upon the surmise that isosteric structures, possibly derived from entirely unrelated compounds, might be endowed with antiviral properties, we searched for similarly active substances.

The xanthates (6) (I) display isosteric features with amidines (II)



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and, therefore, offered themselves as candidates. To our knowledge, these compounds have not yet been introduced into pharmaceutical research. When applied *in vitro* against various taxonomically unrelated viruses, appropriately substituted xanthates were found to be potent viral inhibitors.

MATERIALS AND METHODS

Cells, Viruses, and Media. African green monkey kidney cells (Rita; Italdiagnostics, Rome), CV-1 cells, and bovine papilloma virus type 1 (BPV-1)-transformed hamster embryo fibroblasts [HEF-BPV (7)] were grown in a 5% CO_2/95% air atmosphere in Eagle's basal medium with Earle's salts and 2× the standard concentration of amino acids and vitamins, 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Prior to use the medium (200 ml) had been equilibrated by addition of 2.2 ml of 1 M HCl and incubation in a flat 800-ml plastic flask for 24 hr at 37°C in a 5% CO_2 atmosphere to stabilize the pH at 6.75 ± 0.05.

Cell densities were compared with each other by the following method, which revealed values that were in proportion to the cell number at least between 4×10^5 and 2×10^6 cells per 6-cm dish. After fixation with formaldehyde, the cultures were stained for 2 min with crystal violet (1%). Then they were briefly washed with water and dried. The dye retained by the cells was subsequently eluted twice with 5 ml of 1% acetic acid in ethanol, the eluates were combined, and the A_{550} was determined separately for each culture. The virus strains are referred to in detail in the figure legends.

Nucleic Acid Extraction and Filter Hybridization. DNA and RNA were extracted from cells as described (8). Nucleic acids were electrophoresed in 1.4% agarose gels [the RNA under denaturing conditions as described (9), however, using 15 mM instead of 5 mM CH_3HgOH] and transferred to nitrocellulose filters (10, 11). Viral DNAs cloned in pBR322 were labeled with ^{32}P by nick-translation (12) and used as probes for hybridization as described (13).

Xanthates. The compounds were prepared according to established methods (14).

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Abbreviations: moi, multiplicity of infection; pfu, plaque-forming unit(s); HSV, herpes simplex virus; SV40, simian virus 40; BPV, bovine papilloma virus.

RESULTS

Structural Requirements for the Antiviral Activity of Xanthates. Xanthates with substituents for R^1 and R^2 comprising aliphatic cyclic and noncyclic residues for R^1 and alkali metal or aliphatic residues for R^2 were examined for antiviral activity. Typical examples of such compounds are arranged in Table 1 in decreasing order of antiviral activity against herpes simplex virus type 1 (HSV-1) in monkey kidney cells. The antiviral activity is expressed as the inhibitory concentration (IC) that reduces the virus yield by 50, 90, or 99%. A comparison of the relationship between structure and antiviral function revealed that to confer antiviral activity to the xanthate structure it was mandatory that position R^1 be occupied by a lipophilic substituent. In addition, R^1 should be structurally constrained: mono-, bi-, and tricyclic residues appeared to be superior to corresponding aliphatic chains. D436 displayed a particularly high toxicity, which may account for the apparent antiviral activity. Similar stringent requirements governed the substitution on the R^2 position. Alkali metal was preferred to small alkyl (C_1–C_2) or alkoxy groups. An enlargement of the aliphatic chain did not enhance the antiviral activity to a significant extent.

In particular, D435, D609, and D611 (Table 1) proved to be efficient virus inhibitors. Whether they are capable of inhibiting indiscriminately most if not all viruses in addition to those species that were tested so far remains to be established.

The IC_{50} of D609 compared well with the IC_{50} values that were established for specific herpesvirus inhibitors, such as phosphonoacetic acid [20 μM (1) or 57.5 μM (16) respectively], and phosphonoformic acid [10 μM (1)]. However, the IC_{50} of acyclovir was shown to be lower by two orders of magnitude (16). As has been observed with acyclovir (17), we have also noticed differences in the sensitivity of various herpes strains. HSV-2 appeared to be more sensitive than HSV-1 (Table 2).

