Optimization of the Reaction Conditions for the Synthesis of Neoglycoprotein-AZT-Monophosphate Conjugates†

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Received May 31, 1994®

The coupling of the monophosphate derivative of 3-azido-2,3-dideoxythymidine (AZTMP) to glycoproteins by water soluble carbodiimide (1-ethyl-3-[3-(dimethylamino)propyl]-3-ethylcarbodiimide) was greatly improved, relative to a recently reported method, by using also *N*-hydroxysulfosuccinimide (NHS) in the conjugation reaction. The hydrolysis of the activated AZTMP intermediate, responsible for the low degree of conjugation in the earlier method, could be delayed considerably if the activated phosphate group was converted into an activated ester by addition of NHS. In order to minimize the use of compounds needed for the preparation of AZTMP-protein conjugates, the present study was undertaken to determine if the reaction conditions could be optimized such that a conjugate with 2 AZTMP molecules/mol of neoglycoprotein would result. In addition a low proportion of cross-linked conjugates was desired. Optimization was achieved studying the shape of three-dimensional response surfaces, in which the degree of AZTMP coupling and the percentage of monomeric conjugates were regarded as the relevant responses. It appeared that the optimal conditions for coupling 1-2 mol of AZTMP to 1 mol of glycoprotein were an incubation time of 30 h, an AZTMP amount of 4 mg, an NHS amount between 8 and 15 mg, and a glycoprotein amount of 50 mg.

Introduction

The antiviral drug AZT (3-azido-2,3-dideoxythymidine) is a potent inhibitor of HIV-1 replication and the first clinically successful drug for AIDS and AIDS-related diseases.[1,2] After its phosphorylation to the triphosphate form (AZTTP) by host cell kinases, it can compete with endogenous nucleosides to inhibit HIV-1 reverse transcriptase.[3] In this form it may also act as a chain terminator by incorporation into growing strands of DNA or RNA.[3,4] However, chronic use of AZT by HIV patients can cause severe side reactions, particularly bone marrow suppression.[5,6] In addition, evolvement of virus strains resistant to AZT during therapy renders this nucleoside less effective.[7,8]

To improve the efficacy and reduce the side effects of AZT, the nucleoside can be covalently coupled to a specific macromolecular carrier that may provide cell-specific delivery. Neoglycoproteins have been used before to target nucleoside analogues efficiently to different cell types in the body.[9] Neoglycoproteins can be prepared from human serum albumin (HSA) with various sugars and sugar densities and be employed as macromolecular drug carriers. The presence of sugar-recognizing lectins in the membrane of monocytes/macrophages and T-lymphocytes is the basis of this approach. Lectin-mediated recognition followed by endocytosis of the conjugate and subsequent intracellular release of the drug may provide cell-specific delivery of the drug.[10-15] In the present study the monophosphate derivative of AZT (AZTMP) (Figure 1) was coupled to the neoglycoprotein Lactose$_{28}$-HSA. The advantage of the phosphorylated derivative is that intracellular conversion to the active triphosphate form can occur more readily.[9] In addition, the presence of a monophosphate group provides a chemical handle for coupling to carriers.[16,17]

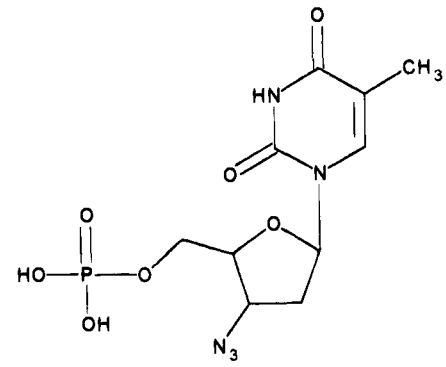

Figure 1. Structure of 3-azido-2,3-dideoxythymidine monophosphate (AZTMP).

