Neoglycoproteins as Carriers for Antiviral Drugs: Synthesis and Analysis of Protein-Drug Conjugates†

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In order to investigate whether neoglycoproteins can potentially act as carriers for targeting of antiviral drugs to certain cell types in the body, various neoglycoproteins were synthesized using thiophosgene-activated *p*-aminophenyl sugar derivatives. These neoglycoproteins were conjugated with the 5′-monophosphate form of the antiviral drug AZT. For a proper characterization of these preparations, both protein and drug content have to be determined. Comparison of the Lowry and the Bio-Rad protein assays revealed that for both the neoglycoprotein carriers themselves and the AZTMP conjugates, the Lowry assay yielded the most reliable and reproducible results. It was demonstrated that both the reagent used for drug conjugation (ECDI) as well as the introduction of phenyl-sugar groups in the protein interfered with the analysis of bound nucleotide as based on spectral differences between protein and protein-drug conjugate. Therefore, we developed a rapid HPLC system for determination of the drug-protein coupling ratio through acid hydrolysis of the covalently bound nucleotide. With the ECDI-mediated conjugation of 5′-monophosphate drug derivatives to neoglycoproteins, products with molar ratios of drug to protein ranging from 1.2 to 5.6 were obtained. The drug-neoglycoprotein conjugates appeared to be fairly stable during storage, in lyophilized form, at -20 °C. The anti-HIV-1 activity of the neoglycoprotein-drug conjugates, as determined in vitro in MT-4 cells, was shown to be dependent on glycosylation of the albumin and also on the kind of sugar present in the neoglycoprotein. The anti-HIV-1 activity of the AZTMP-mannose-albumin conjugate exceeded that of the parent drug by more than 4 times.

Introduction

Human immunodeficiency virus (HIV) is the etiologic agent for the acquired immunodeficiency syndrome (AIDS) and a spectrum of related disorders.[1,2] The HIV virus shows selective tropism for the CD4-molecule bearing helper/inducer T lymphocytes.[3,4] Essential for the interaction between the virus and the CD4-molecule appear to be the carbohydrate chains of the virus envelope glycoprotein gp120.[5-8]

It is suggested that HIV entry in CD4-positive T cells occurs via fusion of the virus envelope with the plasma membrane.[9,10] On the other hand, Pauza et al.[11] published data indicating that HIV enters the T cell by receptor-mediated endocytosis. It is possible, though, that the early events in HIV infection are composed of both a fusion event and a receptor-mediated endocytotic mechanism.[12]

In order to investigate the suitability of neoglycoproteins for drug targeting purposes to HIV-infected lymphocytes, the antiviral agent azidothymidine (3′-azido-3′-deoxythymidine, AZT)[13] is conjugated, in its 5′-monophosphate form, to human serum albumin chemically modified with *p*-aminophenyl sugar derivatives.

The distribution of neoglycoproteins in the body is likely to occur depending on the carbohydrate moiety of the neoglycoprotein.[14-18] We are interested in the possibility of targeting an inhibitor of reverse transcriptase to infected T4 cells, based on the existence of sugar-recognizing lectins on the membrane of lymphocytes.[19-22]

Synthesis of neoglycoproteins was performed with thiophosgene activation of *p*-aminophenyl sugar derivatives.[23] The activated sugars are thought to react with the ϵ-NH_2 groups of lysine in the protein. Depending on the excess and the kind of sugar derivative used, products with different molar ratios sugar to protein can be obtained.

