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ANTIVIRAL ACTIVITY AND METABOLISM OF THE CASTANOSPERMINE DERIVATIVE MDL 28,574, IN CELLS INFECTED WITH HERPES SIMPLEX VIRUS TYPE 2

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Summary: The 6-O-butanoyl derivative of castanospermine (MDL 28,574: BUCAST), an inhibitor of glycoprotein processing, blocked the growth of herpes simplex virus type-2 with the effect markedly enhanced by exposure of cells to the compound pre- as well as post-infection. The effectiveness of the derivative corresponded to an increased uptake with greatest accumulation after virus infection. Gas chromatography/mass spectrometry identified the predominant component in MDL 28,574 treated cells as castanospermine, an inhibitor of α-glucosidase 1. The effects of this compound on the synthesis of viral glycoprotein, gB, was determined with the increased molecular weight of the mannose-rich precursor evidence for the modulation of glycoprotein processing.
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A potential antiviral target in virus replication is the synthesis of virion surface glycoproteins, initially expressed on cells infected with HSV type-1 or type-2. Ten glycoproteins have so far been identified with gB, gD and gH (1,2,3) and most recently gK (4) and gL (5) which complexes with gH, obligatory for HSV replication in cell culture. It is likely that the remainder (gC, gE, gG, gI, and gJ) (6) have functions necessary for virus growth *in vivo*. Both N-linked and O-

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Abbreviations: HSV herpes simplex virus; HIV human immunodeficiency virus; IC_{50} 50% inhibitory concentration; HEF human embryonic fibroblasts; Endo-H endoglycosidase H.

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linked glycoproteins are found on HSV glycoproteins (reviewed in 7) although less is known about the synthesis of the O-linked glycoproteins. Oligosaccharide trimming of the pre-formed glycan attached to N-linked glycoproteins is initiated by hydrolysis of a terminal α-1,2 linked glucose by α-glucosidase 1 prior to the removal of two adjacent α-1,3 linked glucose residues and a number of mannose residues by α-glucosidase-2 and α-mannosidase activities, respectively (8). Restructuring of N-linked glycans to complex or hybrid forms is the ultimate goal in glycoprotein processing. One potent inhibitor of α-glucosidase-1 is the naturally occurring octahydroindolizine, castanospermine, which has good antiviral effects against HIV (8), human cytomegalovirus (9), but with little or no activity against HSV type-1 or type-2 at high concentration (<4mM) (10). The 6-O-acyl derivatives of castanospermine are more potent inhibitors of HIV growth than the natural product with the 6-O-butanoyl derivative, MDL 28,574 (11,12), currently in clinical trials for AIDS. Initial studies using the more lipophilic derivatives, which included MDL 28,574, demonstrated antiviral activity against HSV type-1 and HSV type-2 with IC50s in the order of 100μM (13). This report concentrates on the effect of MDL 28,574 treatment of cells infected with HSV type-2.

MATERIALS AND METHODS

Viruses and cell culture: HEF and monkey kidney cells (vero) were propagated in Eagles MEM plus 10% foetal calf serum (Flow). Monolayers were formed in 24-well cell culture plates or 25cm^2 flasks. Virus stocks were prepared from HSV type-2 (strain HG52) infected cells (14).

Uptake of radiolabelled compounds: Monolayers were incubated in the presence of [^{14}C] MDL 28,574 (1μCi/ml: specific activity 8.27mCi/mMol) or [^{14}C]castanospermine (1μCi/ml: specific activity 10.4mCi/mMol) and samples taken in triplicate at various times. Monolayers were washed three times in chilled PBS with equivalent amounts of "cold" compound, detached, lysed in 1% Triton-X and the radioactivity determined in a β-spectrometer. For infected cells, monolayers were exposed to HSV type-2 at a MOI of 5pfu/cell and incubated for 14 hours prior to the addition of radiolabelled compound.

GC/MS analysis of cell associated octahydroindolizines: Vero cell monolayers formed in 25cm^2 flasks were infected at a MOI of 5pfu/cell with HSV type-2 or left uninfected and incubated for 14 hours before addition of 1mM MDL 28,574. (Lower concentrations had previously shown a lack of sensitivity in the method of analysis). Six hours after the addition of MDL 28,574, monolayers were washed as above and cells scraped off and disrupted by repeated freeze-thawing. Samples were freeze-dried and subsequently extracted into 70% ethanol for 15 hours before centrifugation (5000xg/15 minutes). The supernatant was freeze-dried prior to the addition of Sigma SilA and heated at 50^{o}C for 20 minutes. 1μl of each derivative was injected into the gas chromatograph and the mass spectra obtained on a Finnigan MAT ITD detector.