In a previously adapted method in our laboratory, the AZTMP was activated at a pH of 4.0 by water soluble carbodiimide (EDCI) to react at a pH of 7.5 with the ϵ-NH_2 of the lysine and histidine residues in the neoglycoprotein.[16,18,19] This reaction results in the formation of a phospho-amide bond between the drug and the protein.[20] However the activated intermediate is highly susceptible to hydrolysis into the parent compounds.[17,21,22] Consequently this improved two-step procedure still results in a low degree of AZTMP-coupled conjugates, in relation to the amount of AZTMP added. Besides, the carbodiimide is also able to react with carboxylic acid, groups that are abundantly present in proteins. Although most of the EDCI was allowed to react with the AZTMP before adding the neoglycoprotein, this procedure cannot completely prevent intramo-

† Abbreviations: AZT, 3-azido-2,3-dideoxythymidine; AZTMP, 3-azido-2,3-dideoxythymidine monophosphate; AIDS, acquired immunodeficiency syndrom; EDCI, 1-ethyl-3-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; HIV-1, human immunodeficiency virus-1; HSA, human serum albumin; NHS, *N*-hydroxysulfosuccinimide.

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® Abstract published in *Advance ACS Abstracts*, February 15, 1995.

0022-2623/95/1838-0883$09.00/0

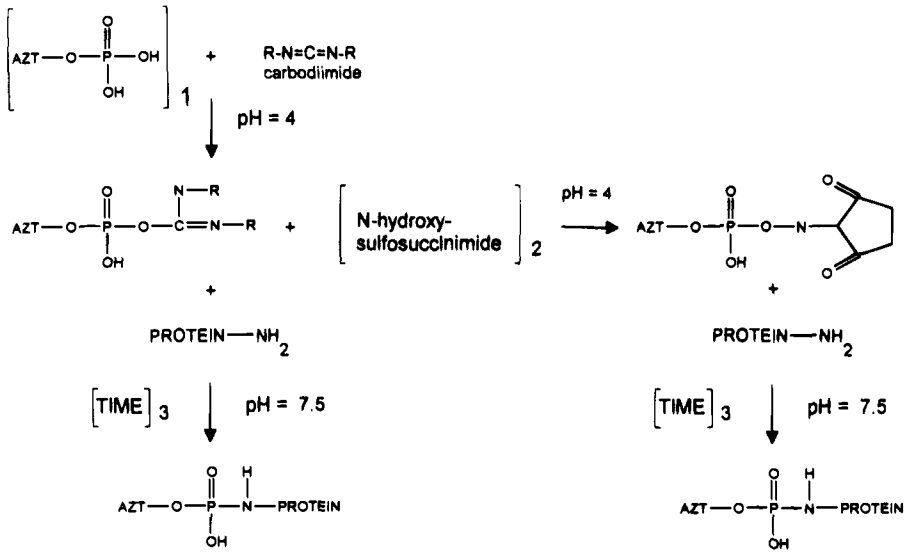

Figure 2. Coupling reaction of AZTMP to neoglycoproteins.

lecular as well as intermolecular cross-linking of the neoglycoprotein molecules. Consequently preparations with a substantial amount of dimeric, oligomeric, and polymeric conjugates are obtained.[17] In the present study, *N*-hydroxysulfosuccinimide (NHS) was used as a second activator in the conjugation reaction. NHS is able to reduce the rate of hydrolysis of the carbodiimide-activated AZT monophosphate by stabilizing the activated intermediate.[21,22] In principle, the use of NHS could lead to both a higher yield and a lower degree of cross-linking of protein molecules.

The circumstances for this coupling reaction were further optimized in order to minimize the use of the starting materials and also with the aim to couple one or two AZTMP molecules to one molecule of neoglycoprotein. This degree of drug loading of the glycoprotein has previously been shown to be sufficient for profound anti-HIV-1 activity *in vitro*.[23] The conjugates, obtained under the different reaction conditions, were analyzed for AZTMP covalently coupled to neoglycoproteins as well as for the percentage of monomeric, not cross-linked, material. The incubation time, AZTMP amount, and NHS amount were systematically varied. The influences of these predictor variables on the responses of AZTMP coupling and percentage of monomeric compounds were described by an empirical model, encompassing the behavior of the reaction mechanism. The graphs of these responses plotted against the predictor variables, the so-called response surfaces, were constructed from this empirical model. Such curves are a visual aid to locate the responses that meet the optimum reaction conditions. The optimization experiments were performed according to a Box–Behnken experimental design. The response surfaces were constructed according to response surface methodology.[24]

It appeared that with the obtained model for coupling AZTMP to neoglycoproteins the variables' reaction time and the AZTMP amount positively influenced the amount of AZTMP coupled. These factors had almost no influence on the percentage of monomeric conjugate. We also demonstrate that while increasing the NHS amount the efficiency of AZTMP coupling is decreased, whereas this positively influenced the yield of monomeric material.

