Synthesis of 2′-Deoxyuridine and 5-Fluoro-2′-deoxyuridine Derivatives and Evaluation in Antibody Targeting Studies

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Derivatives of 2′-deoxyuridine and of the anticancer agent 5-fluoro-2′-deoxyuridine (FdUR) were linked indirectly via a human serum albumin carrier (HSA) to the murine antiosteosarcoma monoclonal antibody 791T/36. Starting from the 2′-deoxyuridines **1a** and **1b**, the new nucleosides containing 5′-succinamic acid **7** and 5′-maleamic acid **8** spacers were synthesized from the key intermediate 5′-aminonucleoside **4**, and the ribofuronamidobenzoic acid **13** from ribofuranuronic acid **10**. These nucleosides were linked via their spacer functionality to HSA. High molar substitution ratios (MSR: moles of drug/mole of HSA) of 25–40 for these derivative–HSA conjugates were achieved. All derivatives were less cytotoxic than the parent drug against both antigen positive osteogenic sarcoma 791T and antigen negative bladder carcinoma T24 cell lines; no IC_{50} was achieved with any derivative against 791T cells. The fluorodeoxyuridine–HSA conjugates were then further linked via a stable thioether bond to the mouse monoclonal antibody 791T/36. The optimum fluorinated 5′-succinamic acid immunoconjugate exhibited an IC_{50} of 1 μM against 791T and T24 cells, slightly better than that of fluorodeoxyuridine. The unconjugated derivative **7** was much less cytotoxic than immunoconjugate, with an IC_{50} of 62 μM on T24 cells, and failed to reach 50% inhibition of 791T cell growth at 290 μM concentration. Derivative **7**–HSA conjugate was 10-fold less cytotoxic than the immunoconjugate against both cell lines. Immunoconjugates synthesized with the other 5-fluoro derivatives were unable to effect 50% inhibition of growth of cell lines. Nonfluorinated derivatives and their HSA conjugates and immunoconjugates exhibited no cytotoxicity.

Introduction

The fluoropyrimidine antimetabolites 5-fluorouracil (FU)[1] and 5-fluoro-2′-deoxyuridine (FdUR)[2] are widely used antineoplastic agents. They indirectly disrupt DNA synthesis through the powerful inhibition of the enzyme thymidylate synthase by fluorodeoxyuridine monophosphate, a metabolite of the drugs. The enzyme supplies the cell with its only de novo source of thymidine.[3,4] In addition, fluoropyrimidines can also exert cytotoxic effects by incorporation of their triphosphate (deoxy)nucleotides into DNA and RNA.[5,6] However, these drugs suffer from a number of disadvantages which include high toxicity, rapid blood clearance, low cytotoxicity, and drug resistance. Such problems may be circumvented by covalent coupling of the drug to antitumour antibodies. An antibody-mediated targeting approach has been investigated with other cytotoxic agents such as methotrexate,[7,8] daunomycin,[9] and fluorouridine.[10,11]

Coupling of FdUR to rabbit serum albumin has been previously reported using water-soluble carbodiimide.[12] In this study, though a molar substitution ratio (MSR) of 28 was demonstrated, efficiency of drug incorporation was reported to be very low. Fluorouridine[10,11] has also been coupled by periodate cleavage of the ribose ring to generate aldehyde groups which were then further reacted with hydrazide-derivatized dextran. Chemically, this method offered fewer possibilities for incorporation of spacer moieties which may produce an improved release of active drug at the appropriate intracellular site. The present study describes the feasibility of synthesizing novel nucleoside derivatives for antibody-directed targeting.

In vitro, FdUR frequently shows superior cytotoxicity to 5-fluorouracil, but in vivo no advantage can be detected,[13] thus offering a potential for improvement. FdUR has two sites, 5′- and 3′-hydroxyl, which can be used for chemical manipulation. The 5′-linkage point has a number of advantages. Derivatives retaining an intact 3′-hydroxyl have shown greater activity in cellular systems, which is thought to be due to recognition by kinases and phosphorylases necessary for activation processes.[14] Also, the 5′-primary alcohol is chemically more reactive, and though not optimal for coupling to protein, can be readily transformed to a more reactive functionality. The latter can be employed to prepare derivatives with carefully chosen spacers to improve coupling efficiency and eventual release of active drug.[15]

FdUR derivatives with appropriate spacers have two potential advantages: increased enzyme accessibility and cleavage points at either the ribose–protein linkage or at the 1-1′ pyrimidine–ribose bond to give active drug. 5-Fluoro-2′-deoxyuridine has a relatively low cytotoxicity compared to other anticancer agents,[16] and so a multivalent carrier, HSA, was used to maximize the MSR.[8,16] It was thought that conjugation of FdUR would probably result in release of 5-fluorouracil to the cytoplasmic target.