In order to conjugate nucleotides to protein, Halloran et al.[24] used water-soluble carbodiimides (e.g. ECDI) as coupling agents. A phosphoamide binding between the phosphate of the nucleoside derivative and the ϵ-NH_2 of lysine as well as the imidazole nitrogens of histidine in the protein is supposed to be formed.[25,26] The 5′-monophosphate derivative of thymidine showed a more efficient degree of substitution per molecule of protein than thymidine itself.[24] Another advantage may be the intracellular delivery of phosphorylated nucleosides, compounds that otherwise enter the cell very poorly because of their polar character. In principle, intracellularly delivered phosphorylated nucleosides may partly overcome a deficient phosphorylation due to the lack of some cellular kinases, that is if diphosphates could also be introduced in this manner. Characterization of the conjugation products includes protein as well as nucleotide analysis. Both the Lowry and the Bio-Rad protein assays were examined in view of potential interference by coupling the phenyl sugar

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† This study was supported by the Dutch Organization for Scientific Research (NWO).

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0022-2623/91/1834-1137$02.50/0

and nucleotide molecules.

Determination of the number of covalently bound drug molecules per mole of protein is generally carried out by using radioactive labeled drugs or measuring absorbance differences between the (neoglyco)protein and the (neoglyco)protein–drug conjugate.[27-30] As can be seen from our present results, the latter type of analysis is influenced by various factors and not suitable for a proper analysis of the particular drug targeting preparations. For this purpose, we developed an assay using acid hydrolysis of the phosphoamide binding between drug and protein in combination with HPLC determination of the released drug.

Antiviral activity of the neoglycoproteins as well as the AZTMP – conjugates was tested in vitro using MT-4 cells infected with HIV-1.[31]

Experimental Section

Chemicals. Human serum albumin (HSA, fraction V), *p*-aminophenyl sugar derivatives, ECDI (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide), thymidine, and thymidine 5′-monophosphate (TMP) were obtained from Sigma Chemical Co. (St. Louis, MO). Thiophosgene and sodium cyanoborohydride were obtained from Janssen Chimica (Beersse, Belgium). [^{3}H]-Thymidine 5′-monophosphate ([^{3}H]-TMP, specific activity 43 Ci/mmol, radiochemical purity > 98%) was purchased from Amersham Int. plc., England. AZT (3′-azido-3′-deoxythymidine) and AZTMP (the 5′-monophosphate derivative of AZT) were synthesized by P. Herdewijn of the Rega Institute, Leuven, Belgium. All other chemicals were of analytical grade or the best grade available.

Synthesis of Neoglycoproteins. The *p*-aminophenyl sugar derivatives were covalently coupled to HSA by using the method of Kataoka and Tavassoli.[23] Purification took place by chromatography with Sephadex G25 or, in case of the mannose–neoglycoproteins, with a Con A affinity chromatography column (Pharmacia Fine Chemicals, Woerden, The Netherlands). With an Amicon stirred cell equipped with a PM10 membrane (Amicon B.V., The Netherlands) the products were washed with distilled water until nonbound sugar was removed. Purified products were lyophilized and stored at 4 °C.

Synthesis of Neoglycoprotein–Nucleotide Conjugates. The method described by Fiume et al.[25] was used for preparing the neoglycoprotein–nucleotide conjugates: 20 mg of AZTMP and 20 mg of neoglycoprotein were dissolved in 1.5 mL of H_2O. After adjustment of the pH to 7.5, 20 mg of ECDI dissolved in 0.2 mL of H_2O was added while the mixture was stirred. Incubation took place for 24 h at 25 °C, in the dark. The reaction was stopped by Sephadex G75 chromatography, using 0.15 M NaCl as eluent. The fractions of the first peak were pooled and extensively washed with distilled water, by using the Amicon stirred cell, as described above, and lyophilized. The second peak was analyzed for protein content and nucleotide: it only consisted of unreacted nucleotide. The lyophilized conjugates were stored at −20 °C.

Using the same procedure we synthesized [^{3}H]TMP–protein conjugates.

Determination of Protein. Protein contents of the various products were determined according to the method of Lowry et al.,[32] a method based on a biuret-type reaction of the protein peptide bonds with copper, followed by the reduction of the phosphomolybdic–phosphotungstic reagent by tyrosine and tryptophan.[33] Also, the commercially available protein assay kit of Bio-Rad (Bio-Rad Laboratories GmbH, FRG), an assay based on the binding of the dye Coomassie Brilliant Blue G250 to protein,[34] was used for protein determination. For both methods we used human serum albumin as the standard protein.