Results

The coupling of AZTMP to neoglycoproteins using NHS in the coupling procedure resulted in conjugates with clearly higher amount of monomeric material, in comparison to the conjugates obtained after reaction without NHS. The latter conjugates consisted for more than 80% of cross-linked material. In addition, the coupling degree of AZTMP was improved. In these new reaction conditions it appeared that the settings of the AZTMP amount could be in between 0.5 and 6.0 mg. These reaction conditions resulted in an appropriate coupling degree (1 or 2 AZTMP molecules/HSA molecule). Without the use of NHS, as previously described, at least 50 mg of AZTMP was needed to obtain a similar result.[16,18]

Optimization of the Degree of AZTMP Coupling. The results of the 15 different reaction conditions, with regard to the coupling degree, are listed in Table 3. On the basis of these results, a model could be calculated with the help of a statistical software package (SAS),[25] which describes the effects of the predictor variables on the coupling of AZTMP. This resulted in the following model (scaled values were used):[24,25] response (AZTMP coupling) = 0.851 + 0.4100 × AZTMP + 0.155 × time − 0.168 × NHS + 0.218 × AZTMP × time − 0.313 × NHS × time − 0.234 × AZTMP × AZTMP. This model was obtained by fitting all possible combinations of variables, quadratic effects, and interactions. The final model was selected which showed the highest R^2_{adj} and the smallest lack-of-fit and contained only significant parameters. The equation shows that from the three predictor variables the AZTMP amount has the largest influence on AZTMP coupling. The model contains the presence of two interaction terms, one positive interaction term between AZTMP and time and one negative interaction term between NHS and time. A positive interaction term means that time positively influences the effect of AZTMP on the response. Higher responses are obtained when both AZTMP and time have the same sign of scaled values (+1 or −1). Consequently with a small amount of AZTMP and a short incubation time or with a large amount of AZTMP and a longer incubation time, higher responses are found. The negative interaction term between NHS and time implies that

Table 1. Settings of the Variables Studied, Together with the Scaled Values (−1, 0, +1)

scaled values	time (h)	NHS (mg)	AZTMP (mg)
−1	6	1.50	0.50
0	18	8.25	3.17
+1	30	15.0	5.84

Table 2. Scaled Reaction Conditions for Each Experiment

exp no.	time	NHS	AZTMP	d			
1	+1	+1	0	9	+1	0	+1
2	+1	−1	0	10	−1	0	+1
3	−1	+1	0	11	+1	0	−1
4	−1	−1	0	12	−1	0	−1
5	0	+1	+1	13	0	0	0
5	0	−1	+1	14	0	0	0
7	0	+1	−1	15	0	0	0
8	0	−1	−1				

higher responses are obtained when both variables differ in sign of scaled values. For instance, higher responses are found with a short incubation time and high amounts of NHS or with longer incubation times and smaller amounts of NHS. Overall, the time negatively influences the effect of NHS on the response.

Figures 3–5 show the response surfaces based on the model, mentioned above. Figure 3 shows the response in relation to time and AZTMP amount for the three amounts of NHS used. It appeared that high amounts of NHS resulted in a low response, thus a low coupling degree of AZTMP. It was also observed that increasing the amounts of NHS gave higher responses with shorter incubation times.

Figure 4 shows the response in relation to AZTMP and NHS amount for the three incubation times used. A longer incubation time always resulted in a higher response. It appeared also that with increasing incubation time and AZTMP amounts, the influence of the NHS concentrations on the coupling of AZTMP to $Lact_{28}$-HSA seems to be very low.

In Figure 5 the responses in relation to time and NHS amount for the three amounts of AZTMP used can be seen. It is shown that reactions with increasing amounts of AZTMP always resulted in higher responses. Large amounts of NHS and long incubation times negatively influenced the coupling reaction, that is, if small amounts of AZTMP are used. However with the use of larger amounts of AZTMP, this influence disappeared.