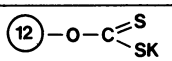
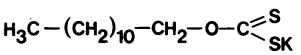
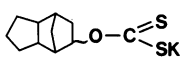
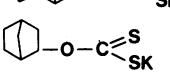
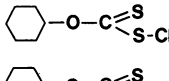
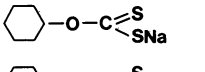
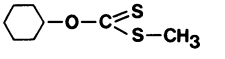
The IC values were subject to fluctuation within certain limits, possibly depending on host cell conditions. The IC_{50} of D609 varied (in six independent determinations) in the case of HSV-1 Ang between 19.3 (Table 1) and 31.5 μM (Table 2), while the IC_{99} ranged between 65.7 and 79.9 μM.

To display antiviral activity, it was essential that the xanthates were assayed *under acidic conditions*. The compound D609 was shown to be ineffective in an alkaline environment. The activity of D609 was reduced within the narrow range of 0.5 pH units (6.8–7.3) in the case of HSV-1 to approximately 1/300th and in the case of the RNA-containing vesicular stomatitis virus to approximately 1/1600th (data not shown). This requirement was found to apply both to the inhibition of all virus species listed in Table 2 and to several other xanthate compounds such as D435, D611, and D416.

Effect on Cellular Proliferation. The ability of cells to proliferate actively represents, in our view, the most stringent criterion for the assessment of cytotoxic effects. Therefore, uninfected primate and murine cell lines in a logarithmically growing state were exposed to various concentrations of the xanthates for several days and the ensuing cell numbers were determined. The cells were seeded at densities that permitted at least one round of mitotic divisions in the untreated cultures.

When plated in 6-cm plastic Petri dishes (10 parallel cultures) at an initial density of $(3.24 \pm 0.36) \times 10^5$ cells, Rita cells had grown in the absence of xanthate compounds to $(6.60 \pm 0.67) \times 10^5$ cells after 2 days at pH 6.75. When the cells were exposed for the same period of time to D609 at its IC_{50} (19.3 μM), the cell number was not significantly altered [$(7.0 \pm 0.64) \times 10^5$ cells]. We also found that both human cells (HeLa) and rodent cells (hamster embryo fibroblasts,

Table 1. Relationship between structure and antiviral activity of various xanthate derivatives

Designation of compound	Chemical structure	M_r	n	Inhibitory effect on HSV-1[a] IC_{50}, μM	IC_{90}, μM	IC_{99}, μM	Conc. of inhibitor required to inhibit growth of cells by 50%,[b] μM
D435		298.54	2	7.5 ± 3.5	29.2 ± 5.9	50.2 ± 21.4	124 ± 5
D436		300.56	2	8.3 ± 2.3	17.7 ± 1.5	63.6 ± 51.3	110 ± 21
D609 *exo*, *endo*		266.46	5	19.3 ± 5	42.9 ± 5.3	74.4 ± 5.3	233 ± 7
D611 *endo*		226.39	1	18.1 ± 1.3	67.1 ± 1.3	94.9 ± 9.2	>350
D614		264.40	1	26 ± 6.1	65.7 ± 11.5	143.7 ± 0	443 ± 40
D416		198.27	2	42.8 ± 0.07	150 ± 23.4	>199	680 ± 51
D607		190.32	1	47.3 ± 4.5	178.2 ± 9.8	>210	>790
D442		206.25	1	80	273.3	>387	ND

For stock solutions (all 1%, wt/vol), compounds D435, D609, D611, and D442 were dissolved in water; compounds D436 and D416, in 80% (vol/vol) ethanol; and compounds D614 and D607, in acetone.

[a]African green monkey kidney cells (Rita) in 6-cm plastic Petri dishes were infected in duplicate with HSV-1 strain Ang (15) at a multiplicity of infection (moi) of 5×10^{-3} plaque-forming units (pfu) per cell. After 1-hr adsorption at 37°C in a 5% CO_2 atmosphere, 8 ml of medium with or without a xanthate was added. The virus yield was determined after 48 hr by plaque assay in Rita cells grown in Linbro plates. The inhibitory concentrations (IC_{50}, IC_{90}, and IC_{99}) were derived from dose–response curves consisting of at least five different concentrations of the compounds that covered the range between 1 and 80 μg/ml. Each concentration was tested in duplicate, and the resulting virus yields were also determined in duplicate. The table contains the mean (±SD) values derived from n dose–response curves.