We describe here the synthesis of 2′-deoxyuridine and FdUR derivatives and their indirect linkage to 791T/36 mAb via an HSA carrier. Cytotoxicities of derivatives and selected conjugates in vitro are also reported.

Results and Discussion

Chemistry. Our earlier attempts to synthesize a nucleoside with a simple 5′-glycine spacer by nucleophilic

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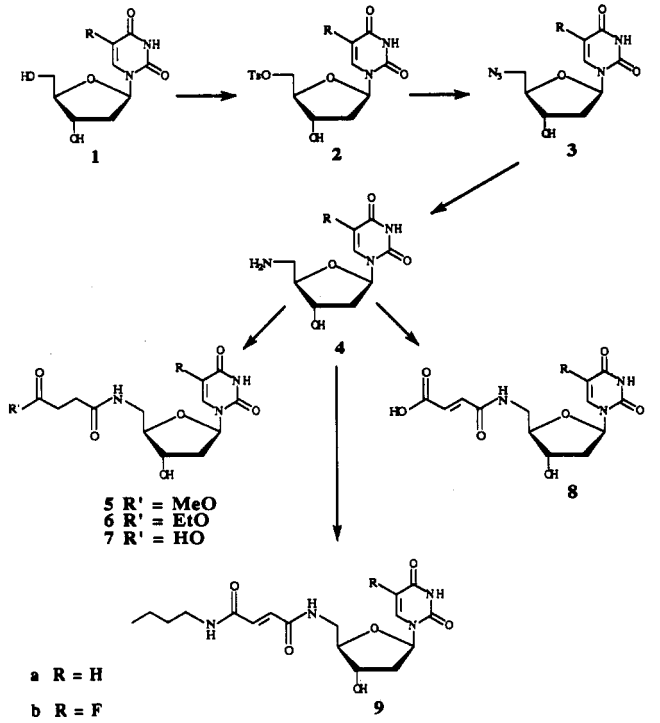
Scheme I



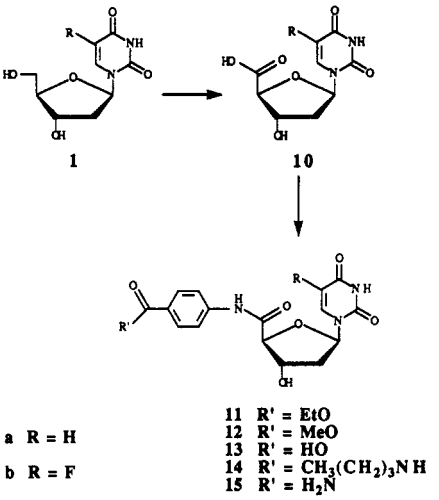
displacement of the 5′-tosyloxy group of **2** with glycine benzyl ester were unsuccessful. A diethyl azodicarboxylate mediated reaction[17] and the phosphorazo method of Grassman and Wünsch[18] (which apparently increases the nucleophilicity of the amine) proved equally barren. We were also unable to produce the potentially useful 5′-cyano, or the 5′-di-*tert*-butyl malonate and 5′-*p*-cyanophenoxy derivatives[19] from **2**. Reactions yielded starting material or breakdown products. This last reaction was particularly disappointing as Pischel and co-workers[19] successfully prepared 5′-*O*-(4-cyanophenyl)-2′,3′-isopropylideneuridine and its corresponding imido ester which was successfully linked to bovine serum albumin.

Conversion of the 5′-hydroxyl to an amine is well documented for both nonfluorinated[20] and fluorinated[21] deoxyuridines. The amine can be used either to link directly to protein or can be alkylated to produce a nucleoside with a 5′-spacer. Synthesis of **4b** was based on Lin and Prusoff's[21] conversion of FdUR to its 5′-amino derivative. In contrast to these authors the conversion of the fluorinated 5′-azide **3b** to the amine **4b** was effected smoothly by catalytic hydrogenation in 80–90% yield without defluorination. We were thus able to dispense with the much less convenient triphenylphosphine method for which we obtained only 50–60% of the target compound **4b** (Scheme I).