Optimization of Prevention of Polymerization. The results of the optimization experiments, with relation to the percentage of monomeric material, are listed in Table 3. Unfortunately, it was not possible, even after transformation of the dependent variable (% monomeric material), to describe the effects of the predictor variables on the percentage of monomeric material with the polynomial model used. Finally, from the measured percentages, seen in Table 3, it became clear that the amount of NHS did have an important influence on the percentage monomer: the larger the amount of NHS used, the higher the percentage of monomeric, not cross-linked, conjugates. The incubation time and AZTMP amount did not influence the percentage of monomeric conjugates significantly.

Conclusions

In order to improve the efficiency of the method for coupling the monophosphate derivative of AZT to neoglycoproteins, the present study was undertaken. The aim of the study was to find optimal conditions for coupling of maximally two AZTMP groups per reacted protein molecule. This coupling degree was found to be sufficient for an anti-HIV-1 effect of the conjugates *in vitro*.[23]

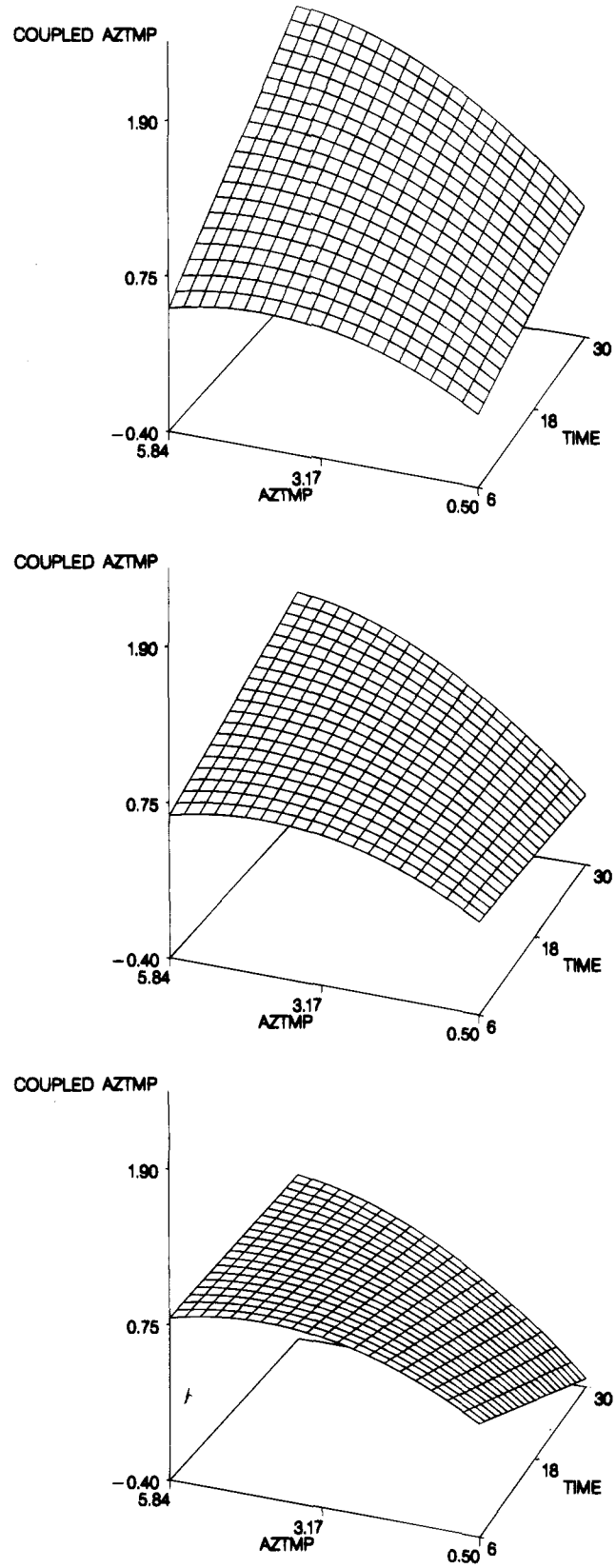

Figure 3. Response as a function of time and AZTMP amount at variable amounts of NHS (−1, 0, +1).