Determination of Sugars. Colorimetric determination of the number of sugars coupled to HSA was carried out by using the phenol–sulfuric acid method.[35]

Spectrophotometric Analysis. All spectrophotometric characteristics of nucleoside analogues and conjugates were determined with a Philips PU 8700 spectrophotometer. This apparatus was also used for measuring the extinctions of protein and sugar assays.

Chromatographic System. The HPLC analyses were performed on a Waters liquid chromatograph (Waters Assoc., Milford, MA) consisting of a 510 pump, an U6K injector, and a Model 440 UV detector set at 254 nm and mostly operating at 0.01 AUFS. A μBondapak C18 column (Waters Assoc., 30 cm × 3.9 mm i.d.) was used guarded with a μBondapak C18 Guard-pak precolumn (Waters Assoc.).

Determination of AZT, AZTMP, Thymidine, and TMP. For determination of the nucleoside analogues AZT and AZTMP the eluent consisted of a mixture of KH_2PO_4 (0.1 M)/$[(CH_3CH_2CH_2CH_2)_4N]^+HSO_4^-$ (8 mM), pH 3.0, and acetonitrile (ACN) (92:8) at a flow rate of 1.5 mL/min.

Retention times were 9.0 min for AZT and 10.4 min for AZTMP. The detection limit of this method was 1.0 ng/mL for AZT and 10.0 ng/mL for AZTMP.

For thymidine and TMP the same elution components were used. The ratio buffer to ACN was 95:5. Retention times were 3.9 and 4.8 min, respectively. Detection limits were 1 ng/mL for both compounds.

Acid Hydrolysis of Conjugates. The conjugates were dissolved in a citric acid (0.1 M)/phosphate (0.2 M) buffer with pH varying from 1.5 to 4.5, at a concentration of 1.0 mg/mL. Immediately after dissolving, 50 μL of the solution was analyzed by HPLC for noncovalently bound nucleotide. After incubation of the solution for 3, 6, 12, and 24 h at 80 °C, another 20 μL was analyzed for released nucleotide by HPLC. Also, the neoglycoprotein carriers and the antiviral drugs were incubated under the same conditions. To determine whether hydrolysis was complete, ^{3}H-labeled nucleotide–conjugates were incubated by using the same procedure. In this case, analysis took place by HPLC with UV detection and by off-line measurement of radioactivity in 0.5-min HPLC fractions. After assessment of the appropriate pH and temperature for complete hydrolysis, these conditions were used for characterization of nonlabeled conjugates.

Stability of the Conjugates. The stability of AZTMP conjugates during storage was determined by HPLC analysis of the products just after synthesis and after a 6-months period of storage at −20 °C.

In Vitro Antiviral Activity. Determination of the antiviral activity of the neoglycoproteins as well as the AZTMP conjugates was carried out by using the MT-4/HIV assay developed by Pauwels et al.[31] Briefly, exponentially growing MT-4 cells were either infected with HIV (strain HTLV-III$_B$) or mock-infected. After resuspension at 4 × 10^5 cells/mL in complete medium [RPMI 1640 DM, supplemented with 10% (v/v) heat-inactivated fetal calf serum and 20 μg/mL gentamycin], 100-μL volumes were added in 96-well microtiter trays to serial 5-fold dilutions of the neoglycoproteins or conjugates in complete medium. The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Five days after infection the viability of mock- and HIV-infected cells was examined by a tetrazolium-based colorimetric assay (the MTT method). The 50% inhibitory concentration (IC_{50}) was defined as the concentration of compound that protected HIV infected cells by 50%, whereas the 50% cytotoxic concentration (CC_{50}) was defined as the concentration of compound that reduced the viability of mock-infected cells by 50%.

All experiments were performed at the Rega Institute, Leuven, Belgium.