Reaction of 5′-aminonucleoside with methyl and ethyl succinyl chloride gave the corresponding 5′-methyl (**5**) and 5′-ethyl (**6**) succinamic esters, which were purified by flash chromatography on silica gel. Alkaline hydrolysis of the esters followed by treatment with Amberlite IR 120 resin (H^+ form) generated free acid **7**. Though we were unable to crystallize **7a** or **7b**, they were found to be analytically pure (NMR, TLC, FAB mass spectrum, and microanalysis). Reaction of **4** with succinic anhydride produced **7** directly, but the reaction was not clean and we were unable to isolate the 5′-succinamic acid in a pure state.

The foregoing reactions did not readily go to completion or yield clean products, and this suggested to us that the use of more rigid spacer molecules may be advantageous both in the synthesis of derivatives and their eventual conjugation to protein. This led to the use of maleamic acid as a spacer. Pure 5′-maleamic acid **8** was obtained by direct reaction of **4** with maleic anhydride. The free acid, sparingly soluble in water, was isolated by careful neutralization of a solution of the sodium salt with dilute hydrochloric acid.

Scheme II



Considering that on conjugation of derivatives to protein the predominant linkage would be via lysyl side chains, the 5′-*N*-butylmaleamide **9** was required as the nearest spectral mimic for estimation of MSRs of maleamic acid derivative conjugates. Analytically pure nonfluorinated test compound **9a** was obtained from **4a** via succinimido ester using dicyclohexylcarbodiimide (DCCI). A similar reaction with the 5-fluoro analogue **4b** was unsuccessful, giving a complex mixture. However, fluorinated 5′-*N*-butylmaleamide **9b** was obtained from **8b** via a mixed anhydride reaction.

We further considered that the ribofurauronaldehyde would be a useful intermediate which could be reacted with a variety of Wittig reagents to produce new derivatives. Unsuccessful attempts were made to convert the 5′-position of 3′-*O*-acetyl-2′-deoxyuridine[22] to its aldehyde derivative by the Pfitzner Moffatt[23–25] method (DMSO, DCCI in the presence of anhydrous phosphoric acid). These workers have oxidized 3′-*O*-acetylthymidine to its aldehyde derivative. Though the presence of the deoxyuridine aldehyde was proved by isolation of the (2,4-dinitrophenyl)hydrazone (3′-ol) derivative, we were unable to isolate the aldehyde and this route was abandoned.

Complete oxidation of the 5′-hydroxymethylene resulted in the formation of a ribofuronuronic acid **10**, a reaction that has been documented with both deoxyuridine[26] and its fluorinated analogue.[27] Reaction of **10a** and its 3′-*O*-acetyl derivative with methyl, ethyl, and *tert*-butyl 4-aminobutyrate failed to give the desired 5′-spacer ester nucleosides; an inseparable mixture always resulted. In analogy to the formation of the 5′-maleamic acid derivative, the more rigid *p*-aminobenzoic acid was selected as a potential spacer. Reaction of **10a** with ethyl or methyl 4-aminobenzoate in the presence of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (ECDI) in a water–dioxan solvent mixture resulted in the formation of *p*-ethoxy and *p*-methoxy ribofuronuronamidobenzoate nucleosides (**11**, **12**; see Scheme II). Hydrolysis of these esters gave the acid **13a**. The fluorinated derivative **5b** was prepared from

the corresponding acid **10b**. We were unable to obtain the spectral mimic *N*-butyl derivative **14**, but the easily accessible benzamide derivative **15** was used as an alternative.

Protein Conjugation. Linkage of 5-fluorodeoxyuridine to HSA using ECDI or the alternative coupling agent carbonyldiimidazole resulted in low substitutions (MSR < 3). The relatively low substitution obtained by us was probably due to use of a lower input of FdUR and EDCI than used by Fiume.[12] However, even with this lower level of reagents the products obtained were highly polymerized and unsuitable for conjugation to antibody. These brief experiments confirmed the difficulty of coupling the unmodified drug and the necessity of appropriate derivatives for conjugation.

For conjugation of derivatives, it was noted that some procedures were more consistent than others in efficiency of conjugation and also that the level of conjugation obtained with any one method was often very sensitive to small changes in the procedure. This necessitated optimization of conjugation procedures for each individual derivative.

