Inhibition of varicella-zoster virus replication by an inhibitor of protein myristoylation

D. R. Harper,¹* R. L. Gilbert,¹ C. Blunt² and R. A. J. McIlhinney²

¹Medical College of St Bartholomew's Hospital, London and ²Medical Research Council Anatomical Neuropharmacology Unit, Oxford, U.K.

Inhibitors of myristoylation and analogues of myristic acid inhibit the replication of some retroviruses including human immunodeficiency virus, but no studies with other virus families have been reported. We have shown that replication of varicella-zoster virus (VZV) in tissue is inhibited by DL-2-hydroxymyristic acid at concentrations similar to those required for inhibition with acyclovir. Protein synthesis is not inhibited, but protein myristoylation is non-specifically reduced. Despite this lack of specificity, DL-2-hydroxymyristic acid inhibits VZV replication without apparent cytotoxicity. This is in agreement with our earlier suggestion that non-specific inhibitors of myristoylation could have antiviral effects without toxicity to cells due to the stability of cellular myristoylproteins. This supports suggestions that myristoylation inhibitors have potential as antiviral drugs against the many viruses that produce myristoylproteins.

Proteins modified by myristic acid are important in both cellular and viral metabolism (Harper & Gilbert, 1992; Schmidt, 1989). The attachment of myristic acid to proteins is catalysed by the enzyme myristoyl-CoA:protein N-myristoyltransferase (NMT) (Harper & Gilbert, 1992; McIlhinney, 1990; Schmidt, 1989; Towler et al., 1988), which attaches myristic acid to an amino-terminal glycine with high substrate specificity. Inhibitors of myristoylation and analogues of myristic acid inhibit replication of some retroviruses, including human immunodeficiency virus (HIV) (Bryant et al., 1989, 1991; Devadas et al., 1992; Saermark et al., 1991; Tashiro et al., 1990) but these studies have not been extended to any other virus family. We have studied the effect of DL-2-hydroxymyristic acid (2-HM) on varicella-zoster virus (human herpesvirus 3, VZV) using a plaque reduction assay together with studies of viral anabolism. VZV produces several myristoylproteins, including the major viral glycoprotein gp1 and the prominent small myristoylprotein Lp7 (lipoprotein 7; Mr 7000) (Harper & Kangro, 1990), suggesting that it might be sensitive to NMT inhibitors. 2-HM was selected for study since it is a competitive inhibitor of protein myristoylation in cultured cells after conversion to the 2-HM-CoA form (Paige et al., 1990).

VZV strain H-551 was grown at 37 °C in MRC5 fibroblasts overlaid with Eagle's MEM containing 5% fetal calf serum. Cells were harvested when cytopathic effect reached 50%. Cells were scraped into the overlay medium and sonicated for 2 min. three times. Cellular debris was then centrifuged at 1000 g for 15 min, and the supernatant (containing 200 to 500 p.f.u./ml of cell-free VZV) then used to infect sub-confluent Mewo cells (Grose & Brunell, 1978) or confluent MRC5 cells in 24-well plates. After 2 h the cells were rinsed and overlaid with MEM containing 2% fetal calf serum, 0.15% agarose, and 2-HM or acyclovir present at 0.8, 8 or 80 µM from 0 to 8 days post-infection. Control cultures containing no inhibitor and/or no virus were also included. Plaques were visualized by staining with crystal violet. All incubations were at 32 °C (Mewo cells) or at 37 °C (MRC5 cells). At concentrations greater than 80 to 100 µM, 2-HM precipitates from aqueous solution, preventing the use of higher concentrations in this assay. VZV is easily grown in MRC5 fibroblasts but forms poorly defined plaques, whereas clear plaques are formed in Mewo melanoma cells (Grose & Brunell, 1978). Inhibition of plaque formation was apparent with both cell lines at all levels tested (Fig. 1). The concentration of 2-HM or acyclovir that produced a 50% reduction in plaque numbers (IC₅₀ value) was determined (Table 1). The IC₅₀ obtained for acyclovir against VZV in these assays was in agreement with results reported elsewhere using MRC5 cells (Boyd et al., 1987) and was similar to that observed for 2-HM. The appearance and stain uptake of cells was unaltered even with 80 µM-2-HM. These results show that inhibition of VZV plaque formation by 2-HM was comparable to that of acyclovir.

The effect of 2-HM on cellular protein synthesis was determined as a further measure of toxicity. Uninfected MRC5 monolayers were incubated for 8 days in the presence of 0.8, 8 or 80 µM-2-HM, and L-[³⁵S]methionine...
Short communication

Fig. 1. Plaque reduction assay of VZV; inhibition of plaque formation by (● and □) 2-HM, or (○ and △) acyclovir, in (——) Mewo cells or in (—-) MRC5 cells. The number of plaques (y-axis) is expressed as a percentage of the number on control monolayers with no inhibitor present.

