Antiviral activity of CTC-8 against herpes simplex virus (HSV-1) in cell culture: evidence for a selective antiviral effect via a cellular mechanism

Tim Fitzmaurice¹, David R Harper² and Hugh J Field³*

¹Centre for Veterinary Science, University of Cambridge, Cambridge, UK
²Charterhouse Therapeutics Ltd, Oxford, UK
*Corresponding author: Tel: +44 (0)1223 330810; Fax: +44 (0)1223 332998; E-mail: hjf10@cam.ac.uk

A synthetic programme produced a series of compounds related to natural prostaglandins, which are known to affect the growth of a number of viruses. Several of the compounds showed potent biological activity including antiviral effects. The compound CTC-8 [(S)-4-tert-butyldimethylsilyloxy-2-cyclopenten-1-one] contains the cyclopentenone ring of prostaglandin A1, but the extended side chains common to the prostaglandin family are truncated. The present study demonstrates that CTC-8 inhibits HSV-1 replication in cell culture at sub-toxic concentrations. The antiviral effect was evidenced by reduction in infectious virus yield, although the compound was not effective in the standard plaque-reduction assay. Time-of-addition studies and other experiments provide a possible explanation for these results by suggesting that the antiviral activity is confined to a single cycle. Under the standard conditions of high-multiplicity infection in BHK cells it was notable that CTC-8 is most effective when added for a short period 6–8 h post-infection. Furthermore, multiple passage of HSV-1 in the presence of CTC-8 did not result in the selection of resistant mutants. The results of these and other experiments are consistent with the hypothesis that the mechanism by which CTC-8 inhibits virus replication involves a cellular target. These results encourage further research into the therapeutic potential of this series of compounds.

Keywords: HSV, prostaglandin, antiviral chemotherapy, cyclopentenone, CTC-8

Introduction

It is now some 40 years since the introduction of the first specific inhibitors of HSV; and the drug acyclovir has been used clinically for almost 25 years. Notwithstanding, no therapy to date has been completely satisfactory and recurrent herpes labialis and herpes genitalis remain important causes of human suffering (Fleming et al., 1997; Vyse et al., 2000). Furthermore, there is still a need for alternative and improved therapeutic approaches.

There have been several reports over the last few years of compounds which are active against HSV via interference with an important cellular component of the virus replication cycle, e.g. inhibition of cyclin-dependent kinases that are known to have a role in HSV replication (Schang et al., 2001). Thus, it has become increasingly evident that many products of the HSV genome interact with cellular proteins and are involved in regulating cellular functions to optimize the efficiency of virus replication in differentiated cells (reviewed by Schang, et al., 2001). While the inhibitors of such non-virus-specific functions may not be potent antiviral therapies when administered alone, they may have a useful role in combination therapy. The use of steroids in combination with specific antiviral nucleosides has long been a therapeutic approach to the management of stromal herpes simplex keratitis (Williams et al., 1977). Recently this approach has been extended to the treatment of herpes labialis and the topical formulation ME-609, which comprises a combination of 5% acyclovir and 1% hydrocortisone, has been tested in a double blind randomized placebo-controlled trial in patients with UV-induced lip lesions (Evans et al., 2002). The treatment was effective and, compared to treatment with a placebo, the combination antiviral-immunomodulatory cream provided significant benefit to patients with experimentally induced herpes labialis, reducing classical lesion incidence, healing time, lesion size and lesion tenderness.

An important disadvantage in the use of steroids to treat HSV lesions is their potential to up-regulate or prolong virus shedding (Kaufman, 1981); thus, the development of non-steroidal anti-inflammatory agents, particularly those having antiviral properties, is an important and novel approach to HSV therapy; this is the rationale that led to the work described here. The present study concerns a member of a series of compounds derived from natural prostaglandins. The development of these compounds is, in
part, aimed at their anti-inflammatory potential. However, the prostaglandin PGF2\(\alpha\) was shown to inhibit HSV-1 (Newton, 1982) and, more recently, it was reported that prostaglandin A1 has antiviral activity against several viruses, including human influenza A and HSV-1 (Santoro, 1997). The current paper presents evidence that CTC-8 is an inhibitor of HSV-1. This compound is one of a series of prostaglandin analogues that had been selected for biological evaluation based on a binding assay for NF\(\kappa\)B. This offers the prospect of combining anti-inflammatory properties with antiviral activity in the same therapeutic compound.