Use of 5-fluororibofuranuronic acid (**10b**) produced a maximum substitution of 11 using a dual-step water-soluble carbodiimide method based on that of Davis and Preston.[28] MSR's of up to 9 were achieved coupling fluorinated 5'-aminonucleoside **4b** (single-step ECDI[8]), but that was dramatically increased to 47 with the use of succinylated HSA.[29] However, the latter conjugates proved unstable and also presented problems with estimation of the substitution ratio due to the heterogeneity of nucleoside linkages to protein.

The spacer–nucleosides permitted reactions to proceed in a more efficient and controllable manner, resulting in highly substituted products which were more stable. Three types of HSA conjugates were synthesized: 5'-succinamic acid (H1), 5'-maleamic acid (H2), and ribofuranuronamidobenzoic acid (H3).

Four methods were assessed for coupling spacer derivatives to carrier: single- and dual-step water-soluble carbodiimide, activated ester using *N*-hydroxysuccinimide in the presence of DCCI,[7] and a mixed anhydride procedure based on that of Pischel and co-workers[30,31] who linked 5'-*O*-(carboxymethyl)uridines to bovine serum albumin. Generally the water-soluble carbodiimide methods were most effective on the more hydrophilic 5'-succinamic acid derivative and the activated ester method with the more hydrophobic benzoic acid derivative **13b**. The mixed anhydride method gave inconsistent products and so was not routinely used.

Nonfluorinated 5'-succinamic acid derivative **7a** consistently gave MSR's up to 25 (ECDI single and dual step) and up to 38 for the fluorinated analogue (dual step). The 5-fluoro-5'-maleamic acid derivative **8b** was no more readily substitutable; MSRs up to 25 were achieved. Expectations that the more rigid spacer may also aid conjugation to protein were not realized. MSR's up to 29 were achieved with the fluorinated and 18 with the nonfluorinated ribofuranuronamidobenzoic acid (**13**) derivatives with the activated ester method.

All the drug–HSA conjugates synthesized (H1–H3) above were then coupled to 791T/36 mAb to give antibody–carrier–drug conjugates (AH1–AH3). Following the method of Garnett and Baldwin in their synthesis of methotrexate-HSA-791T/36 conjugates, we linked antibody acetylated with *N*-(iodoacetyl)succinimide to drug–HSA conjugates which had been selectively reduced at the free cysteine residue.[8] The aforementioned authors used selective precipitation to purify antibody conjugate from free antibody. We were only able to employ this separation technique with the ribofuranuronamidobenzoic acid conjugate (AH3). Other conjugate types were purified by gel filtration chromatography. Even the use of small quantities of heterobifunctional reagent in conjugate synthesis resulted in the formation of higher molecular weight material containing more than one antibody and HSA–drug moieties. Additional gel filtration and hydroxylapatite separations were employed to select a low molecular weight fraction and to remove free antibody. The former technique was more effective but still left some early preparations with up to 70% contaminating free antibody. This figure improved in later batches.

Table I. Cytotoxicity of 5-Fluoronucleoside Primary Derivatives

derivative	cell line	IC_{50} [derivative] μg/mL	IC_{50} [derivative] μM
5-FU	C170	0.3	2.0
5-FdUR	C170	0.1	0.4
5'-aminonucleoside **4b**	C170	26	107
ribofuranuronic acid **10b**	C170	10	41

Biological Evaluation. Antibody binding activity to antigen positive 791T cells, as measured by flow cytofluorimetry,[32] varied with the type of conjugate; up to 57% binding activity was seen for the fluorinated 5'-succinamic acid (AH1), 21% for the 5'-maleamic acid (AH2) series, and 15% for the ribofuranuronamidobenzoic acid (AH3) antibody conjugates. It is noted that these preparations contain varying amounts of free antibody (see the Experimental Section). All of this antibody has also survived the conjugation procedure and is substituted with heterobifunctional reagent. It is not clear whether the free antibody has full activity, some activity, or the same activity as the conjugate. Consequently no correction has been made to these figures with respect to the contaminating free antibody. While these levels of binding activity may appear low, they are similar to that seen with potent and selective 791T/36–HSA–methotrexate conjugates.[7,8]

The key intermediates 5'-aminonucleoside **4b** and ribofuranuronic acid **10b** were assessed using an in vitro [^{75}Se]selenomethionine uptake assay[33] on the recently derived C170 colorectal carcinoma cell line which also shows a low 791T/36 expression.[34] The colorectal line was chosen for this early work, due to the widespread use of fluoropyrimidines in the treatment of this neoplasm. Cytotoxicity data for these intermediates are given in Table I. Cytotoxicities of the remaining FdUR derivatives, derivative–HSA, and derivative–HSA–antibody conjugates were assayed on antigen positive 791T osteosarcoma and control T-24 bladder carconima cells having only 3–5% antigen expression. Data for the other derivatives are presented in Table II and for selected conjugates in Table III.

