The Therapeutic Efficacy of a Xanthate Compound on Herpes Simplex Virus in Skin Lesions of Mice and Guinea-pigs

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SUMMARY

Xanthates have recently been shown to inhibit the replication of both DNA and RNA viruses in vitro. The antiviral activity was exerted only under acidic pH conditions. Curative effects in vivo on herpes simplex virus (HSV)-induced skin lesions were only observed when the xanthate compound was administered in the form of an ointment containing acidic buffer (sodium phosphate pH 5.0). Advanced HSV-2-induced skin lesions in mice were healed by topical treatment with the xanthate compound. HSV-1-induced lesions on skin of guinea-pigs were cured within 2 days even when the treatment was initiated as late as 4 days after infection. Both HSV-1 DNA synthesis and virus production in the skin of guinea-pigs were also shown to be inhibited after treatment with the xanthate compound.

INTRODUCTION

Appropriately substituted xanthates have recently been described as a novel class of antiviral compounds. Analysis in vitro of the antiviral activity of tricyclo-decane-9-yl-xanthogenate (test code D609) demonstrated inhibition of both DNA and RNA viruses (Sauer et al., 1984).

It was possible to interrupt the lytic infectious cycle of the DNA tumour virus simian virus 40 (SV40) at any stage by inhibition of viral transcription and DNA replication. Furthermore, both the transcription and the replication of episomal bovine papillomavirus in transformed cells were selectively inhibited by D609. Herpes simplex virus type 1 (HSV-1) DNA replication was also shown to be selectively inhibited by the xanthate compound D609 (Sauer et al., 1984). All the above-mentioned antiviral effects were seen only in acidic pH conditions. They were not observed in alkaline tissue culture media (> pH 7.0). The biochemical mechanism accounting for the unique properties of the xanthate compounds is still unknown, although we have shown that they function neither as precursors of nucleic acids nor as inducers of interferon (Sauer et al., 1984).

In the present study the efficacy of D609 on HSV-induced lesions on the skin of mice and guinea-pigs was compared with the curative effect of acyclovir. It will be shown that D609 was highly effective in curing fully developed herpetic lesions.

METHODS

Viruses. For the animal experiments, the highly neuropathogenic HSV-1 strain Wal (Munk & Donner, 1963) and the HSV-2 strain HG-52 (Timbury, 1971) were used. The viruses were propagated as described elsewhere (Sauer et al., 1984) and stored in aliquots at −70 °C.

Antiviral compounds. Acyclovir was applied as 5% ointment (Zovirax, Burroughs Wellcome). The xanthate compound D609 was prepared by Merz & Co. (Frankfurt, F.R.G.). Ointments were prepared with an eucerin anhydricum base containing 1% D609 and 10% sodium phosphate pH 5.0 for the treatment of mice and 6% D609 and 10% sodium phosphate pH 5.0 for guinea-pigs (unless otherwise stated).

Infection of animals. Hairless female mice (strain hrs/hrs) were obtained from Jackson Laboratories (Bar Harbor, Me., U.S.A.). The skin of the animals was scarified with an injection needle (in an area of approximately 4 mm²) and 50 µl virus suspension (in the case of HSV-1, 8 × 10⁶ p.f.u./ml unless otherwise stated; in the case of HSV-2, 5 × 10⁶ p.f.u./ml) was applied. Female albino guinea-pigs (260 to 300 g, strain Pirbright White) were
obtained from the Süddeutsche Versuchstierfarm (Tuttlingen, F.R.G.). The hair was removed with electric clippers, and a chemical depilatory (Pilca) was applied and left to take effect for 10 min. A 30-needle spring-loaded injection apparatus (Baunscheidt-Lebenswecker, Kirchner und Wilhelm, Stuttgart, F.R.G.) covering a circular area 2 cm in diameter was used to puncture the skin in four releases, and 100 ~ ml virus suspension (2 × 10^7 p.f.u./ml) was administered. All infected animals developed lesions within 4 days.

**Application of antiviral substances.** The infected skin of the animals was treated twice daily with 0.1 g per mouse or 0.2 g per guinea-pig of the various ointments.

**Assessment of lesion size.** The length and diameter of each lesion was determined and the total area covered with lesions was calculated for each animal.

**Titration of virus in guinea-pig skin samples.** The animals were sacrificed and the infected area of the skin was excised and frozen in liquid nitrogen. The tissue was disintegrated in a frozen state in a 'Mikrodismembrator' (Braun, Melsungen, F.R.G.). The frozen tissue powder was suspended in a ninefold excess (w/v) of tissue culture medium. After low-speed centrifugation (Eppendorf centrifuge, 2 min) the virus content in the supernatant was determined by plaque assay on monkey kidney Rita cells.

