The effects of oligosaccharide trimming inhibitors on glycoprotein expression and infectivity of Junin virus

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Abstract: The effects of specific inhibitors of glycoprotein trimming reactions on Junin virus (JV) replication were investigated. Bromoconduritol, an inhibitor of glucosidase II, significantly reduced infective virus production (DE50: 1.1 mM) and viral protein expression. Neither l-deoxynojirimycin, an inhibitor of both glucosidases I and II, nor l-deoxymannojirimycin and swainsonine, inhibitors of mannosidase I and II, respectively, showed any activity against JV multiplication. These results are the first evidence that the acquisition of a complex form of the envelope glycoprotein oligosaccharide chains is not essential for JV infectivity. The effect of bromoconduritol was reversible and probably due to the formation of an unstable intermediate oligosaccharide structure which may be more sensitive to degradative proteolysis.

Key words: Oligosaccharide trimming inhibitors; Virus infectivity; Glycoprotein expression; Glycoprotein processing; Junin virus

Introduction

Junin virus (JV), the causative agent of Argentine Hemorrhagic Fever, is an arenavirus possessing two envelope glycoproteins, G1 (Mr 45–50 K) and G2 or GP38 (Mr 38 K), derived by proteolytic cleavage from a cell associated precursor GPC (Mr 63–65 K) [1].

Little is known about the biosynthesis, composition and biological functions of JV glycoproteins. GP38, which has been demonstrated to induce neutralizing antibodies [2], is the only glycoprotein exposed on the virion surface [3]. Accordingly, amino acid changes in GP38, detected by peptide mapping are associated with alterations in virus attachment to the host cell receptor and virulence properties [4,5]. In the presence of tunicamycin JV multiplication was significantly blocked [6], suggesting the requirement of N-glycosylation of envelope proteins for virus infectivity. Moreover, by lectin binding assays heterogeneity in carbohydrate chains of GP38 was found, with biantennary complex structures and high mannose units [3].

The final structure of the N-linked oligosac-
charides in glycoproteins is dependent on a series of enzyme reactions which involve the removal or addition of sugar residues. The biological significance of this trimming pathway and the relative importance of high mannose and complex oligosaccharides for the proper function of glycoproteins and viral infectivity can be studied with specific inhibitors of the involved enzymes. We therefore investigated the effects of bromoconduritol, 1-deoxynojirimycin, swainsonine and 1-deoxymannojirimycin, four trimming glycosidase inhibitors which perturb N-linked glycan structure [7], on infectivity and viral protein expression on JV infected cells.

Fig. 1. Effect of glycoprotein trimming inhibitors on JV replication. Vero cells were infected with JV and medium with 2 mM DNJ (●), 2 mM BC (△), 5 μg ml⁻¹ SW (□), 1 mM DMN (▲) or without drug (○) was added. Extracellular (A) and intracellular (B) virus yields were determined by plaque formation. Each point is the average value of duplicate determinations. The error bars representing standard deviations are not seen when they are shorter than the radii of the symbols.

Materials and Methods

Compounds
1-Deoxynojirimycin (DNJ), bromoconduritol (BC), swainsonine (SW) and 1-deoxymannojirimycin (DMN) were purchased from Sigma Chemical Company, USA. Solutions were prepared in culture medium, sterilized by filtration and used immediately.

Cells and viruses
African green monkey kidney (Vero) cells were grown in minimum essential medium (MEM) supplemented with 5% heat-inactivated calf serum and 50 μg/ml gentamicin. The IV4454 strain of JV was propagated in Vero cells and the titer of the stock suspension used in this study was 1 × 10⁶ PFU/ml.

Inhibition of Junin virus replication
Monolayer cultures of Vero cells were infected with JV at a multiplicity of infection of 1 PFU/cell. After 1 h adsorption at 37°C, medium with or without test compound was added. At 24,
48 and 72 h after infection supernatants were harvested and titrated by plaque formation to determine extracellular virus. The remaining cells were washed, frozen and thawed twice, centrifuged at 1000 × g and assayed for intracellular virus.

**Immunofluorescence assays**

Vero cells grown on coverslips were infected with JV and treated with trimming inhibitors as described above. For cytoplasmic immunofluorescence, cell monolayers were washed with cold PBS and fixed in methanol for 15 min at −20°C. Indirect staining was carried out by using anti-JV immunoglobulins purified from hyperimmune rabbit serum reactive against all JV proteins [4] and fluorescein labeled goat anti-rabbit IgG (Sigma Chemical Company). For surface immunofluorescence, infected cells were incubated with the primary anti-JV antibodies before fixation with methanol.