Table 1. IC_{50} values and ratio for 2-HM and acyclovir inhibition of VZV in Mewo cell plaque reduction assay

<table>
<thead>
<tr>
<th></th>
<th>2-HM (µM)</th>
<th>Acyclovir (µM)</th>
<th>Ratio (2-HM:ACV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mewo cells</td>
<td>33.1</td>
<td>23.6</td>
<td>1.40</td>
</tr>
<tr>
<td>MRC5 cells</td>
<td>22.9</td>
<td>17.6</td>
<td>1.29</td>
</tr>
</tbody>
</table>

was added to the overlay medium 2 days before harvesting into reducing PAGE sample buffer (Laemmli, 1970). Proteins were separated on 9% polyacrylamide gels, which were then dried and exposed to X-ray film. Densitometry of the autoradiographs was carried out using a Joyce-Loebl Chromoscan 3 integrating densitometer. There was no alteration in the pattern of proteins synthesized (Fig. 2) and no significant reduction in total protein synthesis at any concentration tested. With 80 µM-2-HM (3.4 x IC_{50}) present for 8 days, methionine incorporation was 97% of that observed with no inhibitor present.

To provide information on the nature of the inhibition observed, the effect of 2-HM on VZV antigen production was assayed using immunoblotting. Cell-free VZV (used in plaque reduction assays) was not used in this work, since the highly cell-associated nature of the virus results in a relatively low titre of VZV in such preparations. In order to allow the use of high virus inocula, confluent monolayers of MRC5 cells were infected with trypsinized VZV-infected MRC5 cells at an infected:uninfected ratio of 1:4 (high inoculum) or 1:250 (low inoculum). Following infection, monolayers were incubated at 37 °C for the times given with the concentrations of 2-HM indicated, and then harvested into reducing PAGE sample buffer when 70 to 80% cytopathic effect was apparent in VZV-infected cultures with no added 2-HM. Samples were separated on 9% polyacrylamide gels, transferred to nitrocellulose by semi-dry blotting, and reacted with high-titre convalescent sera taken from patients with recent secondary VZV infections (Harper et al., 1990). These were then probed with horseradish peroxidase-conjugated anti-human IgG, using enhanced chemiluminescence (Matthews et al., 1985). Total integrals of the X-ray film image were determined by densitometry.

No reduction in antigen synthesis was apparent in the high-inoculum cultures even with 2-HM present at 80 µM (3.4 x IC_{50}). However, when the initial inoculum was reduced to 1:250, allowing detection of antigen produced by the spread of virus within the monolayer, inhibition was clearly apparent (Fig. 3). This suggests that 2-HM may act by inhibiting the spread of VZV from cell to cell. This could be due to an effect of 2-HM on virus assembly since viral myristoylproteins have been shown to be important in the assembly of poliovirus (Marc et al., 1989), polyoma virus (Streuli & Griffin, 1987) and HIV (Bryant & Ratner, 1990).

To measure the effect of 2-HM on protein myristoylation, both high (1:4) and low (1:250) inocula of VZV-infected MRC5 cells were used. Since the effect of 2-HM on viral antigen synthesis is minimal at high infection levels, the high inoculum culture should show any selective effect of 2-HM on viral myristoylprotein synthesis against the background of the general in-
Inhibition of myristoylprotein synthesis. In contrast, the low inoculum cultures allow the effect of inhibition of viral antigen synthesis at low viral inocula (see Fig. 3) to be reflected in viral myristoylprotein synthesis. MRC5 monolayers were infected and incubated as described above. [9,10(n)-3H]Myristic acid was added to the overlay medium 2 days before harvesting the cells into reducing PAGE sample buffer. Proteins were separated on 10.5% polyacrylamide gels containing 50% sucrose (Harper & Kangro, 1990). The gels were then impregnated with 2,5-diphenyloxazole in dimethyl sulphoxide (Bonner & Laskey, 1974), dried, and exposed to X-ray film. Densitometry of the fluorographs was carried out as described above with, in addition, quantification of the Lp7 band.

In contrast to the results obtained with 2-HM using [35S]methionine, the total incorporation of [3H]myristic acid decreased with increasing levels of 2-HM (Fig. 4). At high virus inoculum, the effect of 2-HM on viral Lp7 production was consistent with the overall decrease in general protein myristoylation, indicating that the in-
hibition of myristoylation was non-specific. However, with a lower virus inoculum, Lp7 production was selectively inhibited, in accordance with our results showing a more pronounced effect of 2-HM on viral antigen synthesis at lower viral inocula.

The results presented here show that, as we had previously suggested (McIlhinney, 1990), a general inhibition of protein myristoylation can inhibit the replication of a virus without apparent toxicity to cells. The limited solubility of 2-HM in aqueous solution prevented the determination of a 2-HM concentration causing a 90% inhibition of plaque formation. However, there was no apparent effect with 2-HM at 80 μM (3.4 × IC50) on cell viability as assayed by protein synthesis, cellular appearance or stain uptake, even after incubation of cells with 2-HM for 8 days. This lack of apparent toxicity, together with an observed IC50 comparable to that of acyclovir, suggests that myristoylation inhibitors may be worthy of further investigation as antiviral agents, although studies of whole organism toxicity will be essential. Such inhibitors will also be useful in lipoprotein and viral research.

In conclusion, a compound which is a general inhibitor of cellular protein myristoylation has been shown to inhibit replication of a new class of viruses without apparent toxicity in the cell culture systems used.

The authors wish to thank Dr Derek Kinchington for facilitating this research. This work was supported by grants from the Wellcome Trust (R.L.G.) and the MRC AIDS-directed Programme (C.B.).

References