**Southern blot analysis of guinea-pig skin.** The infected skin area was frozen in liquid nitrogen and the DNA was isolated as described (Krieg et al., 1983). The DNA samples were run after restriction enzyme digestion on agarose gels (3 V/cm, 16 h) and then transferred to nitrocellulose filters. After hybridization to 32P-labelled, cloned HSV-1 DNA fragments coding either for immediate early genes (BamH1 n) or the early thymidine kinase gene (BamH1 p) (Knopf et al., 1983), X-ray films were exposed to the filters.

**RESULTS**

**Treatment of HSV-1 and HSV-2 in hairless mice early after infection**

We used various treatment regimens in hairless mice, starting either 1 or 14 days after infection. As was found in vitro, unbuffered ointments had no curative effects at their alkaline pH (pH 9-1). Only a 1% D609 eucerin ammonium citrate/sodium phosphate-buffered ointment (pH 6-8) had antiviral activity. For comparison, a placebo containing no D609 and the commercially available ointment containing acyclovir (5%) were applied.

Following initiation of the topical treatment either 1 or 2 days after infection, different therapeutic effects were obtained with D609 and acyclovir. The latter was found to be superior, in particular when a high titre of HSV-1 (2 × 10^7 p.f.u./ml) was used for infection: 10 out of 10 animals failed to develop clinical symptoms following treatment with acyclovir, while D609 protected only two of 10 animals. Only at a lower titre (8 × 10^6 p.f.u./ml) did D609 have a therapeutic effect (eight of 10 animals protected). Comparable results were obtained after infection with HSV-2. Both the untreated and the placebo-treated groups developed extensive skin lesions, which persisted over several weeks (in some cases up to 2 months). Again, acyclovir was more efficient in preventing the appearance of skin lesions (9/10) than was D609 (7/10) (data not shown).

**Treatment of extended HSV-2-induced lesions in hairless mice**

In contrast to the results described above, D609 proved to be superior to acyclovir when applied topically at later, advanced stages. Reduction of the size of extensive lesions was accomplished with D609, while acyclovir had no significant effect. This result is consistent with data showing a reduced therapeutic efficiency of acyclovir during late stages of HSV infection (Park et al., 1980; Hill et al., 1982; Landry et al., 1982; Corey et al., 1982; Spruance et al., 1982).

Hairless mice were infected with HSV-2 after scarification, and 2 weeks later animals bearing extensive lesions were selected and randomly distributed to three groups of 10 animals each. The lesions varied in size between 50 and 350 mm^2 per animal. One group was treated twice daily with placebo (mean size of lesions 131 mm^2 ± 60 mm^2), one with 1% D609 (mean size of lesions 101 mm^2 ± 64 mm^2), and one with 5% acyclovir (mean size of lesions 129 mm^2 ± 66 mm^2). The size of each lesion in each animal was determined at the time points indicated in Fig. 1, and compared individually with its size at the beginning of treatment. The growth or regression of the lesions of each animal is expressed as a percentage of the initial size. The mean percentage and standard deviation for each group is shown in Fig. 1. Student's t-test was used to determine the significance of the effect of each treatment. After as little as 4 days of treatment the increase in size of the placebo- and acyclovir-treated lesions was significant (P < 0.001), while the D609-
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Fig. 1. Curative effect of D609 on HSV-2-induced skin lesions. Two weeks after infection of hairless mice with HSV-2, animals with extensive lesions were selected, and treatment (three times a day for the first 5 days, then twice a day) of groups of 10 animals was initiated. The lesion size at the beginning of treatment is taken as 100%. Error bars indicate standard deviation. O, Treated with eucerin anhydricum + 10% sodium phosphate pH 5.0; ●, treated with eucerin anhydricum + 10% sodium phosphate pH 5.0 + 1% D609; ▲, treated with 5% acyclovir (Zovirax).

Treated lesions had significantly regressed in size ($P < 0.01$) (Fig. 2). After 7 days of treatment the placebo- and acyclovir-treated animals were killed because of the severity of the lesions and the appearance of neurological symptoms. Four animals in the placebo group died between 4 and 7 days after the beginning of the treatment.

Treatment of HSV-1-induced skin lesions in guinea-pigs

HSV infection of the skin of guinea-pigs is considered to bear a closer resemblance to the disease in humans than the mouse model system (Hubler et al., 1974; Collins & Oliver, 1982). Therefore, we studied the influence of D609 ointments both early and late after infection of the scarified, depilated skin of guinea-pigs with HSV-1.