Results

Synthesis of Neoglycoproteins. By varying the molar ratio sugar derivative:protein during the synthesis of the

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Table I. Results of Syntheses of Neoglycoproteins Using *p*-Aminophenyl Sugars Activated by Thiophosgene and Molar Ratios AZTMP:Protein after ECDI-Mediated Coupling

sugar	molar ratio sugar: protein in reaction mixture	molar ratio bound sugar: protein in neoglycoprotein product	AZTMP:protein molar ratio covalently bound	AZTMP:protein molar ratio noncovalently bound
mannose	10:1	7:1	2.4:1	0.4:1
	25:1	22:1	1.4:1	0.1:1
	420:1	40:1	1.2:1	0.1:1
galactose	420:1	32:1	1.7:1	0.2:1
fucose	420:1	10:1	3.2:1	0.3:1
glucose	420:1	26:1	5.4:1	0.2:1
–	–	–	5.6:1	0.2:1

neoglycoproteins we were able to obtain products with different molar ratios sugar:protein as summarized in Table I.

Synthesis of Conjugates. Results of the conjugation reaction of various neoglycoproteins with AZTMP are also shown in Table I. As can be seen, the molar ratio of nucleotide to protein varies depending on the protein used. It was not possible to completely remove noncovalently bound drug even by extensive washing of the product with distilled water. Presumably, this is caused by high affinity of the drug for the albumin. Dialysis for 48 h against flowing water was inferior compared with this washing procedure.

Protein Determination. The protein assays commonly used are the Lowry and the Bio-Rad assay. To investigate which assay would be the most appropriate in view of potential interference by the sugars coupled to the proteins, we determined the protein content of HSA in the presence of a known excess of *p*-aminophenyl sugar derivative and of a known excess of (di)saccharides. Also, protein content of the neoglycoprotein products and their conjugates were measured by both methods.

The results (data not shown) reveal a substantial influence of the sugars on both the Lowry and Bio-Rad assays. This influence consists of a 10–30% increase in color formation. At the same time, the Bio-Rad assay was shown not to be suitable for determining the protein content after conjugation with the nucleotide AZTMP: only 45–85% of the protein present was actually detected, depending on the preparation tested. Using the Lowry method, approximately 100% of the protein present was assayed, generally with less variability.

Determination of Bound Nucleotide. We examined the influence of ECDI on the UV-absorption characteristics of human serum albumin by carrying out the ECDI-mediated synthesis without adding the nucleotide. The spectrum of the resulting product is shown in Figure 1. It is clear that this spectral change may have considerable implications for the quantification of bound nucleotide by absorption measurement.

Since the introduction of a phenyl group in the linkage between sugar and protein causes a considerable increase in UV absorbance, an acid hydrolysis/HPLC assay was developed in order to have a reliable method for determination of bound nucleotide in this class of neoglycoproteins. By using ^{3}H-labeled TMP we validated the required pH (2.5) and incubation time (24 h) needed for total hydrolysis. Table II shows the results of determination of nucleotide to protein ratio using the three different methods described (UV, ^{3}H-label, and acid hydrolysis/HPLC measurement).

A typical chromatogram of a freshly prepared solution of AZTMP conjugated to $Man_{22}HSA$[41] is shown in Figure 2a. The AZTMP detected is noncovalently bound. After acid hydrolysis for 24 h at 80 °C the covalently bound

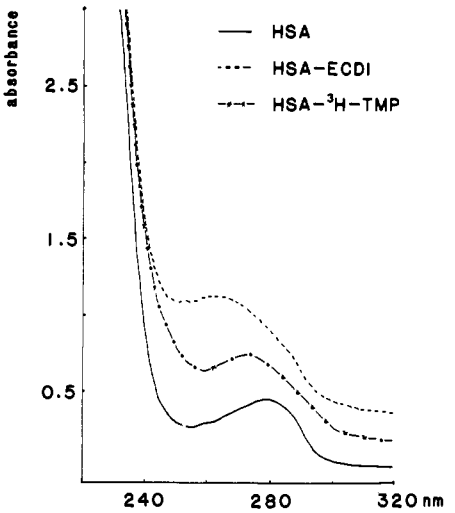

Figure 1. Spectrophotometric analysis of HSA, HSA reacted with ECDI, and HSA conjugated with [^{3}H]TMP, with ECDI as coupling agent.