[b]Extrapolated from dose–response curves. Duplicate cultures of Rita cells (1.3×10^6) in 6-cm plastic Petri dishes were exposed 1 day after seeding for 48 hr to various concentrations (1, 2, 5, 10, 20, 30, 40, 80, and 150 μg/ml) of the xanthates in 8 ml of medium as described above at pH 6.75 ± 0.05. As a control, 6 or 7 cultures remained untreated. Cell densities were compared with each other as described in *Materials and Methods*. ND, not determined.

Table 2. Range of antiviral activity of D609

Virus	*n*	IC_{50},[a] μM	*P*[b]
Vaccinia[c]	7	9.7	<0.02
Vesicular stomatitis[d]	4	6.5	<0.01
Coxsackie B5[e]	2	2.8	<0.01
SV40[f]	3	9.0	<0.05
Herpesvirus strains[g]			
HSV-1 Ang[h]	20	31.5	<0.01
HSV-1 Wal[i]	4	21.3	<0.01
HSV-1 MacIntyre[j]	4	33.0	<0.02
HSV-2 HG-52[k]	2	4.5	<0.02

[a]Determined from *n* assays as described in the legend to Table 1. Unless otherwise stated, plaques were scored 2 days after plating.
[b]Comparison with untreated controls by Student's *t* test after logarithmic transformation of the data so that variances were comparable.
[c]Strain Elstree (Bayer. Landesimpfanstalt), assayed on HeLa cells (moi, 0.02 pfu per cell), harvested 48 hr after infection.
[d]Assayed on Rita cells (moi, 0.005 pfu per cell), harvested 96 hr after infection.
[e]Obtained from the American Type Culture Collection, assayed on Rita cells (moi, 0.02 pfu per cell), harvested 24 hr after infection. Plaques were scored 1 day after plating.
[f]Simian virus 40 strain SP 776, assayed on CV-1 cells (moi, 8 pfu per cell), harvested 96 hr after infection. Plaques were scored 23 days after infection.
[g]Assayed on Rita cells.
[h]As described in the legend to Table 1.
[i]Described in ref. 18 (moi, 0.04 pfu per cell), harvested 72 hr after infection.
[j]Described in ref. 19 (moi, 0.03 pfu per cell), harvested 72 hr after infection.
[k]Described in ref. 20 (moi, 0.007 pfu per cell), harvested 48 hr after infection.

primary mouse embryo fibroblasts) responded in a similar fashion under the influence of D609 and D435 at the IC_{50} (data not shown).

As a convenient measure for cytotoxicity, we have indicated in Table 1 the concentrations of the various xanthate compounds that are required to inhibit cellular growth by 50%. These data were derived from dose–response curves covering a range of concentrations of the compounds over two orders of magnitude. It may be seen that compounds with little antiviral activity were tolerated at higher concentrations by the cells.

On the basis of these data, we chose D609 for further detailed investigation. Although the compound falls at the third position in Table 1, with an IC_{99} of 74.4 μM, its 50% growth inhibitory concentration, 233 μM, appeared to be quite favorable. In addition, the IC_{50} values against various virus species indicated that further detailed studies with this compound would be useful.

This is particularly evident from the comparison of the dose–response curves in Fig. 1. While the inhibitory effect on the growth of HSV-1 encompassed a reduction of the titer of almost 3 logs within the tested range (between 1 and 20 μg/ml) there was no significant influence on the growth of uninfected cells.

Effect on Viral Macromolecules. In the presence of D609 at its IC_{50} the unimpaired mitotic activity suggested a specific interaction of the compound with viral growth processes. HSV DNA and cellular DNA can be readily separated by virtue of their different specific densities by CsCl density equilibrium centrifugation. We have therefore studied the influence of D609 on the DNA synthesis in HSV-1-infected Rita cells. A low moi was chosen such that the host DNA synthesis was not entirely quenched after infection (21), to visualize the effect of the compound not only on the viral but also on the residual host DNA synthesis.

A peak of labeled HSV DNA was obtained (Fig. 2*A*, fractions 12–18) in uninhibited infected cells 2 days after infection and after 1 day of radioactive labeling. In addition, some residual host DNA replication was still detectable (fractions 19–23). The exposure of infected cells to D609 at 15 μg/ml (corresponding to an IC_{97}) for 2 days almost abolished viral DNA synthesis (Fig. 2*B*). Quite unexpectedly, rather than the synthesis of the host DNA being curtailed, the incorporation of [^{3}H]thymidine into the latter occurred to a greater extent than was the case in the untreated infected culture. This observation confirms at the molecular level the high antiviral specificity of the compound.