The primary derivatives **4b** and **10b** were 50–150-fold less cytotoxic than the parent compound on the colorectal carcinoma cell line which was very sensitive to fluoropyrimidines. The secondary nucleoside derivatives **6b**, **7b**, **8b**, **9b**, **11b**, and **13b** were at least 1 order of magnitude less cytotoxic than the parent drug on the 791T and T-24 cell lines used as the experimental and control pair for the remaining cytotoxicity tests. While the paired cell lines

Table II. Cytotoxicity of 5-Fluoronucleoside Secondary Derivatives

derivative	cell line	IC_{50} [derivative] μg/mL	IC_{50} [derivative] μM
5-FU	791T	0.7	5.5
	T24	0.5	4.1
5-FdUR	791T	0.8	3.2
	T24	1.0	4.1
5′-ethyl succinamate **6b**	791T	≫100[a]	≫280
	T24	≫100	≫280
5′-succinamic acid **7b**	791T	>100[b]	>290
	T24	21	62
5′-maleamic acid **8b**	791T	~110	~320[c]
	T24	79	231
5′-*N*-butylmaleamide **9b**	791T	≫100	≫300
	T24	≫100	≫300
ethyl ribofuranuronamidobenzoate **11b**	791T	≫100	≫300
	T24	≫100	≫300
ribofuranuronamidobenzoic acid **13b**	791T	≫100	≫300
	T24	100	263

[a] ≫, less than 20% inhibition achieved. [b] >, 20–40% inhibition achieved. [c] 49% inhibition achieved at 100 μg/mL derivative.

Table III. Cytotoxicity of 5-Fluoronucleoside Conjugates

conjugate	cell line	IC_{50} [derivative] μg/mL	IC_{50} [derivative] μM
5′-Succinamic Acid (Type H1: MSR 33)			
H1.11	791T	7.1	20.6
	T24	5.7	16.6
AH1.11	791T	0.4	1.1
	T24	0.4	1.1
5′-Maleamic Acid (Type H2: MSR 26)			
H2.8	791T	≫10[a]	≫30
	T24	≫10	≫30
AH2.8	791T	≫0.5	≫1.5
	T24	>0.5	>1.5
Ribofuranuronamidobenzoic Acid (Type H3: MSR 28)			
H3.2	791T	≫3.0	≫7.9
	T24	≫3.0	≫7.9
AH3.2	791T	≫1.0	≫2.6
	T24	>1.0[b]	>2.6

[a] ≫, less than 20% inhibition achieved. [b] >, 20–40% inhibition achieved.

showed similar sensitivity to the parent drug and FU, T-24 cells appeared more sensitive than 791T to all the derivatives. An IC_{50} was not achieved with any derivative on the osteosarcoma cell line. The 5′-succinamic acid derivative showed the highest cytotoxicity, with the 5′-maleamic acid and ribofuranuronamidobenzoic acid derivatives both about 4-fold less cytotoxic. The other derivatives, which were prepared as spectral mimics, demonstrated no appreciable cytotoxicity. Similarly 791T/36 antibody has no cytotoxic activity.[35]

Only 5′-succinamic acid conjugates (H1.11 and AH1.11, Table II) were sufficiently cytotoxic to achieve 50% inhibition of cells. The immunoconjugate AH1.11 was approximately equicytotoxic to both cell lines with an IC_{50} of 1.1 μM, slightly more cytotoxic than that of FdUR and FU (5.5–3.2 μM). The corresponding 5′-succinamic acid–HSA conjugate (H1.11) was 4–6-fold less cytotoxic than the free base and nucleotide. The small increase in cytotoxicity of the antibody targeted drug compared to the free drug is especially noteworthy. There are few similar reports in the literature,[8,36] most drugs becoming much less active on conjugation to macromolecules.[15]

The cytotoxicity of the conjugate (AH1.11) was 16–20-fold more cytotoxic than the corresponding HSA–derivative (H1) preparation but showing no significant specificity on conjugation of antibody. It has been shown previously that conjugation of 791T/36 antibody to HSA–methotrexate greatly increases the cytotoxicity of the conjugate[7,8] and that these results can be explained by the increased uptake through endocytosis of the antibody (or conjugate).[37] While T-24 cells do express a low level of antigen, this level is not as effective for endocytosis as the higher level on 791T cells. It is not yet clear, therefore, why the antibody conjugate AH1.11 has the same cytotoxicity on both 791T and T-24 cell lines and why it is greater than that for the HSA conjugate H1.11.