Table II. Analysis of Molar Ratio [^{3}H]TMP:HSA by Various Methods

method	molar ratio covalently plus noncovalently bound [^{3}H]TMP:HSA
UV	2.4 ± 0.2
^{3}H	1.6 ± 0.1
acid hydrolysis/HPLC[a]	1.5 ± 0.1

[a] With HPLC, it is also possible to measure noncovalently bound nucleotide: in the case of [^{3}H]TMP–HSA the molar ratio noncovalently bound TMP:HSA was 0.1:1.

AZTMP is released from the phosphoamide binding. HPLC analysis of the product now includes covalently as well as noncovalently bound AZTMP (figure 2b).

Stability Experiments. During a 6-month period of storage of the conjugates, in lyophilized form at −20 °C, the amount of drug noncovalently present has increased from 3% to 10%.

In Vitro Antiviral Activity. Table III shows that the neoglycoproteins themselves do not have any significant cytoprotective activity in the HIV-1 assay. In case of the AZTMP conjugates, $Man_{22}HSA$–AZTMP is far more antivirally active against HIV-1 than HSA- and $Gluc_{26}HSA$–AZTMP, in spite of the fact that the drug load of the former compound is much smaller. Comparison of the potency (per unit AZTMP) of the neoglycoprotein–AZTMP conjugates with the potency of HSA–AZTMP reveals a remarkable difference depending on the kind of sugar present in the conjugate: the $Gluc_{26}HSA$–AZTMP is only slightly more potent than the HSA–AZTMP conjugate, whereas the mannosylated conjugate is more than

Table III. Anti-HIV-1 Activity of Neoglycoproteins, Neoglycoprotein–AZTMP and HSA–AZTMP Conjugates, AZTMP, and AZT, as Determined by a MT-4/HIV Assay[31]

	molar ratio AZTMP: protein	n^a	CC_{50},[b] μM	IC_{50}[c] × 10^{-3} μM	IC_{50} expressed as AZTMP present in conjugate (×10^{-3} μM)	potency (per unit AZTMP) of neoglycoprotein–AZTMP conjugate vs HSA–AZTMP conjugate
$Gluc_{26}HSA$		2	>3.3	>3300		
$Man_{22}HSA$		6	>13.3	>3300		
HSA–AZTMP	5.8:1	4	>1.4	5.9 ± 1.8	34.4 ± 10.7	1.0
$Gluc_{26}$HSA–HZTMP	5.6:1	4	>1.2	1.8 ± 1.5	10.4 ± 8.2	3.3
Man_{22}HSA–AZTMP	1.5:1	2	>6.6	0.6 ± 0.2	0.9 ± 0.3	38.2
AZTMP			25.9	3.7		
AZT			13.0	5.0		

[a] Number of experiments. [b] 50% cytotoxic concentration. [c] 50% inhibitory concentration.