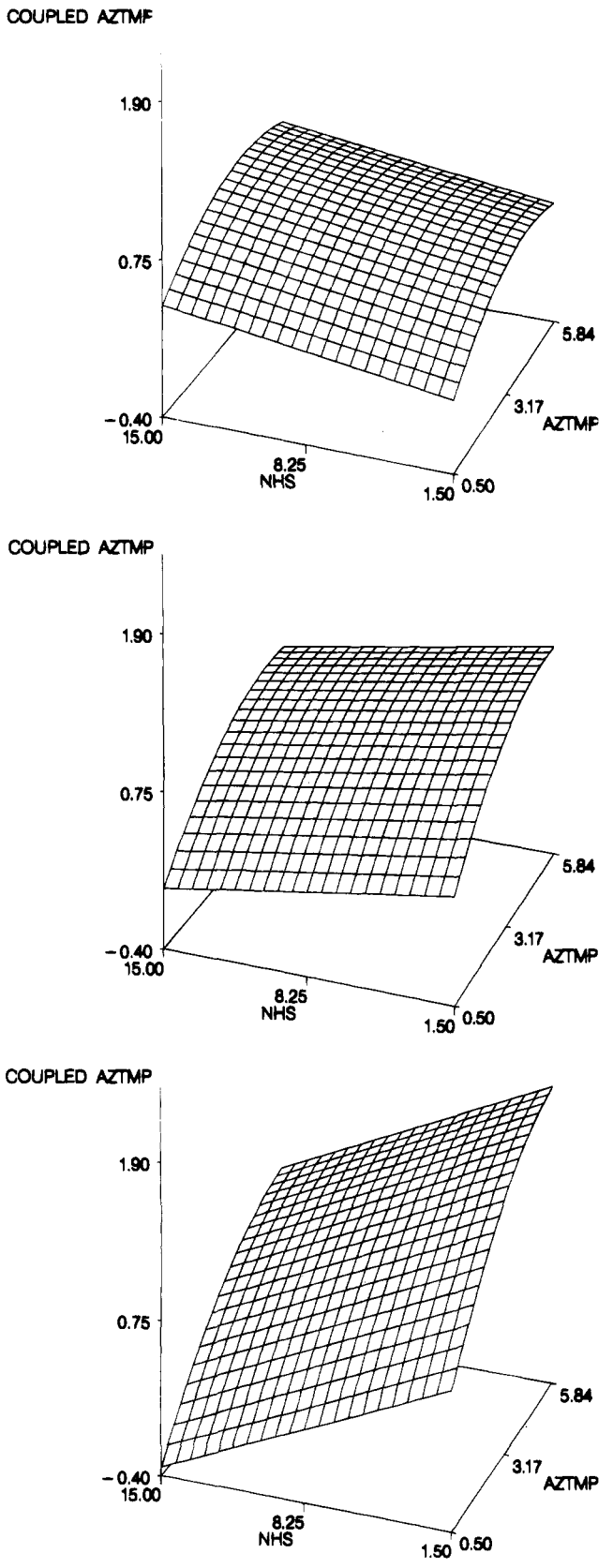

Figure 4. Response as a function of AZTMP and NHS amount at variable incubation times (−1, 0, +1).