**Results**

The lack of toxicity of the four inhibitors for Vero cells was first investigated by assessing their effects on cell growth. Vero cells were maintained in the presence of medium containing varying concentrations of each drug or were left untreated for several days and the number of viable cells was recorded. Maximal noncytotoxic concentrations were 2 mM for DNJ and BC, 1 mM for DMN and 5 μg/ml for SW, respectively.

The effects of inhibitors of glycoprotein processing on the replication of JV are presented in Fig. 1. The data indicate that BC, a potent inhibitor of glucosidase II, significantly reduced both extracellular and intracellular virus yields by 60–90%, whereas DNJ, an inhibitor of both glucosidase I and II, was not active against JV replication. The other two compounds DMN and SW, inhibitors of mannosidase I and II, respectively, also did not affect the formation of infectious virus.

The dose response effect of BC against JV replication is depicted in Fig. 2 and the $ED_{50}$ (effective dose required to inhibit virus replication by 50%) determined from these data was 1.1 mM.

To examine reversibility of BC inhibition on JV growth, cells were infected and treated with compound for 24 h, then culture fluid was replaced with fresh medium without drug, cells were incubated for a further 24 h and supernatant virus yields were assayed. The reversible effect of the BC induced changes was demonstrated by the raised levels of virus infectivity once the compound was removed (Table 1).

In order to determine the effect of trimming inhibitors on the expression of viral proteins, drug treated and untreated cells were analyzed by cytoplasmic and membrane immunofluorescence assays. The pattern of cytoplasmic fluorescence seen in permeabilized cells was almost unaffected by DNJ, SW and DMN, whereas almost no viral proteins were detected on BC treated cells (Fig. 3). When immunofluorescence staining of viral proteins on the membrane of infected cells was performed, BC treated cells were again negative for viral antigen expression. Infected cells treated with the other inhibitors were positive for surface JV proteins although the fluorescence signal intensity was slightly weaker in DNJ treated cells in comparison with control JV infected cells (data not shown).

**Discussion**

The role of the oligosaccharide maturation process in the biosynthesis and biological proper-
ties of viral glycoproteins is not clear. Studies with trimming inhibitors have demonstrated considerable variability among different viruses in their requirement for complex type N-glycosylation for glycoprotein function and the consequent formation of infectious viral particles. It has been reported that glucosidase inhibitors, but not mannosidase inhibitors, impaired virus production in most retrovirus infected cells, including human immunodeficiency virus (HIV) infected lymphocyte lines [8-10], suggesting an essential role for glucose trimming and the potential of these compounds as anti-HIV therapeutic agents. By contrast, in vesicular stomatitis virus it has been shown that although viral glycoprotein structure is altered, neither glucosidase nor mannosidase inhibitors affect infective virus formation [11]. This is the first report on the effect of blocking glycoprotein processing on JV replication and the results obtained clearly indicate that addition of complex carbohydrate to the glycoprotein is not essential for virus infectivity. Inhibitors such as DNJ, DMN and SW, which increase high mannose content and block complex formation do not affect protein expression on cell surface and consequently do not interfere with infective viral production. BC, the only inhibitor affecting viral infectivity, directly abrogates detection of viral protein as measured by immunofluorescence assay. At present the reason for the differences between both glucosidase inhibitors, DNJ and BC, is unknown. However, their action on arenavirus infected cells described here corresponds with observations made with the influenza A fowl plague virus, which has an external cleavable hemagglutinin. The influenza protein glycosylated in the presence of BC has oligosaccharides of the structure Glc$_4$Man$_7$$_9$GlcNAc$_3$ and is metabolically unstable, preventing the release of infectious virus [12,13]. When fowl plague virus is grown in the presence of DNJ, the oligosaccharide has the composition Glc$_3$Man$_7$$_9$GlcNAc$_2$, but the altered protein is stable and infectivity is not reduced [14]. Thus, the inhibitory effect of BC on JV infected cells could be due to an increased proteolysis of the intermediate glycoprotein structure as a result of lack of protection by appended oligosaccharides or improper folding of the protein. When protein stability is not affected, the modification only of its glycosylation state, produced by the other inhibitors, is not enough to affect JV infectivity, indicating that its replication is not dependent on the carbohydrate chain structure of the envelope glycoproteins.

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**References**