Antiviral assays: Antiviral activity was assayed by plaque-reduction assay principally as described previously (14). With pre-treatment protocols, cell

monolayers were formed and virus absorbed in the presence of compound and subsequently incubated for plaque development in similar concentrations of compound.

HSV glycoprotein analysis: HEF monolayers (25cm^2 flasks) were formed in the presence or absence of compound and infected at a high MOI. At pre-determined intervals, cells were recovered, lysed and subjected to SDS-PAGE analysis (Pharmacia Phast-system) under non-reducing conditions. Some samples were digested with Endo-H (Oxford Glycosystems). Separated proteins were transferred electrophoretically to nitrocellulose (Phast-blot) and viral glycoproteins identified with a monoclonal antibody to gB (Mab1817), followed by a biotinylated anti-mouse antibody and a streptavidin alkaline phosphatase conjugate (Amersham). Glycoproteins were visualised using the chromagenic substrate 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) (Gibco). Biotinylated standards (Biorad) were used as markers.

RESULTS

The effect of pre-conditioning cells with MDL 28,574 prior to infection compared to treatment post-infection alone was investigated as a means to increase antiviral potency. HEFs were seeded in the presence of different concentrations of compound to produce near confluent monolayers by 48 or 72 hours before infection with HSV type-2 at low MOI. Cells were maintained in the presence of compound during virus adsorption and during the assay for plaque formation. When treatment was post-infection only, IC_{50} values were 105 and 110μM with a more marked suppressive effect (IC50 <4μM) when cells were exposed to compound pre- as well as post-infection (Figure 1). Pre-treatment alone with MDL 28,574 (300μM) for 72 hours was insufficient to protect HEF cells from plaque formation by HSV type-2. In general, results were similar in HSV type-2 infected vero cells with a 20-fold decrease in IC50 values seen after pre-treatment (results not shown).

Experiments were carried out to compare the uptake of [^{14}C]MDL 28,574 or [^{14}C]castanospermine by uninfected HEFs (Figure 2) or vero cells (results not shown) or after infection of either cell with HSV type-2 at high MOI. In both instances, substantially more [^{14}C]MDL 28,574 accumulated when compared to [^{14}C]castanospermine, with a significant increase in levels of the derivative in virus infected cells (Figure 2).

To determine the structural integrity of MDL 28,574 in cells exposed to the compound, pre-formed monolayers of vero cells were infected with HSV type-2 and incubated for 24 hours. Monolayers were then exposed to 1mM MDL 28,574 for 6 hours at 37^{o}C and washed well in PBS. Cells were harvested and lysed by freeze/thaw and ultrasonication then derivitized prior to analysis by GC/MS (Figure 3). Initial tests carried out with cell homogenates spiked with 1mM 6-O-butanoyl castanospermine (MDL 28,574), prior to processing and