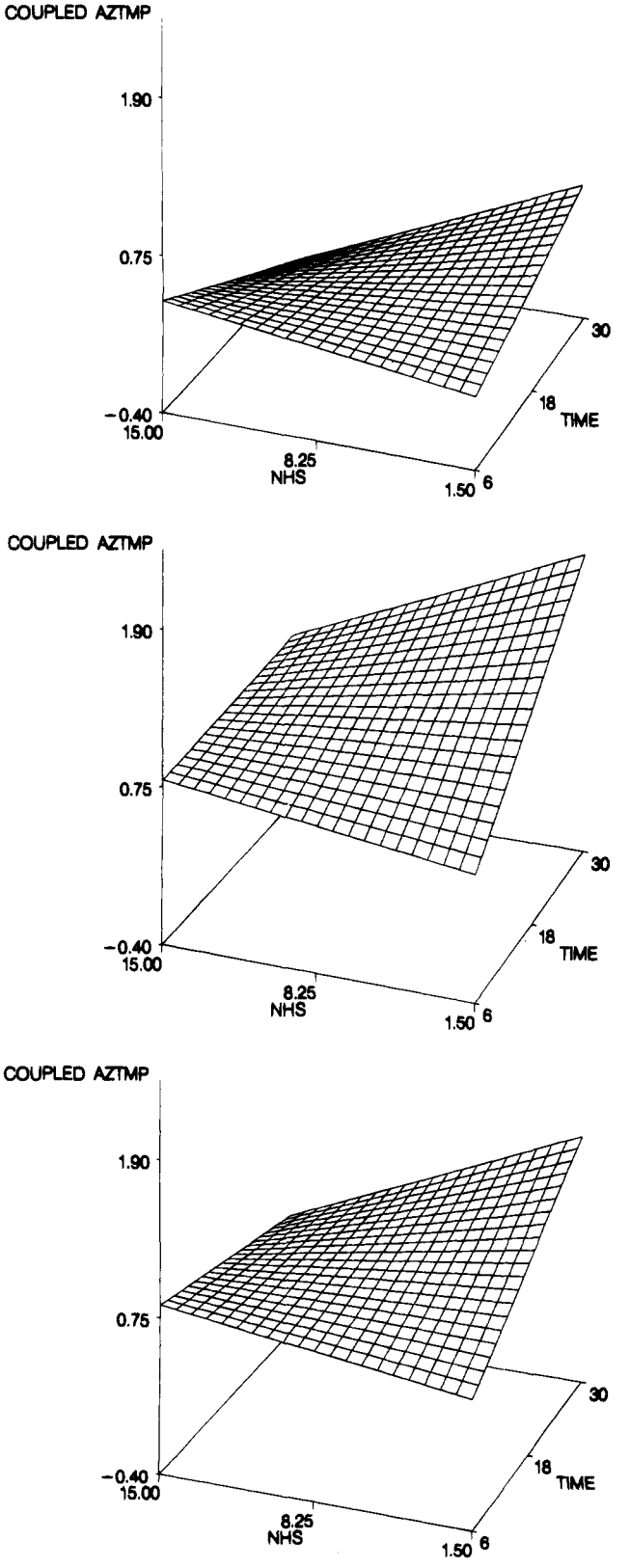

Figure 5. Response as a function of time and NHS amount at variable amounts of AZTMP (−1, 0, +1).

Polymerization of proteins can have an important influence on homogeneity of the drug-targeting preparation. Cellular recognition and uptake of the neoglycoprotein can largely differ between monomeric and polymeric fractions.[26] Therefore it is necessary to prepare drug conjugates with minimal amounts of polymeric material. Simply excluding protein during the initial carbodiimide activation of the nucleoside, as was done in a recently adapted method, did not improve the monomeric percentage of the conjugates sufficiently.[16,18] Besides, the amount of AZTMP that actually became coupled in this procedure was very low, caused by the instability of the activated intermediate

Table 3. Actually Measured Values Together with Theoretically Predicted Values

exp no.	AZTMP coupling measured	AZTMP coupling predicted	measured % monomeric compounds
1	0.31	0.53	81.5
2	1.50	1.49	65.8
3	0.79	0.84	69.6
4	0.73	0.55	71.2
5	1.07	0.86	72.5
6	1.13	1.20	51.6
7	0.14	0.04	82.9
8	0.29	0.38	46.1
9	1.41	1.40	49.6
10	0.50	0.65	79.2
11	0.22	0.14	70.6
12	0.18	0.27	67.5
13	0.88	0.85	64.9
14	0.97	0.85	67.8
15	0.78	0.85	64.6

at pH 7.5, at which it was necessary to add the protein. In the present method, in addition, NHS was used in the conjugation reaction. NHS, as a second activator of the reaction, is able to delay the hydrolysis of the activated intermediate, obtained after reaction of AZTMP and EDCI. This occurs by formation of an active ester, which is also able to couple with the ϵ-NH_2 of the lysines and the imidazole nitrogens of histidines in the albumin.[20,27] By using NHS in the first step of the coupling reaction at pH 4, the activated phosphate group was immediately converted into a more stable intermediate, and this resulted in a greatly improved coupling efficiency. Less then one-tenth of the original AZTMP concentration was needed for a coupling of one to two molecules of AZTMP to protein.

The reaction conditions were further optimized according to an experimental design. The advantages of working with a design instead of testing different reactions circumstances by trial and error lie in the extra information that is obtained. With such an experimental design, it is also possible to study the effects of variables and the interactions of the variables independent of each other.

