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The HIV replication inhibitor 3′-fluoro-3′-deoxythymidine blocks sialylation of N-linked oligosaccharides

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Summary

The ability of 3′-fluoro-3′-deoxythymidine (FLT) to interfere with glycosylation was investigated in an experimental system, where the effects on the herpes simplex virus type 1-specified glycoprotein gC were determined. By adding FLT to HSV-infected cells after the peak of DNA synthesis, it was possible to segregate possible effects on nucleic acid metabolism from the effects on glycosylation of gC. It was found that FLT treatment of HSV-infected cells at concentrations of 20–500 μg/ml resulted in a significant increase in the electrophoretic mobility of gC, indicating a reduction of the amount of carbohydrates incorporated into gC. Lectin-binding assays demonstrated that the FLT treatment blocked addition of sialic acid to complex type N-linked glycans. The effects on glycosylation were observed in cells infected with an HSV mutant, deficient in thymidine kinase (TK), but not in cells infected with wild type virus. The cells infected with the wild type virus contained five times more total FLT metabolites than the cells infected with the TK-deficient mutant, whereas the latter cell type contained significantly higher amounts of unmetabolized FLT. This result indicates that FLT itself, and not a metabolite, was responsible for the effects on glycosylation.

HIV; Sialylation; Oligosaccharides; 3′-Fluoro-3′-deoxythymidine

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Introduction

The thymidine analog, 3′-fluoro-3′-deoxythymidine (FLT) has been found to inhibit human immunodeficiency virus (HIV) replication in cell culture more efficiently than 3′-azido-3′-deoxythymidine (AZT) (Matthes et al., 1988; Bazin et al., 1989). In addition, FLT is more efficient than AZT against simian immunodeficiency virus in vivo (Lundgren et al., 1990). FLT, akin to AZT, exerts its antiviral activity after being phosphorylated to the triphosphate level, acting as an inhibitor of the HIV- specified reverse transcriptase (Mitsuya et al., 1985; Bazin et al., 1989). Due to this higher antiviral effect, FLT has been considered as an alternative or complement to AZT for treatment of HIV infections, and is now being evaluated in patients (P.A. Barditch-Crovo et al.; Human pharmacokinetic prophile of 3′deoxy-3′-fluorothymidine (FLT), a potent anti-HIV nucleoside. Abstract 1335. Interscience Conference on Antimicrobiological Agents and Chemotherapy, Chicago, 1991).

Very little is known about the possible interference of FLT or its metabolites with other processes than DNA synthesis, such as glycosylation of proteins. Thus, several antimetabolites and antiviral compounds, interfering with nucleic acid metabolism, have been found also to affect the peripheral steps of protein glycosylation, such as galactosylation and sialylation (Olofsson et al., 1985; Datema and Olofsson, 1989; Peters et al., 1990). Such relatively small changes in the carbohydrate composition may not be readily detectable in the HIV-infected cells, where the inhibition of HIV-specified reverse transcriptase is the main event. Using the highly glycosylated herpes simplex virus type 1 (HSV-1) glycoprotein gC-1 as a reporter protein, we have established HSV-1 infected cells as a sensitive model system for detection of interference with the terminal steps in protein glycosylation (Olofsson et al., 1985, 1988). This glycoprotein is glycosylated by the host cell glycosylation machinery, and its precursor, designated pgC, which contains high mannose glycans, has a considerably lower apparent molecular weight (105 K) than the fully glycosylation product (130 K) (Spear, 1985; Campadelli-Fiume and Serafini-Cessi, 1985). Consequently, even minor differences in the cellular system for peripheral glycosylation are detected as significant shifts in the electrophoretical mobility of gC-1 (Olofsson et al., 1985). Using this system, we here describe that FLT, in addition to the effects at low concentration on reverse transcription, may interfere at high concentrations with late steps in glycosylation of viral and possibly also cellular membrane proteins.