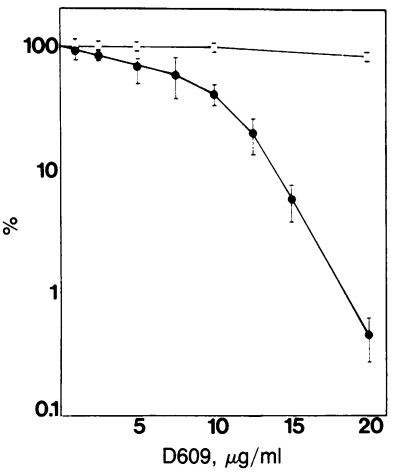


FIG. 1. Comparison of cytotoxic and antiviral effects of D609. ○, Influence of D609 on growing Rita cells. The experimental details were as in the legend to Table 1. ●, influence of D609 on the yield of HSV-1 Ang in Rita cells. Experimental details were as in the legend to Table 1, except that all data were derived from 9 or 10 infected plates, the yield of which was examined individually by plaque titration in duplicate. Error bars indicate SD. The titer at 48 hr after infection in the absence of D609 (mean value of 10 samples) was 2.03×10^7 pfu/ml.

We have chosen another DNA virus, SV40, which, unlike HSV, requires cellular DNA polymerase for its replication. We found that the antiviral xanthates are able to interrupt the infectious cycle at any given stage, even when several rounds of viral DNA replication had preceded the administration of the compound and when late viral gene products were already synthesized. When administered between 0

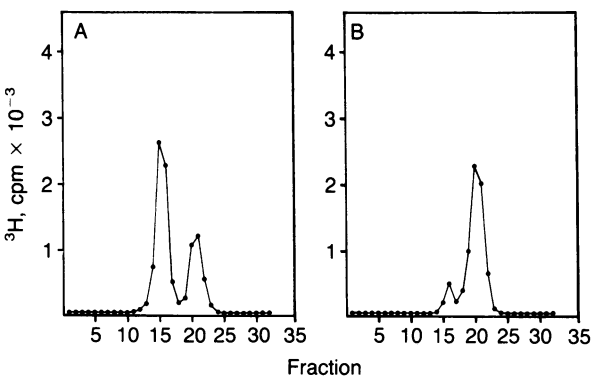


FIG. 2. Preferential inhibition of herpesvirus DNA synthesis after treatment with D609. Density equilibrium centrifugation of DNA from HSV-1-infected untreated Rita cells (*A*); and infected Rita cells that had been treated with D609 at 15 μg/ml between 1 and 48 hr after infection. Between 1 and 2 days after infection (10^{-3} pfu per cell) the treated and untreated cultures were exposed to [^{3}H]thymidine at 2.5 μCi/ml (1 Ci = 37 GBq). The DNA was extracted from the cells as described (8) and centrifuged in a 50 Ti rotor of the Spinco centrifuge at 30,000 rpm for 3 days in a CsCl gradient (initial density 1.72 g/ml). Fractions were collected from the bottom.

and 24 hr after infection, D609 (and D435, data not shown) abolished both SV40 DNA replication and transcription (Fig. 3, lane a). The same observation was made (Fig. 3, lane b) when D609 was added to the culture medium between 24 and 48 hr, a period of time during which substantial amounts of SV40 DNA (Fig. 3, lane e) and mRNA (data not shown) were already synthesized in untreated infected CV-1 cells. Between 2 and 3 days after infection, when the SV40 DNA replication was climaxing (lane f) and SV40 RNA transcripts were available in large amounts (lane h), D609 still arrested both the viral DNA replication, (lane c) and the transcription of the SV40 genomes (lane g). This conclusion was further substantiated by immunofluorescence data. As detected with SV40–polyoma subgroup-specific antisera (22), SV40 DNA capsid antigens disappeared from the nuclei of cells that were treated 2 days after infection with D609 after an exposure for 8 hr to the inhibitor at 30 μg/ml.