There are a number of factors which may have reduced the specificity in these conjugates. The amount of antibody required to achieve IC_{50} was above saturation level thus increasing the nonspecific uptake of conjugate. This point is particularly important in the assay employed where cells are continuously incubated with drug or conjugate. Also, the presence of contaminating antibody in the preparation may have a 2-fold effect. Firstly, to raise the "apparent" antibody binding activity and secondly to decrease conjugate cytotoxicity against the antigen-bearing 791T cells by competing for antigen binding sites. It is worth noting that many direct conjugates do not show any evidence of specificity in this continuous incubation type of assay, but a difference between antigen negative and positive cells is seen when assayed by a short exposure of cells to conjugate. Unfortunately these assays require considerably higher drug concentrations which was not possible here due to the limited availability of derivative and conjugate.

The protein-bound 5′-succinamic acid derivative was 20–50-fold more cytotoxic than the corresponding nucleoside derivative and even more cytotoxic than the corresponding spectral mimic with the blocked spacer, suggesting that the conjugation to a macromolecule confers a new mechanism of delivery. These conjugates would be expected to act via a lysosomotropic mechanism.[38] Studies by Garnett et al.[37,39] have indicated the involvement of lysosomes in the cytotoxicity of 791T/36–HSA–methotrexate conjugates. It is presumed that the 5-fluoro-5′-succinamic acid nucleoside **7b** conjugates behave similarly and are degraded in the lysosomes to release FU intracellularly. Release of active drug derivative intracellularly or FU at the cell surface followed by uptake cannot, however, be ruled out at present.

None of the nonfluorinated derivatives or conjugates showed any cytotoxicity, demonstrating that cytotoxicity was mediated by the activity of the metabolic inhibitor.

The other two immunoconjugates, AH2 and AH3, both showed some evidence of greater cytotoxicity than their respective HSA–derivative conjugates (H2 and H3), although IC_{50} was not reached in either case. The very poor cytotoxicity of these conjugates probably results from a reduced intracellular release of active drug from the maleamic acid and ribofuronamidobenzoic acid **13b** nucleoside HSA conjugates compared to the succinamic acid derivative. Insufficient product to allow an IC_{50} to be reached prevents a firm conclusion on the relative effectiveness of the immunoconjugate compared to the derivative HSA–conjugate, although the factors discussed above will also apply to these conjugates.

The cytotoxicity of the 5′-succinamide carrier conjugate was of similar potency to the rabbit serum albumin–FdUR conjugates of Barbanti–Brodano.[12] The 5′-succinamide immunoconjugate was also comparable to the fluorouridine conjugates reported by Hurwitz and co-workers.[10,11] The

latter authors linked periodate oxidized fluorouridine via hydrazide-modified dextran to an antileukemia IgM recognizing B-leukemia 38B cells. Their immunoconjugate was more potent than carrier conjugate and similar to FU, but not as potent as their starting material fluorouridine.

Due to the limited availability of derivative, drug–carrier and antibody conjugate preparations were performed on a small scale. Thus the final purified and concentrated preparations only permitted cytotoxicity testing over a limited range. With an improved synthesis and purification one would expect an increase in binding, cytotoxicity, and selectivity of antibody conjugates. Even with these improvements, cytotoxicity would probably only be modest compared with the high values obtained with other chemoimmunoconjugates and immunotoxin conjugates.[40] Further investigation of the correct spacer groups for effective release of drug appears to be necessary for development of fluoropyrimidine conjugates,[37] but nevertheless, we feel the present study gives some valuable indications for future work.

There has also been a resurgence of interest in nucleoside derivatives because of their potential in antiviral therapy. Coupling of antiviral nucleoside derivatives to neoglycoproteins as drug carriers has already been reported.[41] Our studies may contribute to the development of better delivery systems for these agents.