Acyclovir was again found to be superior to D609 in the early stages of infection: when the treatment was started 18 h after infection acyclovir prevented the appearance of lesions in 10 of 10 animals, while D609 (6%) prevented the appearance of lesions in four of six animals. The number of lesions was smaller, however, than in the placebo group and they appeared to be less severe. In accordance with this observation we found an inhibition of HSV-1 production in the D609-treated skin by 82% 1 day after infection (Table 2). That the replication of HSV-1 DNA was indeed inhibited by D609 is supported by the data shown in Fig. 4. Part of the skin samples that were used for the titration (Table 2) was used for the extraction of DNA. The relative amount of viral DNA was determined in the Southern blot after hybridization with a HSV-1-specific probe. The difference between the relative amount of HSV-1 DNA in the placebo-treated and in the D609-treated skin samples is evident, with some of the latter displaying only faint signals, if any (Fig. 4).
Fig. 2. Curative effect of D609 on HSV-2-induced skin lesions in representative animals from the experiment described in Fig. 1. From each group a typical animal is shown at the beginning, after 4, and after 7 days of treatment.

Table 1. Effects of D609 on HSV-1-induced lesions in guinea-pigs*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of animals treated</th>
<th>Total number of lesions</th>
<th>% Persisting lesions (mean value)†</th>
<th>Significance of curative effect‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Beginning of treatment</td>
<td>After 2 days of treatment</td>
<td></td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>5</td>
<td>108</td>
<td>119</td>
<td>112 ± 16</td>
</tr>
<tr>
<td>6% D609</td>
<td>5</td>
<td>110</td>
<td>31</td>
<td>32 ± 13</td>
</tr>
<tr>
<td>Zovirax</td>
<td>5</td>
<td>81</td>
<td>87</td>
<td>111 ± 10</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>6</td>
<td>82</td>
<td>83</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>6% D609</td>
<td>6</td>
<td>152</td>
<td>23</td>
<td>15 ± 9</td>
</tr>
</tbody>
</table>

* Four days after infection with HSV-1, guinea-pigs were treated twice a day with various ointments. The number of lesions was determined for each animal at the beginning of treatment and 48 h later.
† Change in number of lesions was expressed as a percentage for each animal and the mean value (± standard deviation) for each group was calculated.
‡ The significance of the change in the number of lesions for each group after 2 days of treatment was determined by Student’s t-test. NE, No significant effect detectable.

A dramatic curative effect of D609 was observed when the treatment was initiated 96 h after infection, by which time the herpetic lesions were fully developed. Within 2 days (with two applications of the ointment per day) most lesions were cured in all D609-treated animals (Table 1). The remaining lesions, which had almost regressed by this time (see Fig. 3) disappeared
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Fig. 3. Curative effect of D609 on HSV-1-induced skin lesions in guinea-pigs. Typical animals (described in Table 1) at the beginning of experiment 1 and after 2 days of treatment are shown.

Table 2. Inhibition of virus production in guinea-pig skin*

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment</th>
<th>Titre (p.f.u./g tissue × 10^-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Placebo</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>Placebo</td>
<td>11.5</td>
</tr>
<tr>
<td>3</td>
<td>Placebo</td>
<td>2.3</td>
</tr>
<tr>
<td>4</td>
<td>Placebo</td>
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<tr>
<td>5</td>
<td>Placebo</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>Placebo</td>
<td>1.8</td>
</tr>
<tr>
<td>1</td>
<td>6% D609</td>
<td>0.45</td>
</tr>
<tr>
<td>2</td>
<td>6% D609</td>
<td>0.55</td>
</tr>
<tr>
<td>3</td>
<td>6% D609</td>
<td>0.85</td>
</tr>
<tr>
<td>4</td>
<td>6% D609</td>
<td>0.55</td>
</tr>
<tr>
<td>5</td>
<td>6% D609</td>
<td>1.7</td>
</tr>
<tr>
<td>6</td>
<td>6% D609</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Twelve guinea-pigs were infected with HSV-1 at a single site each. The animals were treated three times with a phosphate-buffered, D609-containing ointment (6%) or with a phosphate buffer placebo (4, 8 and 18 h after infection). Six h after the last application the animals were sacrificed and the infected skin was removed. From each tissue sample virus was extracted and the virus concentration was titrated (in duplicate) on Rta cells.

within the next 16 h, although treatment was terminated after a total of four applications. No new lesions appeared during an observation period of several weeks.

The placebo- and Zovirax-treated animals displayed no curative effects (Table 1); the number of lesions and their severity remained unaffected (see Fig. 3). Spontaneous healing of these lesions was noted between 10 and 12 days after infection. It should be added that in all groups one or two cases of temporary paralysis of the hind legs occurred independently of the curative effects on the skin lesions.
Placebo D609

Fig. 4. Inhibition of viral DNA replication in guinea-pig skin. Twelve guinea-pigs were infected with HSV-1. After three applications (4, 8 and 18 h after infection) of phosphate-buffered 6% D609 or phosphate placebo the animals were sacrificed (24 h after infection) and the infected skin was removed. Total DNA was extracted separately from each tissue sample. Ten μg of each DNA preparation was cleaved with *Bam*HI and analysed by electrophoresis on a 1% agarose gel (3 V/cm, 16 h). The DNA was transferred to a nitrocellulose filter which was then hybridized to 32P-labelled HSV-1 DNA (*Bam*HI fragment n, cloned in pBR322). An X-ray film was exposed to the filter for 10 days. Each lane represents a single animal.