The expression of the SV40 tumor antigen was also inhibited by the addition of 20 μg/ml of D609 between 1 and 48 hr after infection of permissive CV-1 cells. In contrast, the antiviral xanthate compounds D609 and D435 failed to inhibit the expression of SV40 tumor antigen in SV40-transformed established cell lines (SV40-3T3 and SV80) that were exposed to the compounds for up to 6 days. This suggests that the xanthate compounds interact with free viral genomes rather than with integrated ones.

Reversion of Papilloma Virus-Induced Transformed Phenotype. In contrast to SV40, BPV persists in transformed cells as a free unintegrated molecule (23). The episomal state of the viral genomes may account for a most striking effect which was observed after exposure of BPV-transformed hamster embryo fibroblast (HEF) cells (7) to D609. When treated with D609 at 15 μg/ml at pH 6.75, BPV-transformed HEF cells assumed within 24 hr the same growth pattern as untransformed HEF cells. Most important, treatment of HEF cells with the same amount of D609 over the same period of time did not influence their growth pattern. Hence, after 5 days, BPV-transformed HEF cells had grown to a relative density that exceeded by approximately 3-fold the relative density attained by the D609-treated and the untreated HEF cultures. In addition, the D609-treated transformed

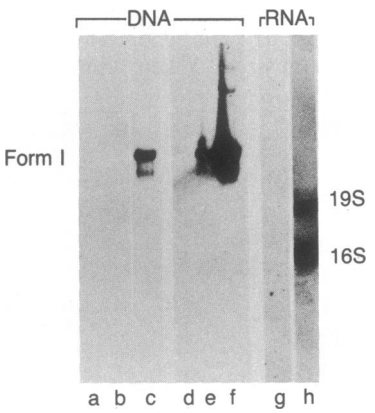


FIG. 3. Inhibition of SV40 DNA and RNA synthesis by D609. CV-1 cells were infected 24 hr after seeding with SV40 at 0.1 pfu per cell and D609 was added at 20 μg/ml either 1 hr after infection (lanes a and b) or 2 days later (lane c). The DNA was isolated 24 hr (lane a), 48 hr (lane b), and 72 hr (lane c) after infection. The DNA from untreated infected cultures was isolated 24 hr (lane d), 48 hr (lane e) and 72 hr (lane f) after infection. The RNA that had been extracted together with the DNA (8) shown in lane c is contained in lane g, and the RNA from the culture whose DNA is depicted in lane f is shown in lane h. The DNA (5 μg per lane) and the RNA preparations (20 μg per lane) were electrophoresed in 1.4% agarose gels. ^{32}P-labeled nick-translated (12) cloned SV40 DNA was used as a probe to reveal homologous sequences (13).

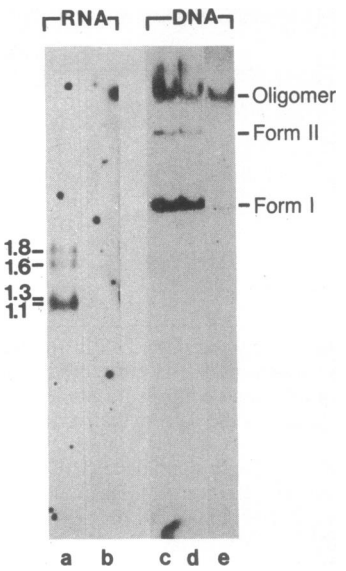


FIG. 4. Effect of D609 on BPV genomes in transformed cells. BPV-1 transformed HEF cells were treated with D609 at pH 6.75. The cultures were passaged at weekly intervals (1:4 split ratio). The medium was changed every 48 hr. Nucleic acids were extracted (8); 10 μg of DNA and 20 μg of RNA per lane were subjected to electrophoresis on agarose gels, and BPV-1-specific RNA and DNA sequences were visualized as described (7). Lanes a and c, untreated culture; lanes b and d, culture treated with D609 at 30 μg/ml for 48 hr; lane e, culture treated for 3 weeks with D609 at 20 μg/ml. Numbers on left are RNA lengths in kilobases.

cells displayed a reverted phenotype by assuming a flat morphology in conjunction with the reappearance of contact inhibition (unpublished data). Whether or not such treatment can eventually lead to "cured" cells after further passages is not yet known.