Experimental Procedures

Chemistry. Melting points were determined on a Reichert melting point apparatus type 7905 using a Levell type DTIK and are uncorrected. Thin-layer chromatography (TLC) was performed on plastic plates precoated (0.25 mm) with silica gel 60/F_{254} (Merck), and preparative TLC on 25- × 25-cm glass plates coated with silica gel GF_{254}. Flash chromatography was conducted under medium pressure of nitrogen in 1.8- and 2.5- × 80-cm flash columns using Merck silica gel 60 [0.040–0.063 μm (230–400-mesh ASTM)] and activity II silica gel with UV indicator. UV data was recorded on a Pye Unicam SP400 UV/vis spectrophotometer, and IR spectra were measured on a dual-beam Perkin-Elmer 257 grating IR spectrophotometer. ^{1}H NMR spectra were measured on a Varian EM390 (90 MHz) spectrometer using TMS as an internal standard except for the compounds dissolved in D_2O where sodium 3-(trimethylsilyl)propanesulfonate was used as an external standard. FAB-MS analysis was performed on an AEI MS902 spectrometer. All evaporations were carried out at circa 15 mmHg using a rotary evaporator. The following TLC systems were used: (1) chloroform–methanol (6:1), (2) acetic acid–butanol–water (5:2:3), (3) chloroform–methanol–acetic acid (12:2:1), (4) ethyl acetate–acetic acid (6:1), (5) chloroform–ethanol (6:1).

Unless otherwise stated, all reactions and operations involving HSA and mAb and their conjugates were carried out in phosphate buffered saline (PBS) (0.05 M phosphate buffer pH 7.2 containing 0.1 M sodium chloride). UV spectra of derivatives were also determined using this buffer to allow calculation of molar ratios of drug to protein.

5′-*O*-Tosyl-2′-deoxyuridine (2a). To an ice-cold stirred solution of 2′-deoxyuridine (3.60 g, 15.8 mmol) in anhydrous pyridine (15 mL) was added *p*-toluenesulfonyl chloride (3.03 g, 15.8 mmol), and the reaction mixture was left at 4 °C for 4 h. Solvent was removed under reduced pressure (at 40 °C), the resultant syrup was washed with ether (5 × 30 mL) and water (5 × 60 mL) and digested with chloroform (150 mL), and the insoluble product was collected by filtration and recrystallized (ethanol) to afford the title compound as beige crystals (2.9 g). A second crop (0.61 g) was obtained by slow addition of petroleum ether (bp 40–60 °C) to the mother liquor. The combined yield (3.51 g, 70%) was dried overnight in a desiccator: mp 154–156 °C (lit.[42] mp 156–157 °C); ν_{max} (KBr)/cm^{-1} 3480 (OH), 1720 and 1670 (C=O); *m/z* (FAB) 383 [M$^+$ + 1, 41], 271 (3), 193 (6), 139 (10), 113 (30), 81 (100); δ [$(CD_3)_2SO/D_2O$] 7.70 (2H, d, *J* = 7.5 Hz, Ar-H), 7.38 (3 H, d, *J* = 7.5 Hz, Ar-H and 6-H), 6.03 (1 H, t, *J* = 6 Hz, 1′-H), 5.55 (1 H, d, *J* = 7.5 Hz, 5-H), 4.13 (3 H, m, 3′-H and 5′-H_2), 3.86 (1 H, m, 4′-H), 2.40 (3 H, s, Ar-CH_3), 2.12 (2 H, t, *J* = 6 Hz, 2′-H_2).

5-Fluoro-5′-*O*-tosyl-2′-deoxyuridine (2b). Use of (FdUR) (**1b**) in the above procedure afforded the 5-fluoro analogue as beige crystals which was purified by recrystallization from ethanol (277 mg, 63%): mp 159.5–162 °C (lit.[20] mp 163–164 °C); ν_{max} (KBr)/cm^{-1} 3490 (OH), 1720 and 1680 (C=O); δ [$(CD_3)_2CO/D_2O$] 7.80 (1 H, d, *J* = 6 Hz, 6-H), 7.65 (2 H, d, *J* = 7.5 Hz, Ar-H_2), 7.43 (2 H, d, *J* = 7.5 Hz, Ar-H_2), 6.17 (1 H, td, *J* = 6 and 1.5 Hz, 1′-H), 4.32 (3 H, m, 3′-H and 5′-H_2), 4.07 (1 H, m, 4′-H), 2.43 (3 H, s, Ar-CH_3), 2.25 (2 H, m, 2′-H).