Materials and Methods

Virus and cells

The HSV-1 strain F was used for standard experiments, whereas the thymidine-kinase-deficient strain R9C2 and its parent wild type strain SC16 (kindly supplied by Dr. H. Field, Cambridge, UK) were used in some

experiments. African Green Monkey Kidney cells, strain AH1 (GMK), were used throughout the study. The cells were cultivated in Eagle's minimal essential medium, supplemented with 10% fetal calf serum, for which the suitability of each batch was tested on a panel of sensitive, exponentially growing diploid and transformed cell lines. For maintenance, the same medium without serum but with antibiotics (penicillin [100 U/ml] and streptomycin [100 μg/ml]) was used.

Drugs, chemicals and radiochemicals

3′-Fluoro-3′-deoxythymidine (FLT) was obtained from Medivir AB, Huddinge, Sweden. HSV-1 infected cells were radiolabelled with D-[6-^{3}H]glucosamine hydrochloride (GlcN; specific activity 29 Ci/mmol) obtained from Amersham International, UK. All reagents and solvents were of reagent grade.

Monoclonal and polyclonal antibodies

The rabbit antiserum, specific for gC-1, was prepared by immunizing rabbits with immunosorbent-purified (monoclonal antibodies coupled to CNBr-activated Sepharose) gC-1, as previously described (Olofsson et al., 1983).

FLT treatment of HSV-infected cells

Petri dish (50 mm) cultures of GMK-AH1 cells were infected with 10 PFU (plaque-forming units)/ml of HSV-1. The cells were treated with the nucleoside analog from 6 h post-infection (pi) (i.e., after the peak of DNA synthesis), radiolabelled with [6-^{3}H]GlcN at 7 h pi and the cells were harvested at 18 h pi. The cells were lysed in 1 ml TBS (Tris-buffered saline; 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5) containing 1% Triton X-100 and the nuclei and cellular debris were removed by centrifugation for 2 min at 11 000 × *g*. The cytoplasmic extract was subjected to radioimmunoprecipitation, as previously described, with a polyclonal rabbit antiserum, specific for gC-1 (Olofsson et al., 1983). The precipitates were separated on SDS-PAGE and the gels were subjected to fluorography.

Extraction and HPLC analysis of FLT metabolites

About 10^6 cells in a 50-mm Petri dish were infected with the TK^+ or the TK^- strain of HSV. At 8 h pi, the cells were labelled with 1 μM (total volume: 1.5 ml) of [^{3}H]FLT (1400 dpm/pmol). Following incubation at 37°C for 4 h, the cells were placed on ice and washed twice with ice-cold phosphate-buffered saline (PBS). The cells were extracted for 10 min with 1.5 ml ice-cold 60% (v/v) methanol under gentle agitation. To facilitate a quantitative recovery of the extract, the plate was tilted to an elevation of about 30° for 10 min. Thereafter, the methanol extract was aspirated and stored on dry ice. The cells were re-extracted by the same procedure with 1 ml of 60% methanol and both extracts were pooled, and lyophilized for HPLC analysis of FLT metabolites.

Intracellular [^{3}H]FLT metabolites were determined by HPLC on a Partisil-

SAX column (Whatman, NJ, USA) (Karlsson et al., 1989). The separation started with 20 mM ammonium phosphate buffer, pH 3.8, 7% methanol for 3 min. Between 3 and 6.5 min a linear gradient changed to 0.5 M ammoniumphosphate buffer, pH 3.8, 7% methanol that continued isocratically from 6.5 to 25 min. The flow was 1.5 ml/min throughout the run. The radioactivity was determined simultaneously with a flow detector (Radiomatic, Tampa FI, USA). The retention times for the FLT metabolites were: FLT 4 min, FLT monophosphate (FLTMP) 9 min, FLT diphosphate (FLTDP) 12 min, and FLT triphosphate (FLTTP) 17 min.