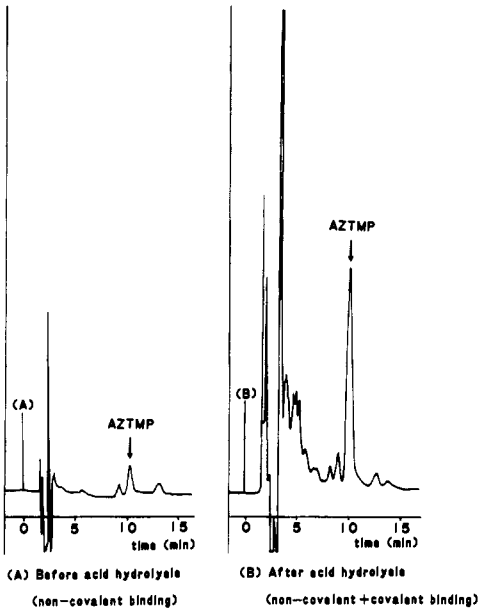

Figure 2. Determination of noncovalently bound AZTMP (A) and AZTMP released after acid hydrolysis (B) as described in the Experimental Section: (A) 50 μL of a 1 mg/mL solution of Man_{22}HSA–AZTMP conjugate in buffer pH 2.5, analyzed immediately after dissolving, (B) 20 μL of that same solution, analyzed after a 24-h incubation at 80 °C.

30 times as potent. Cellular toxicity of the compounds was not observed up to a concentration range of 1.2–13.3 μM, the limit that is imposed by the solubility of the compounds.[36]

Discussion

The present study deals with the synthesis and characterization of neoglycoproteins and their conjugation products with the nucleoside analogue AZTMP. These products were prepared in order to investigate whether the neoglycoproteins can act as potential carriers for nucleoside analogues to HIV-infected T4 lymphocytes, depending on the kind and number of sugars coupled. For interpreting the results of activity and pharmacokinetic studies, a precise knowledge of the amount of drug bound covalently as well as noncovalently to the protein carrier is required. This implies the necessity of proper quantification of both the protein and drug content in the particular preparations.

Synthesis of Conjugates. Under the same coupling conditions, there is a considerable variation in the molar ratio of AZTMP to protein in the various conjugates (Table I). This may be a consequence of the fact that the antiviral drug is covalently bound to one of the imidazole nitrogens of histidine and the ϵ-NH_2 group of lysine. The latter group is also taking part in the coupling of the sugar molecules to the protein.[23] So, in general, the more ϵ-NH_2 groups that have reacted with the sugar molecules, the fewer that should be available for the nucleotide molecules.

Protein Determination. Commonly, Lowry and Bio-Rad assays are used for determining protein, with HSA used as the standard protein. From our results, it can be concluded that with the Lowry as well as the Bio-Rad assay, some corrections have to be made because of an elevation in color formation due to the sugar component present in the neoglycoprotein. These results are in accordance with those published by Thorne[33] and Bensadoun[37] with regard to the Lowry assay. Data about sugar influences on the Bio-Rad assay were not available. Our present study shows that a similar increase in color formation occurs with this assay. A factor that only influences the Bio-Rad assay seems to be coupling of the drug to the protein. Coupling of the drug results in considerably lower protein values, in spite of the fact that the NaCl-containing elution medium used in the Sephadex purification was completely removed (experimentally established using HSA, data not shown). With Lowry's method, the protein contents of the products approximated 100% after correction for sugar influences, depending on the water content of the lyophilized conjugates. In the case of Bio-Rad, however, the binding of the dye Coomassie Brilliant Blue G250 to the protein is likely to be impaired by the conjugated drug, because neither the reaction of ECDI with the protein nor the presence of (noncovalent) AZTMP interfered with the assay.

In conclusion, it can be said that the Lowry assay is the most reliable method for protein determination in neoglycoprotein preparations, although small corrections have to be made as a result of the influence of the sugars present.

Determination of Bound Nucleotide. (a) Methods Based on Spectral Changes. Figure 1 shows the influence of the reagent ECDI on the absorption characteristics of HSA. It is obvious that an increased absorption occurs in the range of 240–320 nm. This increase also exists after coupling TMP (Figure 1) as well as AZTMP to HSA or *p*-aminophenyl sugar–glycoproteins. Likewise, this phenomenon occurs after coupling ara-AMP to lactosylated HSA (L_x-HSA: spectrum not shown), performed as described by Fiume et al.[25,26,38,39] In some of their studies, these authors determined the molar ratio of *ara*-AMP to L_x-HSA by measuring such spectral differences. At the same time, with the spectral method, it is not possible to

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discriminate between noncovalently and covalently bound drug. These factors may endanger a proper interpretation of the actual drug targeting efficiency.