**Effect of D609 and acyclovir on transcription in vitro**

Rita cells infected with HSV-1 were treated with D609 or acyclovir, RNA was extracted and hybridized after electrophoresis and transfer to nitrocellulose with probes prepared from specific fragments of HSV-1 DNA. The results are shown in Fig. 5.

**DISCUSSION**

Xanthates, which have not yet been introduced into pharmaceutical research, are capable of inhibiting both DNA and RNA virus species. None of the stages that are affected by classic antiviral compounds, such as virus adsorption, penetration or uncoating, is the target of the xanthates. Furthermore, the xanthates do not function as precursors in nucleic acid synthesis (Sauer *et al.*, 1984).

Although the biochemical mechanism of their antiviral activity is not yet understood, we
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Fig. 5. Inhibition of HSV-1 transcription. Rita cells were infected with HSV-1 (0.1 p.f.u./cell, strain Wal). After 1 h adsorption, tissue culture medium (equilibrated to pH 6-8) was added. One culture was treated with 5 μg/ml acyclovir or 30 μg/ml D609 and one culture remained untreated. After 12 h incubation total RNA was extracted from the cells (Amtmann & Sauer, 1982) and 10 μg of each preparation was electrophoresed on a denaturing (10 mM-CH₃HgOH) 1.4% agarose gel (3 V/cm, 16 h). After transfer to nitrocellulose the RNA was hybridized to ³²P-labelled, cloned HSV-1 DNA fragments (a to c, BamHI n or d to f, BamHI p). The filters were exposed to X-ray film for 4 days. (a, d) Untreated; (b, e) treated with 5 μg/ml acyclovir; (c, f) treated with 30 μg/ml D609.

know that both viral DNA replication and transcription are blocked by these compounds. For example, HSV DNA replication was shown to be selectively inhibited. In addition, the DNA and RNA synthesis of the DNA tumour virus SV40 could be blocked at any stage of the lytic growth cycle, and a similar specific antiviral effect was noted in the case of papillomavirus-transformed tissue culture cells, in which the virus genomes persist as non-infectious episomes (Sauer et al., 1984). Experiments in vitro revealed that in HSV-1-infected cells the virus-specific mRNA synthesis was inhibited by D609 treatment. We tested both immediate early (BamHI n) and early viral genes (BamHI p) the expression of which is known to be unaffected by inhibitors of viral DNA synthesis (Wagner, 1984). The transcriptional activity of the viral thymidine
kinase gene was completely suppressed. In the case of an immediate early gene a strong although incomplete inhibition was observed. Acyclovir, in contrast, had no effect on the expression of either gene at concentrations that prevented virus production completely.

The ability of the xanthates to suppress not only viral DNA replication but also the expression of viral genes appears to be of particular importance for their therapeutic efficacy in vivo. Thus, it is conceivable that the continued synthesis of at least some of the viral mRNA species and proteins despite arrested viral DNA replication in the presence of acyclovir may be responsible for the persistence of the HSV lesions. At later stages after infection (4 days) virus replication, which is the target affected by acyclovir, has almost ceased.

The failure of D609 to inhibit virus spread along the nerves at the early stages of infection may be explained by its pH dependence, which confines the therapeutic effect of the compound to the body surface, which is immediately accessible. (It should be added that we have also been unable to prevent or cure latent HSV infections in mouse ganglia; unpublished data.)

The healing of HSV-induced skin lesions at advanced stages of the infection, in particular in guinea-pigs, is considered to be probably the most accurate model available for experimental simulation of the human disease (Hubler et al., 1974; Collins & Oliver, 1982). This observation together with the low toxicity of D609 (an oral LD50 of 1300 mg/kg in rats; an intraperitoneal LD50 of 175 mg/kg; 4 weeks of daily administration of 2 to 20 mg/kg intraperitoneally with no subchronic effect) renders the xanthate compound D609 a promising candidate for application in humans.

We thank Dieter Baumgartl for expert technical assistance and K. Knopf for providing the HSV-1 plasmids.

Note added in proof. After this work was completed, we have found that the limitation of the acidic pH dependence of the antiviral activity of the xanthate D609 can be circumvented. The simultaneous application of certain bipolar compounds renders D609 antivirally active under physiological pH conditions (manuscript in preparation).

REFERENCES


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