Choice was made for a second-order design, in order to obtain sufficient information to control the conjugation reaction, on the basis of a minimal amount of reactions. The chosen design allows the use of at most three variables. Since it was the intention to minimize the amount of AZTMP in this reaction, AZTMP was one of the variables. As NHS was the new activator in this reaction, it would be interesting to determine the influence of this compound on the coupling reaction. The pilot experiments already showed that NHS could have a drastic influence on the percentage of monomeric compound; therefore NHS was the second predictor variable. The incubation time was chosen as the third variable in order to check if the influence of the incubation time on the coupling degree and on the percentage of monomeric material could be conflicting. A constant protein concentration of 20 mg/mL was used. In fact, the addition of 0.5–5.8 mg of AZTMP to 50 mg of albumin implies a fair excess of available ϵ-NH_2 groups and imidazole nitrogens over the entire range. Raising the protein concentration to a large extent would certainly lead to more polymerization, even if the total overall yield of conjugate would increase. It is unlikely that modest variations in the protein concentration around the albumin concentration as used in this study would be a crucial factor in the degree of polymerization. Through optimization of the incubation time and NHS concentration, a minimal amount of the expensive reaction component AZTMP material could be used in order to produce the required low-density conjugate with minimal polymerization, still maintaining an acceptable reaction yield.

With the chosen design, more optimal reaction circumstances for coupling AZTMP to the neoglycoprotein could be inferred. Some of the experimental data showed scattering compared to the predicted values. However this can be anticipated realizing that reaction conditions can slightly vary and that determinations of coupling degree and polymerization also exhibit some variability. It can therefore be concluded that with the present reaction conditions the chosen model predicts the optimal AZTMP coupling conditions with an acceptable reliability, that is, if the entire pattern of trends is taken into account.

The optimization procedures indicates that for an optimal coupling the AZTMP amount and the incubation time should be large but that the NHS amount should be low. That NHS, apart from stabilizing the activated AZTMP, negatively influences the AZTMP coupling is probably caused by the lower affinity of the activated ester for the reactive sites in the protein. Consequently, using larger amounts of NHS in the reaction mixture, more activated ester is formed but less coupling does occur. An almost identical phenomenon was seen in the experiments of Staros et al.,[21] in which increasing amounts of NHS resulted in a decrease in coupling of hemocyanin and glycine.

Unfortunately the experimental design could not be used for describing the influences of the three predictor variables on the percentage of monomeric material. The value of the pure error (pe), estimated from the triplicate determination, appeared to be much smaller than the rmse (which is correlated to the R^2_{adj}), and therefore a proper fit could not be reached. Probably, this coupling procedure contains at least one unknown factor that has an influence on the percentage of monomeric material.

In spite of this fact, it was quite clear that the amount of NHS significantly influenced the percentage of cross-linking between the conjugates. Reactions without NHS revealed a relatively large polymeric fraction, and reactions with a larger amount of NHS always provided the best monomeric material. This is in line with the explanation for the low coupling yield using large amounts of NHS. Since the NHS efficiently transforms the hydrolysis sensitive intermediate to a far more stable reaction product, less native EDCI is being retransformed, and this may cause less intra- and intermolecular cross-linking in the protein fraction. Although the optimal reaction circumstances found with this model are, in principle, only valid for the neoglycoprotein used in this study ($Lact_{28}$-HSA), it is likely that similar trends will be found with related sugar derivatives of HSA, that is, if sugar density and overall charge of the protein is not largely different from the lactosylated HSA.

Materials and Methods

Chemicals. Human serum albumin (HSA), consisting for more than 95% of monomeric albumin, was obtained from the Central Laboratory of the Blood Transfusion Service of the Red Cross (Amsterdam, The Netherlands). EDCI (1-ethyl-3-[3-

(dimethylamino)propyl]-3-ethylcarbodiimide) and sodium cyanoborohydride ($NaBH_3CN$) were obtained form Janssen Chimica (Beerse, Belgium). *N*-Hydroxysulfosuccinimide (NHS) was obtained from Pierce (IL). 3-Azido-2,3-dideoxythymidine (AZT) was obtained from Sigma Chemical Co. (St. Louis, MO). The 5'-monophosphate derivative of AZT (AZTMP) was 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 lactosylated neoglycoprotein ($Lact_{28}$-HSA) was synthesized by reductive amination according to Schwartz et al.[28] The disaccharide lactose was attached to HSA during a 72 h reaction in 0.25 M sodium phosphate buffer, pH 8.5, at 37 °C in the dark, in the presence of the reducing agent $NaBH_3CN$. The product was purified using an Amicon stirred cell (Amicon, Danvers, MA) equipped with a Filtron omega-membrane (Filtron Technology Corp., MA) and using Sephadex G-25 gel chromatography (Pharmacia, Uppsala, Sweden) eluted with distilled water. The purified product was lyophilized (Lyolab A, LSL Secfroid, Aclens, Switzerland) and stored at −20 °C.