Another complicating factor appears to be the molar absorptivity of the covalently bound drug. Deviations from the linear relationship between absorbance and concentration can be encountered when the charge distribution of the drug is affected by neighboring molecules or large organic ions or molecules (e.g. proteins).[40]

(b) Acid Hydrolysis/HPLC Method. The above-mentioned factors should be taken into account if the characterization of the drug-targeting preparations is warranted. Yet, the relative contributions of these various factors to the errors in the estimation of the drug:protein ratio are difficult to establish. Therefore, we developed a method which uses a combination of acid hydrolysis of the phosphoamide bond, formed by ECDI coupling,[25,26] and determination of the released drug by HPLC. The conditions for complete hydrolysis were ascertained by using [^{3}H]TMP. By coupling this compound to HSA it was possible to determine bound nucleotide by using three different methods: (1) spectral differences between carrier and conjugate (2) radioactivity measurement, and (3) acid hydrolysis/HPLC. The data, shown in Table II, indicate that the results of the acid hydrolysis/HPLC method are consistent with the ^{3}H measurement. The spectral method leads to overestimation of the molar ratio nucleotide:protein, probably caused by the ECDI reagent, as discussed above.

Under the conditions determined by [^{3}H]TMP–HSA analysis, noncovalently as well as covalently bound (nonradioactive) AZTMP in various conjugates was assessed. By means of this method the molar ratio of drug to protein could be determined more accurately.

Stability of the Conjugates. The conjugates seemed to be fairly stable: during 6 months of storage at −20 °C in lyophilized form, only up to 7% of covalently bound drug was released from the protein.

In Vitro Antiviral Activity. A pronounced anti-HIV-1 activity of Man_{22}HSA–AZTMP was observed, whereas $Gluc_{26}$HSA and HSA conjugates exhibited a much lower potency. None of the conjugates showed cytotoxic effects at the highest possible concentration. The neoglycoprotein carriers themselves tested in the present study, were virtually inactive.

It remains to be established whether the marked potency of the Man_{22}HSA–AZTMP conjugate can be explained by sugar-specific recognition and subsequent endocytosis of the conjugate into the MT-4 cells or alternatively by some synergistic action of the glycoprotein carrier and the AZTMP molecules. Experiments to elucidate this sugar-specific antiviral activity of the AZTMP–neoglycoprotein conjugates in more detail are in progress.

General Conclusions

Characterization of antiviral drug–neoglycoprotein conjugates requires proper analysis of both protein and drug content. This study shows that protein determination according to Lowry is a reliable method for characterization of the neoglycoproteins as well as the nucleotide conjugates. However, corrections should be made to account for the interference by the sugar molecules present in the neoglycoprotein preparations. The Bio-Rad protein assay was demonstrated not to be suitable for analysis of the glycoprotein–drug conjugates.

The present study indicates that drug analysis can be easily performed by incubating the conjugates at pH 2.5 for 24 h at 80 °C, resulting in complete hydrolysis of the phosphoamide bond between the protein and the drug, followed by HPLC analysis of the released drug.

It was possible to covalently couple 5′-monophosphate nucleosides in a molar ratio of drug to neoglycoprotein ranging from 1.2 to 5.6, depending on the number of sugar molecules in the neoglycoprotein carrier molecule.

The neoglycoprotein–AZTMP conjugates, in the lyophilized form, were shown to be stable when stored at −20 °C for at least 6 months.

A pronounced anti-HIV-1 activity of the mannose-AZTMP-neoglycoprotein conjugate was observed, whereas the glucose-containing conjugate did not show any activity, indicating sugar specificity.

(40) Shoog, D. A.; West, D. M. *Principles of Instrumental Analysis*, 2nd ed.; Saunders College: Philadelphia, 1981; p 153.

(41) Abbreviations: Man = mannose, Gluc = glucose. The number denotes the number of sugar molecules per protein molecule.