Lectin chromatography procedures

Glycoprotein C-1 was purified from [^{3}H]GlcN-labelled HSV-infected cells by an immunosorbent, containing a polyclonal gC-1 specific antibody coupled to Protein A-Sepharose. Purified gC was digested by pronase as described by Olofsson et al. (1983) and the digests were subjected to lectin chromatography in mini columns eluted under the following conditions: lentil lectin (LCA; 50 mM α-methyl mannoside), ricinus communis lectin (RCA; 50 mM galactose) and wheat germ lectin (WGA; 50 mM GlcNAc; *N*-acetylglucosamine) (Goldstein and Poretz, 1986). The pronase digests (200 μl) were applied onto columns containing 1 ml lectin gel, and washed with 10 bed volumes of TBS to reach a baseline level of cpm. Thereafter, specifically bound glycopeptides were eluted by addition of five bed volumes of relevant sugar. All wash and eluate fractions were subjected to liquid scintillation counting to ensure complete recovery of the added radiolabel. In some experiments pronase digests treated with sialidase (Olofsson et al., 1983) were analyzed.

Results

1. Demonstration of FLT-induced modification of glycosylation in HSV-infected cells

The HSV-1 specified glycoprotein C (gC-1) was used as a glycoprotein probe to evaluate the effects of potential glycosylation inhibitors, because this glycoprotein is heavily glycosylated both with respect to N-linked and O-linked oligosaccharides (Olofsson et al., 1983; Lundström et al., 1987a). Even small modifications in the glycosylation capacity of HSV-infected cells therefore give rise to significant shifts in the electrophoretic mobility of gC-1 (Olofsson et al., 1985). To determine if FLT was able to modify glycosylation, we treated HSV-infected cells with FLT and labelled the cells with [^{3}H]GlcN from 6 h pi. This period of time was chosen to minimize effects of FLT on DNA replication (Olofsson et al., 1985). At 18 h pi, the cells were harvested and glycoproteins were extracted with TBS, containing 1% Triton X-100. Following immunoprecipitation with a gC-1 specific antiserum, the resulting precipitates were subjected to SDS-polyacrylamide electrophoresis (Fig. 1).

We found that FLT-treatment (1875 μM) of cells infected with a TK$^-$ strain

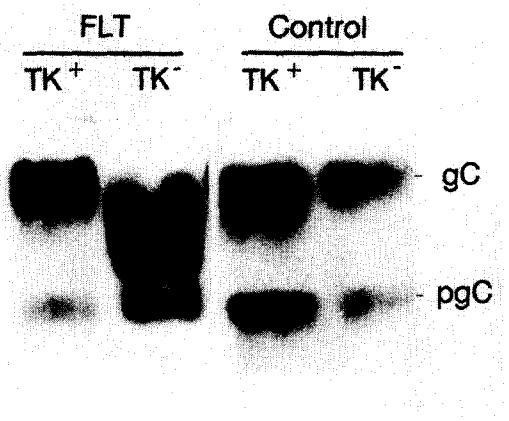

Fig. 1. FLT-induced increase in the electrophoretic mobility of gC-1. Extracts from HSV-infected and FLT-treated (1875 μM) cells were subjected to radioimmunoprecipitation with a polyclonal gC-1-specific rabbit antiserum. FLT was added at 6 h pi and [^{3}H]GlcN was added at 7 h pi. The immunoprecipitates were subjected to SDS polyacrylamide gel electrophoresis and fluorographed. Cells infected with a TK$^-$ HSV strain and cells infected with the corresponding wild type virus were analyzed. Positions of precursor gC-1 and completely glycosylated gC-1 (indicated as pgC and gC, respectively) are indicated.