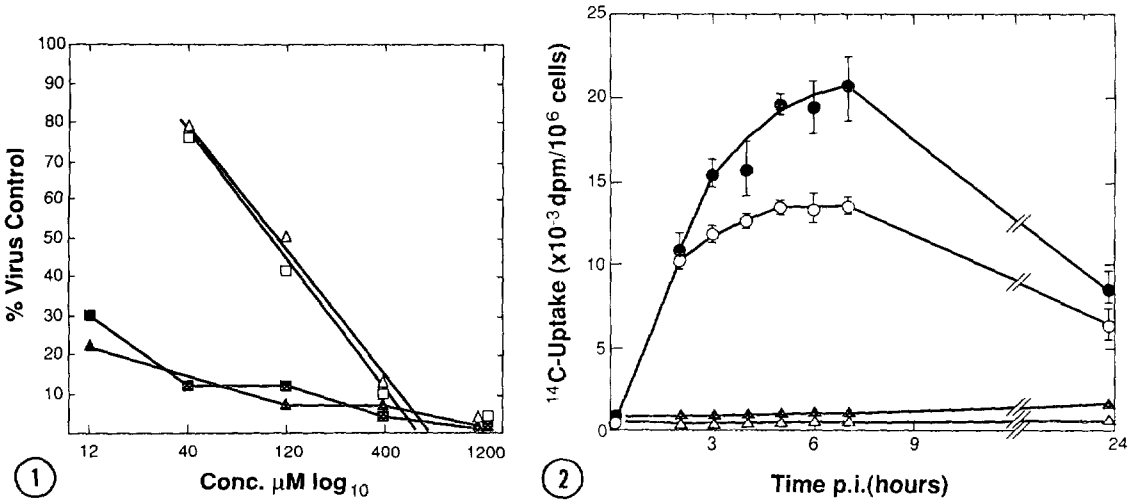

Fig. 1. Plaque-reduction assays from monolayers of HEF cells formed in the presence of different concentrations of MDL 28,574 (closed symbols) or without pre-treatment (open symbols) prior to infection with HSV type-2, strain HG52: cells were subsequently maintained in the presence of the respective concentration of compound. HEF cells pre-treated for 48 hours (Δ–Δ) or 72 hours (■—■) prior to infection.

Fig. 2. HEF monolayers were infected with HSV type-2 strain HG52 at high MOI (solid symbols) and incubated for 14 hours at 37°C prior to exposure to 100μM [^{14}C]MDL 28,574 (circles) or 100μM [^{14}C]castanospermine (triangles) and sampled at various times 24 hours post-exposure. Uninfected HEF monolayers (open symbols) were treated in parallel as described above. Levels of radioactivity (+ S.D.) are plotted as a function of time.

freeze-drying, showed the derivative was readily detected under these conditions (Figure 3a). The mass spectrum of the pertrimethylsilyl-6-O-butanoyl castanospermine from this analysis is shown in Figure 3b. The pertrimethylsilyl-derivative of castanospermine was readily separated from the pertrimethylsilyl-derivative of 6-O-butanoyl castanospermine by GC using an OV-1 column: a shorter retention time was recorded for castanospermine (Figure 3c). This trace is from analysis of HSV type-2 infected cells after exposure to 6-O-butanoyl castanospermine. The mass spectrum of the pertrimethylsilyl-derivative of castanospermine is shown in Figure 3d. Castanospermine was therefore identified as the major octahydroindolizine associated with infected cells after exposure to MDL 28,574.

To provide evidence for effects on glycoprotein processing, we investigated HSV type-2 infected HEF cells after exposure to MDL 28,574 (300μM) by non-reduced SDS-PAGE and western blot analysis, using the monoclonal antibody reactive to gB and the pre-treatment protocol. In untreated preparations (figure 4), glycoproteins were identified which represent the monomer of the gB precursor (104KDa) and its mature form (119KDa) (15): the multiple bands of