To explore the interaction of D609 with the viral genomes residing in the transformed cells, DNA and RNA were extracted from treated and untreated cultures and the BPV-specific nucleic acid sequences were revealed in Southern blots by hybridization with a nick-translated BPV DNA probe. After 2 days of treatment, the viral mRNA species, properties of which have been detailed elsewhere (7), were reduced beyond the level of detection (Fig. 4, lanes a and b). BPV-1 DNA sequences persisted in an apparently undiminished amount as superhelical (form I), relaxed circular (form II), and oligomeric (24) forms (Fig. 4, lanes c and d). However, during three passages in the presence of D609 the viral DNA content was gradually reduced (Fig. 4, lane e). The monomeric form I and form II DNAs were lost preferentially, probably as a result of segregation.

These effects—namely, reversion of the transformed phenotype and disappearance of viral transcripts and of viral DNA—were strictly pH dependent. They could not be observed at pH 7.5.

DISCUSSION

The broad range of antiviral activity of the xanthates is shared by interferon, while the former are not limited by species specificity. That they are not stimulators of interferon synthesis is demonstrated by a number of observations. Unlike interferon (25), D609 is capable of blocking late transcripts in the SV40 growth cycle. The inhibitory effect of D609 on the RNA-containing vesicular stomatitis virus also remained unimpaired by the presence of actinomycin D (unpublished), which is known to block interferon. Furthermore, the medium from Rita cell cultures that had been exposed for 24 hr to D609 at 20 μg/ml was devoid of nondialyz-

able antiviral macromolecules as tested in HSV-1 plaque assays.

Lysosomotropic agents (26) displaying antiviral activity were shown to inhibit the entry of viruses into the cell (27, 28) or the release of enveloped viruses from the lysosomes into the cytoplasm (29). In contrast, the xanthates are capable of inhibiting the viral growth cycle at any stage, as shown with SV40, and they block both the replication and the transcription of persistent intranuclear BPV genomes.

Although the pH dependence of the antiviral activity is not yet understood, we think it likely that cellular compartments might be affected. For example, lowering the pH of the medium to 6 or below was shown to influence the interaction between viruses and lysosomes (29). The xanthates did not suffer irreversible alterations rendering them inactive in consequence of a temporary incubation at pH 7.4. After shift-down to the acidic pH, their antiviral potential was found to be unaltered.

By exposing both normal and virus-infected cells to the radiolabeled xanthate compound D609 (custom labeled [^{3}H]tricyclodecan-9-yl-xanthogenate, 6.4 Ci/mmol, radiochemical purity 95%, Amersham, Buckinghamshire, England) and monitoring the localization of the label, we found that the radioactivity was confined essentially to the cytoplasmic fraction (data not shown).

Accordingly, no incorporation into the DNA was detected. After exposure of 10^7 SV40-infected CV-1 cells (2 pfu per cell) 2 days after infection for 6 hr to 0.2 mCi of [^{3}H]D609 no label was incorporated above the background into either the viral or the host DNA.

The data presented in this study suggest an interaction of the xanthates with cellular mechanisms that appear to be more crucial for the viral growth than for the host itself. Therefore, it is not surprising that *we have failed to isolate drug-resistant mutants*. After four passages of HSV-1 in D609 at the IC_{98}, no measurable loss of sensitivity was encountered (unpublished data).

Both the low cytotoxicity and the acute toxicity (LD_{50}, 1.39 g/kg of mouse, oral application) are favorable characteristics for D609 as an antiviral drug. The requirement of an acidic milieu appears to preclude a systemic application in view of the pH of the blood, 7.3–7.4. This apparent handicap may, however, turn out to be an advantage, since tissues displaying a low pH can be interacted with selectively.

To assess the utility of D609 *in vivo*, we have treated hairless mice which were infected with HSV-1 or HSV-2 after scarification. In agreement with the results obtained *in vitro*, preliminary results have shown that only acidic ointments (1% D609) displayed a curative effect. We have noticed the regression of extended herpetic lesions when the therapy was initiated no earlier than 6 days after infection.

In contrast, lesions in untreated animals progressed, eventually covering both the dorsal and ventral area of the body. We did not find harmful side effects such as inflammatory responses during the experiments.

Treatment of the depilated skin of guinea pigs for 2 weeks with the D609 ointment did not disturb the growth of hair. This shows that neither growth nor differentiation processes were interfered with by the xanthate compound.

We thank Otto Westphal for valuable discussion.

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