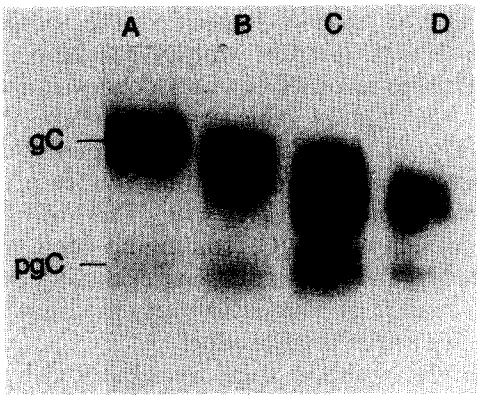

Fig. 2. Effects of FLT concentration on the mobility of gC-1, produced in cells infected by the TK$^-$ strain of HSV. Extracts from HSV-infected cells, treated with 0 (A), 75 (B), 375 (C) and 1875 (D) μM of FLT were radiolabelled and processed as described in the legend to Fig. 1.

of HSV resulted in a significant shift in the electrophoretic mobility of gC-1, indicating a modified glycosylation, whereas no such shift was observed for FLT-treated cells infected with a TK$^+$ strain of HSV. These results indicated that (i) FLT blocked one or more steps in the terminal glycosylation of gC-1 and that (ii) this effect was not dependent on HSV-1 specified TK, as was reported for the interference with protein glycosylation caused by 5-*n*-propyl-2′-deoxyuridine (PdU) and (E)-5-(2-bromovinyl)-2′-deoxyuridine (BVdU) (Olofsson et al., 1988; Olofsson and Datema, 1990). In fact, our results

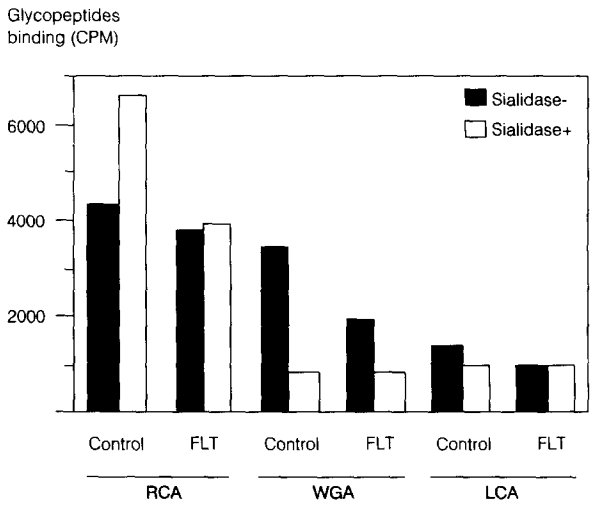

Fig. 3. Lectin binding of [^{3}H]GlcN-labelled glycopeptides from purified gC-1. About 30 000 cpm of purified gC-1 glycopeptides were analyzed in each chromatogram. Control cells and FLT-treated cells (190 μM), infected with the TK$^-$ strain of HSV were analyzed. In some experiments the glycopeptides were treated with sialidase, as indicated in the figure, prior to lectin chromatography.

demonstrate that the activity of HSV-1-specified TK abolished the ability of FLT and/or its metabolites to interfere with glycosylation. The shifts in the electrophoretic mobility were recorded for FLT concentrations from 75 μM to 1875 μM, which was the highest concentration where the cells remained morphological intact (Fig. 2). The change in electrophoretic mobility of gC-1 produced at the highest concentrations was much more prominent than that recorded for gC-1, produced in cells treated with 75 μM of FLT. We found that the electrophoretical mobility of pgC, containing high mannose N-linked glycans, was not affected by the FLT treatment, excluding proteolytic cleavage or interference with early glycosylation as a cause for the altered electrophoretical mobility of the final product.