**Materials and methods**

**Antiviral compounds**

CTC-8 (Figure 1) was synthesized in the laboratories of Professor S Roberts, Department of Chemistry, University of Liverpool by a stereospecific silyl group transfer reaction process (Danishevsky, Cabal & Chow, 1989). Purity was determined by NMR spectroscopy. CTC-8 was stored at \(-20^\circ\)C as a lyophilized powder in small, weighed aliquots of typically 0.5–3 mg. Working stock solutions (100 \(\mu\)M) were stored in small aliquots at \(-20^\circ\)C. Acyclovir was a gift from (the former) Smithkline Beecham.

**Virus**

The HSV-1 used in this study was strain SC-16 (Hill et al., 1975). Virus working stocks were grown in BHK-21 cells infected at low multiplicity of infection (m.o.i.). This strain has been widely used for assessing anti-herpesvirus compounds both in vitro and in vivo (Field et al., 1979; Field et al., 1995). The acyclovir-resistant strain, Cl(101)TK- (Field et al., 1981) was grown under the same conditions.

**Figure 1. Structure of CTC-8 in relation to parental prostaglandin A1**

Cell culture

The baby hamster kidney cell line (BHK-21 cells) and the human cell line (HEp-2) were cultured in Eagles’ minimal essential medium (EMEM) supplemented with antibiotics and 10% foetal calf serum. The green monkey kidney cell line (VERO) was cultured in Dulbecco’s modified Eagles’ medium (DMEM) supplemented with antibiotics and 10% foetal calf serum. The concentration of serum was reduced to 1% for virus infection or the addition of the antiviral compound.

**Plaque reduction assay**

HSV-1 was adsorbed to confluent BHK-21 cells contained in 24-well plates for 1 h, so as to produce approximately 50 plaques per well. Virus infection medium (EMEM) containing 0.3% carboxymethylcellulose was then added. At 48 h p.i., monolayers were fixed and stained by means of crystal violet in 20% ethanol and plaques enumerated microscopically.

**Virus yield assay**

Confluent monolayers of BHK-21 cells in 24-well plastic tissue culture plates were infected with HSV-1 at a multiplicity of infection (m.o.i.) 10 p.f.u./cell. After 1 h adsorption, surplus inoculum was removed from the wells and media appropriate to the cell type containing various concentrations of test compounds was added to the plates to duplicate wells. After 24 h incubation, supernatant was removed from wells and titrated individually by a plaque assay in Vero cells. Studies on effects of multiplicity of infection were conducted by varying that parameter.

**Growth curve**

A number of 6-well plates were seeded with BHK cells and allowed to grow to confluence. They were then infected with HSV-1 using m.o.i.=10 p.f.u./cell. After 1 h adsorption, the inoculum was removed and medium containing either 0.1 \(\mu\)M or 10 \(\mu\)M of CTC-8 was added to all wells. At various times (1, 2, 6, 12, 18, 24, 36, 48 and 72 h p.i.) supernatant and cells were harvested from separate wells. These samples were then frozen to \(-70^\circ\)C for later titration by plaque assay in Vero cells.

**Cell toxicity assay (proliferating cells)**

BHK-21 cells were seeded at low density (5×10^5 \(\text{cells/well}\)) in 24-well plates and incubated for 24 h. The cells were counted in representative wells and the medium was then removed and replaced with fresh EMEM containing CTC-8 or ACV at concentrations of 0.1, 1, 10 or 100 \(\mu\)M in duplicate. Medium without compound was added to control wells. The cells were assessed visually for obvious cytopathic effects after 48 h incubation, then were harvested and cells counted in a haemocytometer using Trypan Blue exclusion as a marker for viability.
Cell toxicity assay (non-proliferating cells)

BHK-21 cells were seeded at similar density of 5×10^4 cells/well in 24-well plates as above but were incubated until they had become confluent (>48 h). The number of cells/well was counted and growth medium was replaced with medium containing compounds as above. After a further 48 h incubation in the presence of the compounds the number of viable cells in each well was determined using Trypan Blue exclusion.

Time-of-addition

These studies were based on the standard infectious virus assay. Thus, cells were infected at m.o.i.=10 p.f.u./cell and infectious virus yield was measured at 24 h p.i. CTC-8 (10 µM) was added at various times before, during or after infection. If the compound was added at or before infection, fresh CTC-8 was also added during and after virus adsorption. In some cases, after a period of treatment, the CTC-8-containing medium was removed and replaced with drug-free medium. Control cells were treated in an identical way with compound free medium.

Antiviral resistance

The selection of HSV mutants with acquired resistance to nucleoside analogues is a well-described phenomenon for HSV in cell culture (Darby & Field, 1984) and is a recognized problem in immunocompromised patients (Coen, 1996). In an attempt to select resistant strains, virus was serially passaged in the presence of 10 µM CTC-8 for 22 passages. Virus was also passaged a similar number of times without inhibitor. Stocks were obtained from the final passage with or without inhibitor, stored at –70°C and titrated. A virus yield inhibition assay was performed, as described above, to determine relative sensitivities to CTC-8.