The product was characterized by protein determination according to Lowry et al.[29] The degree of sugar coupling to HSA was determined using the phenol−sulfuric acid method by Dubois et al.[30] The percentage of monomeric conjugate was determined using an FPLC chromatography system equipped with a Superose-12 column (30 cm × 1 cm) (Pharmacia) and UV 280 nm detection. Elution was performed with PBS at a flow rate of 0.5 mL/min.

Synthesis of Neoglycoprotein−AZTMP Conjugates. The method used was an adaption of the method described for the coupling of the antiviral drug ara-AMP to proteins by Jansen et al.[18] In order to rule out the influences of buffer on the coupling procedure, three stirring solutions (PBS, Tris/HCl (0.1 M, pH 7.4), and distilled water) were tested first. It appeared that the stirring solution did not influence the efficiency of the coupling procedure, and therefore it was decided that the optimization reactions were performed in a PBS buffer.

Secondly other pilot experiments were performed to determine the upper and lower settings of the predictor variables' incubation time and AZTMP and NHS amounts. This was performed in order to execute the optimization experiments in the variable space of the design which were chosen to be a coupling degree of one or two AZTMP molecules to HSA and a high amount of monomeric conjugates. These experiments revealed that variance in NHS amount should be in between 1.5 and 15 mg and AZTMP amount in between 0.5 and 6 mg, both in the reaction volume used, and that the incubation time could be varied from 6 to 30 h.

The optimization reactions were performed as follows (see Figure 2): 50 mg of EDCI and variable amounts of NHS were dissolved in 0.5 mL of H_2O. This was added to a solution of variable amounts of AZTMP in 0.5 mL of H_2O. The pH was set to 4 with 1 N HCl, and the mixture was stirred for 15 min at room temperature. Hereafter the pH was adjusted to 7.5 with 1 N NaOH, and subsequently 50 mg of $Lact_{28}$-HSA in 1.5 mL of PBS was added dropwise. After incubation for variable hours in the dark at room temperature, the sample was purified using Sephadex G-75 gel filtration (Pharmacia). Elution was performed with distilled water. The protein fraction was lyophilized and stored at −20 °C. The conjugates were characterized as described above (protein, sugar, and percentage of monomeric conjugate). The amount of AZTMP coupled to the neoglycoprotein was determined using the acid hydrolysis/HPLC method according to Molema et al.[31] The HPLC analyses were performed on a Waters liquid chromatograph (Waters Assoc., Milford, ME) consisting of a 510 pump, a U6K injector, and a model 440 UV detector set at 254 nm and operating at 0.02 AUFS. A μBondapak C18 column (Water Assoc.; 30 cm × 3.9 mm i.d.) was used guarded with a μBondapak C18 guard−pak precolumn (Waters Assoc.).

Optimization of the Coupling Method. Optimization was performed according to a Box−Behnken experimental design, which is a second-order design, requiring only three levels.[24] This Box−Behnken experimental design is a combination of a factorial design, varied on two levels, and an incomplete block design. Polynomial models were used to describe the effects of the predictor variables on the response within the design space. Effects caused by interactions between the predicted variables were also taken into account. The response surfaces were constructed from the determined empirical model.

The values of the predictor variables used in these experiments are listed in Table 1. The model parameters used to describe the effects of the variables on the responses were based on the scaled values for the variables. These latter variables are also listed in Table **1**. With this design it was sufficient to perform each experiment in singular, except for the center point of the design (0,0,0) which was performed in triplicate, to get an estimate of the pure error of the measurements. The variance in the center point measurements provides a reference for detecting the potential lack of fit. The experiments were performed randomly to extract systematic effects of uncontrollable factors on the responses. Conditions for each experiment are given in Table 2.

Acknowledgment. This work was supported by Grant No. 900-521-148 from the Dutch Organization for Scientific Research (NWO) and the council for Health Research (RGO).

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JM940341L