To determine which glycosylation steps were blocked by FLT, we used a panel of lectins consisting of LCA, RCA and WGA. In our system, these lectins bind to the following defined carbohydrate structures of gC-1: diantennary complex type N-linked glycans (LCA); terminal β(1-4)-linked galactose of N-linked glycans (RCA) terminal sialic acid (WGA) (Olofsson et al., 1983, 1985, 1990; Lundström et al., 1987a;b). For these experiments [^{3}H]GlcN-labelled gC-1 was purified from FLT-treated (190 μM) or control cells and digested with pronase. This treatment liberates the altogether 9 N-linked glycans of gC-1 (Spear, 1985; Campadelli-Fiume and Serafini-Cessi, 1985) from the majority of peptide material (Olofsson et al., 1983, 1985; Lundström et al., 1987a;b). The resulting glycopeptides were analyzed by lectin affinity chromatography on mini columns as previously described and the results are presented in Fig. 3.

The most interesting results were obtained with WGA and RCA. As

TABLE 1

FLT metabolites in uninfected and HSV-infected cells

*Exponentially growing and stationary phase uninfected cells as well as stationary HSV-infected were analysed. **The content of various FLT phosphates was determined as described in Materials and Methods. ***Total amount of FLT and its phosphates (pmol/10^6 cells).

Cell type*		FLT		FLTMP**		FLTDP**		FLTTP**		Σ FLT***
		pmol/10^6 cells	%	pmol/10^6 cells	%	pmol/10^6 cells	%	pmol/10^6 cells	%	
Uninfected	Stat	37	54	11	16	2	3	18	26	68
	Exp	32	73	6	13	2	5	4	9	44
HSV (TK^-)		35	55	9	14	6	9	14	21	64
HSV (TK^+)		14	4	157	41	69	18	140	37	380

previously reported (Olofsson et al., 1985), the amount of WGA-binding activity of glycopeptides from control cells decreased after sialidase treatment, indicating the presence of oligosaccharides with terminal sialic acid. As expected, the amount of RCA-binding glycopeptides increased after this treatment, indicating exposure of penultimate galactose residues by removal of terminal sialic acid. We have previously shown that the RCA-binding activity of sialidase-treated gC is abolished by β-galactosidase treatment and reconstituted by treatment with UDP-galactose and galactosyl transferase, adding galatose to N-linked glycans in the configuration Galβ(1-4)GlcNAc (Olofsson et al., 1990).

In FLT-treated HSV-1-TK^- infected cells, however, we found no reproducible difference in the amount of RCA-binding glycopeptides between sialidase-treated and untreated glycopeptides, but a small difference between sialidase-sensitive and sialidase-insensitive WGA-binding glycopeptides was encountered. No change in the amount of LCA-binding glycopeptides was detected. These results, which were found in repeated experiments, demonstrate that FLT treatment is associated with a significant decrease in sialylation of oligosaccharides with terminal, RCA-binding galactose.

2. FLT metabolites in HSV- and uninfected cells

To determine the metabolic fate of FLT and possibly define the FLT metabolite responsible for inhibition of sialylation, we labelled HSV-infected and mock-infected cells with tritiated FLT. FLT and its phosphates were extracted with 60% methanol and analyzed by HPLC. Uninfected stationary phase cells had a low capacity to accumulate FLT, most likely due to a low thymidine kinase activity, and exponentially growing cells showed higher levels of phosphorylated metabolites. For cells infected with a TK^- mutant of HSV, the amount of extractable FLT and FLT phosphates were similar to that of exponentially growing cells, whereas 4–5 times more FLT metabolites were detected in the cells infected with the TK^+ strain of HSV (Table 1). However, in the cells infected with the TK^+ strain, the majority of FLT was recovered as

FLT mono-, di-, or triphosphates. Therefore, despite their relatively low total content of FLT and its metabolites, the cells infected with the TK^- strain of HSV contained about 3 times as much unphosphorylated FLT as did cells infected with the TK^+ strain. Glycosylation inhibition was detected only in cells infected with the TK^- strain. We have not determined the pattern of FLT phosphorylation at 500 μg/ml, but interference with glycosylation was detected also at 75 μM, which is in the same order of magnitude as used in this experiment, and only cells infected with the TK^- strain showed altered glycosylation. Therefore our data suggest that FLT itself, and not a metabolite thereof, exerted inhibition of sialylation and that FLT phosphates have no effect on glycosylation.