The sensitivity of an ACV-resistant thymidine kinase defective mutant was tested by means of virus yield assay. Activity of ACV and CTC-8 against HSV-1 (strain SC16) and the ACV resistant strain Cl(101)TK– was assessed under standard conditions for the virus yield assay.

Results

Virus yield assay

When increasing concentrations of CTC-8 were added to BHK cells after virus adsorption, a dose-dependent reduction of virus yield was measured at 24 h p.i. (Table 1). The dose of compound that reduces the yield of infectious virus to 10% of that in the absence of compound (ED_{50}) was found to be approximately 4 µM. The response to a different strain of HSV-1 [Cl(101)TK–] was similar. In HEp-2 cells, HSV-1 was less sensitive to CTC-8 with an ED_{50} of approximately 10 µM (data not shown).

Table 1. Effect of compound concentration on yield of infectious virus at 24 h p.i., showing evidence for lack of cross-resistance of an HSV-1 TK-deletion mutant to CTC-8

<table>
<thead>
<tr>
<th>Comp. conc. (µM)</th>
<th>Virus yield (mean log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C101(TK)</td>
</tr>
<tr>
<td>0</td>
<td>7.0</td>
</tr>
<tr>
<td>10</td>
<td>6.7</td>
</tr>
<tr>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td>0.1</td>
<td>6.8</td>
</tr>
<tr>
<td>0</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Plaque reduction assays

When CTC-8 was tested by means of the plaque-reduction assay, no evidence of antiviral activity was obtained; there was no measurable reduction in the number or size of plaques. This was true even at higher drug concentrations that produced morphological evidence of toxicity. When the nucleoside analogue ACV was tested under identical conditions, the ED_{50} in BHK cells was approximately 0.1 µM. Tests were carried out using BHK cells in which fresh CTC-8 was added at regular intervals to the medium overlay during the 48 h incubation period required for plaques to develop. No reduction in the number or size of plaques was observed; this ruled out the possibility of rapid breakdown of CTC-8 during the assay but the results remained negative.

Cytotoxicity cell monolayers incubated in the presence of CTC-8 showed obvious morphological changes at high drug concentrations (>100 µM). Quantitative data were obtained by means of a dye-exclusion method. Cells were seeded at low density and the effects of CTC-8 on proliferating cells were tested. Confluent monolayers of non-proliferating cells were also used. The data from proliferating cells show evidence of reduced growth rate using 50% cell loss as a cut-off point (Figure 2). It may be seen from the figure that CTC-8 had less effect on confluent cells. The ID_{50} and ID_{90} values were calculated to be approximately 4 and 10 µM in proliferating cells and 10 and 100 µM in non-proliferating cells, respectively, giving a selectivity index of the order of 10, depending on conditions. Similar results were obtained in HEp-2 cells (data not shown).

Multiplicity of infection

The effect of m.o.i. on inhibition of virus yield was tested over the range 0.1 to 100 p.f.u./cell. While there is a trend to improved virus inhibition by CTC-8 as the m.o.i. decreases, the magnitude of the effect is small (Figure 3), suggesting that the compound is effective over a wide range of multiplicity.
Virus growth curve
In order to examine the kinetics of virus inhibition during the course of virus infection under one-step conditions, cells were infected at m.o.i.=10 and incubation carried out in the presence of CTC-8. Cells and supernatant were analysed separately for infectious virus yield. The results (Figures 4 a and b) indicate that CTC-8 at 10 µM reduced virus yield at all time points up to 24 h p.i. and the response was greatest in the cell-free fraction with a reduction in yield of up to 4 log10. However, virus production did occur over the next 48 h and, at 72 h p.i., the levels of virus were recovered similar to untreated wells. The reduction in yield was less marked at 1 µM CTC-8 with significant reduction only visible at 24 h p.i. (data not shown).

Time of addition
In order to further elucidate the mechanism of action of CTC-8, the effects of varying the time of addition of CTC-8 to infected BHK cells was examined using the conditions for the normal virus yield assay. Thus, cells were infected at m.o.i.=10 and infectious virus yield was measured at 24 h p.i. When compound was added before infection, it was maintained at the same concentration during virus adsorption and after adsorption by replenishment. In some cases, after a period of treatment, the compound-containing medium was removed and replaced with compound-free medium. The results (Figure 5) were unexpected. Addition of CTC-8 at, or shortly after, infection (up to 12 h p.i.) was effective. When compound was added at >12 h p.i the inhibitory effect was attenuated (data not shown). An important finding was that delaying addition of compound to 6 h p.i. produced a marked reduction in virus yield.