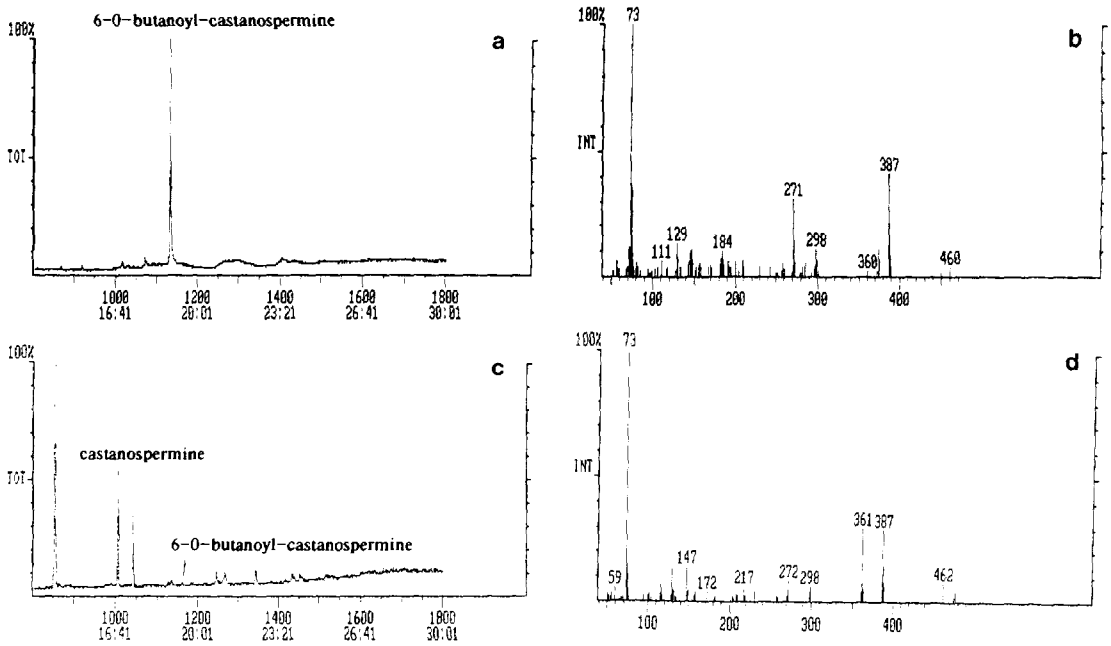

Fig. 3. Vero cell monolayers were infected with HSV type-2 strain HG52 at high MOI and incubated for 14 hours prior to exposure to 1mM MDL 28,574 for 6 hours at 37°C. (a) Gas chromatogram of MDL 28,574 spiked into a cell extract to show its retention time and (b) identity by mass-spectrometry. (c) The gas chromatogram of octahydroindolizines recovered from treated, infected cells and (d) the mass-spectrum of castanospermine.

about 200KDa represented dimeric forms. Cells treated with MDL 28,574 contained similar monomeric forms of gB but with a precursor molecule of decreased electrophoretic mobility that, in some gels, co-migrated with the mature form (Figure 4a). When both samples were treated with endo-H prior to separation and western-blot analysis, the mature forms and precursor molecules appeared as two bands of similar size (Figure 4b): an increased electophoretic mobility was apparant for both forms, with the greatest reduction in size evident for the mannose rich precursors. It was concluded that the increased size of the precursor, seen after MDL 28,574 treatment, was related to the retention of the terminal glucose and, consequently, mannose residues associated with N-linked glycans, the size being normalised after removal of mannose rich glycans by endo-H treatment.

DISCUSSION

Inhibitors of viral glycoprotein synthesis per se, have been of clinical benefit when used topically in patients with genital herpesvirus infection (16). A block in post-translational modification of viral glycoproteins by the action of MDL 28,574 on glycoprotein processing, should provide a more selective target.

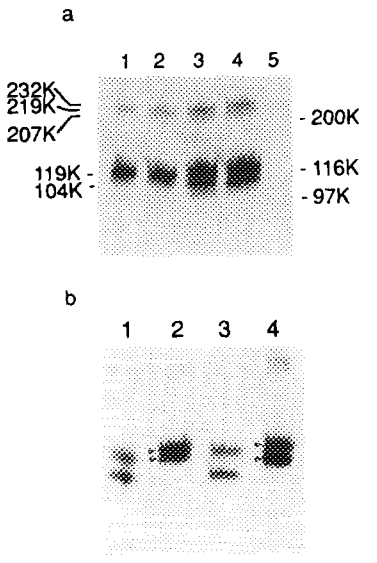

Fig. 4. HEF monolayers were infected with HSV type-2 strain HG52 at high MOI and treated with MDL 28,574 (300μM) using the pre- and post-treatment protocols. Cells were harvested at 14 hours post-infection and analysed by SDS-PAGE and western blot analysis without **(panel a)** or with **(panel b)** endo-H treatment. Glycoprotein products were identified using monoclonal antibody reactive for gB of HSV type-2. **a**: MDL 28,574 treated (tracks 1 & 2), untreated virus controls (tracks 3 & 4), acyclovir (10μM) negative control (track 5). **b**: MDL 28,574 treated (track 2) or untreated control (track 4) and after endo-H digestion (tracks 1 & 3, respectively).

This first report of an inhibitor of glycoprotein processing that affects the growth of HSV *in vitro* relates to the increased potency of the 6-O butanoyl derivative of castanospermine. This effect, which corresponded to the greater access of MDL 28,574 to infected cells, was similar to studies on HIV infection (11,12) where α-glucosidase 1 was identified as the target enzyme. The data presented here shows that castanospermine was the active intracellular component consistent with the observation that MDL 28,574 is a poor inhibitor of purified α-glucosidase 1 (12). The increased uptake of [^{14}C] MDL 28574 in HSV type-2 infected cells could relate to the highly modified infected cell surface, allowing for better membrane up-take or transport of the 6-O-acyl derivative. The antiviral mechanism responsible for the activity of MDL 28,574 against HSV infection remains to be elucidated and could involve modification of one or more of the ten HSV type-2 encoded glycoproteins. The current study was only concerned with the synthesis of gB as an indicator of altered viral glycoprotein processing. This provided evidence that the intracellular product of MDL 28,574, castanospermine, was indeed targeting α-glucosidase 1. It was of note that the the sizes of multiple dimers, which appeared to represent different combinations of precursor and mature forms, were altered in response to MDL

28,574 treatment and could represent important changes to higher-order structures of gB.

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