Discussion

The data in the present paper demonstrate that FLT, a potent anti-HIV agent, which as FLTTP inhibits reverse transcriptase, may act as an inhibitor of terminal glycosylation. It was not possible to detect this effect in HIV-infected cells due to the strong FLT-induced inhibition of reverse transcription, which is necessary for production of HIV glycoproteins. Our data (obtained with HSV-infected cells) indicate that unmetabolized FLT is responsible for the inhibition, and that no activation by viral or host cell enzymes is necessary for the FLT-induced effects on glycosylation. This suggests that the interference with glycosylation could also occur in FLT-treated cells, infected with an FLT-resistant mutant of HIV.

The most significant, and probably the sole, effect of FLT on glycosylation was the inhibition of terminal sialylation. This conclusion was supported both by the changes in the WGA- and RCA-binding activities of gC-1 glycopeptides and by the influence of sialidase treatment on the lectin-binding properties of these glycopeptides. The relatively small decrease in RCA-binding and PNA-binding activity after sialidase treatment between gC-1 from FLT-treated and -untreated cells indicates that no significant inhibition of galactosylation took place, which is in contrast to the inhibitory pattern for the HSV TK-dependent BVdU-induced inhibition of glycosylation (Olofsson et al., 1988). We found large amounts of RCA-binding glycopeptides from gC-1 of FLT-treated cells but no sialidase-inducible RCA-binding glycopeptides. This shows that shortage of suitable galactosylated acceptor oligosaccharides is not a major cause for the decrease in sialylation.

The results presented here suggest that FLT, and not an FLT phosphate, interferes with sialylation. This is in accordance with results obtained for several antitumor agents, where 3-deazauridine interferes with sialylation by depleting the CMP-sialic acid pool and D-arabinofuranosylcytosine by competitive inhibition of sialic acid synthetase and of sialyl transferase (Hindenburg et al., 1985). At present we do not know which of these mechanisms is responsible for the FLT-induced inhibition of sialylation.

Because these terminal steps in glycosylation, as far as known, are not essential for the infectivity of enveloped viruses (Datema et al., 1987), and because the FLT-induced inhibition of sialylation is demonstrable only at concentrations of FLT that are considerably higher than those used in vivo (Lundgren et al., 1990), it can be concluded that the ability to interfere with glycosylation does not contribute to the anti-HIV effect of FLT. However, terminal sialic acid has been reported to mask antigenic epitopes of glycoproteins of several enveloped viruses, making them resistant to normally neutralizing monoclonal antibodies (Huso et al., 1988; Olofsson et al., 1987). If this phenomenon also occurs during HIV infection of humans, there is a theoretical possibility that FLT increases the susceptibility to immune responses of those HIV strains that are resistant to the normal FLT-induced antiviral effects.

The effects of FLT on glycosylation take place at concentrations that are close to the toxic ones. It could, therefore, be argued that the effects on glycosylation only reflect the general toxic effects of FLT. There are, however, reasons to believe that the directly toxic effects of FLT and its effect on glycosylation are separate phenomena. Thus, FLT as well as its triphosphate contribute to the total toxicity of FLT (Matthes et al., 1987, 1988; Cox and Harmenberg, 1991). However, the glycosylation capacity was not impaired in FLT-treated cells infected with the TK^+ strain of HSV, despite the fact that these cells contained 4–5 times as much of FLT and FLT metabolites as do the cells infected with the TK^- strain. These data suggest that the inhibition of terminal glycosylation and the general toxicity of FLT are separable properties. The increase of FLTTP in cells infected with TK^+ HSV (Table 1) could also have in vivo implications if cells were infected with both HIV and HSV, and a transactivation of HIV by HSV (Laurence, 1990) could thus be counteracted.

Acknowledgements

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