Absence of resistance to CTC-8
After 22 serial passages of virus in BHK cells in the presence of an inhibitory concentration (10 µM) of CTC-8, no difference in the sensitivity was observed in the yield reduction assay. The ED90 values were found to be identical to those of the original working stock of virus. Similar methods readily yield virus with acquired resistance to ACV. It was noted (Table 1) that a thymidine-kinase defective mutant of HSV-1 which has acquired resistance to ACV remained sensitive to CTC-8.

Discussion
Our most important finding is that CTC-8 is an inhibitor of HSV-1 and evidence was obtained showing that the inhibition is selective. The observation of antiviral activity, reported previously with the parent compound PG-A1, was thus demonstrated using this synthetic derivative. Although CTC-8 proved to be inactive in a classical plaque-reduction assay, this is readily explained. It was found that early addition of compound abrogated the
Effects of CTC-8 on HSV in cell culture

antiviral effect and that optimum inhibition of virus yield occurred for a short period several hours after virus infection, conditions that do not prevail in the low multiplicity, asynchronous infection of the plaque reduction assay.

While we observe reproducible antiviral activity in virus yield assays, the lack of effect in plaque assays must be addressed as its use is widespread for preliminary characterization of novel compounds. The fact that CTC-8 was
effective at low m.o.i. suggests that this is not the reason for lack of detectable activity in the plaque reduction test, which is by definition carried out under low multiplicity conditions for the first round of virus replication. Our contention is that the plaque reduction assay is in general unsuitable for use with CTC-8 and similar compounds, due to the likely cellular target for of their mechanism of action. Data published elsewhere provide evidence that compounds in this series, due to their relationship to prosta glandin A1, may be modulators of NFκB release (Santoro and Roberts, 1999). This transcription factor pathway is a candidate for mediating antiviral activity in tissue culture; producing a dose-dependent reduction the rate of replication of virus. Furthermore, our time-of-addition studies indicate that cells that are exposed to compound several hours prior to infection become ‘tolerant’ and the effect of virus inhibition is lost. The virus yield assay, performed under one-step conditions, involves a single round of synchronous replication and appears to be the best method of demonstrating the compound’s antiviral activity. It remains to be seen how these specific antiviral effects involving critical events in the virus replication cycle with time will translate into the milieu of asynchronously virus-infected cells that exist in vivo.

When considering the potential cellular toxicity of CTC-8 (as determined by cell viability), a marked difference was observed between the effects on proliferating and non-proliferating cells; this may be important. Prosta glandins have been shown to have effects on the proliferation and differentiation of cells and this prosta glandin-derived compound, CTC-8, may display similar activity. It was notable that rapidly dividing cells were most sensitive to the compound. Virus yield assays were performed using confluent monolayers comprising non-proliferating cells; this is arguably be a closer model for the cells at the mucocutaneous junction that are typically infected in vivo during primary herpes and recurrences.

Resistance to antiviral compounds is a route to defining antiviral targets. The detection of acquired drug-resistance provides unequivocal evidence for specific antiviral mechanism of action. Such evidence was not forthcoming for CTC-8. An ACV-resistant TK-defective strain of HSV-1 was equally sensitive to the compound and we found that prolonged passage of HSV-1 in the presence of CTC-8 produced no evidence for selection of resistant mutants. The conditions used were those that would readily allow the section of mutations giving resistance to nucleoside analogues. Lack of resistance development to CTC-8 is not surprising given the proposed mechanism of action. Similar results have been reported for other antiviral compounds which involve cellular target, such as the cyclin-dependent kinase (CdK) inhibitors (Schang, 2002). Further evidence of virus specificity comes from considering the variety of different mammalian herpes viruses, several of which were shown to be sensitive to CTC-8. However, it was also shown that two laboratory strains of HSV-2 were insensitive to CTC-8, although they were sensitive to other compounds in this series (data not shown). These results will be the subject of a future publication.

While CTC-8 is a representative of a class of compounds that displays fairly modest specific antiviral activity against HSV-1, and the proposed mechanism of action implies that cells may vary in susceptibility according to the stage in the virus replication cycle, the development of this series of compounds may still lead to valuable addition to the antiviral armoury. Further work will be carried out to explore their potential, both alone and in combination with nucleoside antivirals, using in vivo infection models.

Acknowledgements

This work was supported by a grant from Charterhouse Therapeutics Ltd. Compounds were supplied by Professor S Roberts of the University of Liverpool. CTC-8 is the subject of patents lodged with the European Patent Office and the US Patent Office.

References