5′-Azido-2′,5′-dideoxyuridine (3a). Lithium azide (0.25 g, 5.1 mmol) was added to a stirred solution of 5′-*O*-tosyl-2′-deoxyuridine (**2a**) (2.5 g, 6.5 mmol) in *N*,*N*-dimethylformamide (DMF) (10 mL) at 60–70 °C (oil bath). After 3 h a further amount of lithium azide (0.25 g) was added and the reaction stirred overnight at 50 °C. After cooling, the solution was filtered, and the filtrate was evaporated in vacuo (oil pump) at 50 °C; the resultant solid was dissolved in 50% aqueous ethanol (20 mL), stirred for 45 min with Zerolit 325 resin (H^+ form, 5.0 g) to remove lithium ions, and filtered. After complete removal of solvent the resultant semisolid was crystallized and recrystallized from ethanol–ether at low temperature to afford 5′-azido-2′,5′-dideoxyuridine (**3a**) (0.96 g, 60%): mp 138–140 °C (lit.[42] mp 139.5–140.5 °C); ν_{max} (KBr)/cm^{-1} 3480 (OH), 2105 (azide) and 1680 (C=O); δ [$(CD_3)_2CO/D_2O$] 7.77 (1 H, d, *J* = 7.5 Hz, 6-H), 6.30 (1 H, t, *J* = 6 Hz, 1′-H), 5.72 (1 H, d, *J* = 7.5 Hz, 5′-H_2), 4.40 (1 H, q, *J* = 4.5 Hz, 3′-H), 4.02 (1 H, q, *J* = 4.5 Hz, 4′-H), 3.63 (2 H, d, *J* = 4.3 Hz, 5′-H_2), 2.33 (2 H, t, *J* = 6 Hz, 2′-H).

5-Fluoro-5′-azido-2′,5′-dideoxyuridine (3b). Use of **2b** in the foregoing procedure yielded analytically pure product **3b** as colorless crystals (65 mg, 60%): mp 133–135 °C (lit.[20] mp 134–135 °C); ν_{max} (KBr)/cm^{-1} 3480 (OH), 2105 (azide), 1725 and 1670 (C=O); δ [$(CD_3)_2CO/D_2O$] 7.88 (1 H, d, *J* = 6 Hz, 6-H), 6.28 (1 H, td, *J* = 6 and 1.5 Hz, 1′-H), 4.40 (1 H, q, *J* = 4.5 Hz, 3′-H), 4.00 (1 H, q, *J* = 4.5 Hz, 4′-H), 3.67 (2 H, d, *J* = 4.5 Hz, 5′-H_2), 2.32 (2 H, dd, *J* = 6 and 4.5 Hz, 2′-H_2).

5′-Amino-2′,5′-dideoxyuridine (4a). (i) Via Catalytic Hydrogenation. A solution of 5′-azido-2′,5′-dideoxyuridine (**3a**) (0.3 g, 1.19 mmol) in ethanol (50 mL) was hydrogenated (3 h) in the presence of 10% Pd–C catalyst (40 mg). The progress of the reaction was followed by TLC (R_f 0.01, system 1; 0.67, system 2). The catalyst was removed by filtration through a Celite pad and the filtrate evaporated to dryness affording 5′-amine **4a** as a pale solid (0.243 g, 90%). A small sample was recrystallized from ethanol–water–ether: mp 210 °C dec [lit.[42] mp 230 °C dec]; λ_{max} (PBS) 261 nm (log ε 3.98); ν_{max} (Nujol)/cm^{-1}, 3520 (OH) and 1730 (C=O); δ (D_2O) 7.46 (1 H, d, *J* = 7.5 Hz, 6-H), 6.05 (1 H, t, *J* = 7.5 Hz, 1′-H), 5.68 (1 H, d, *J* = 7.5 Hz, 5-H), 4.23 (1 H, q, *J* = 4.5 Hz, 3′-H), 3.86 (1 H, m, 4′-H), 2.91 (2 H, m, 5′-H_2), 2.26 (2 H, t, *J* = 4.5 Hz, 2′-H_2).

(ii) Via the Triphenylphosphine Method.[21] Triphenylphosphine (1.038 g, 3.96 mmol) and 5′-azide **3a** (0.5 g, 1.97 mmol) were stirred together in pyridine (10 mL) for 1 h at room temperature. Solvent was removed under reduced pressure, the resultant residue dissolved in ammonia solution (35%, 2 mL), and the solution kept at room temperature for 2 h. After evaporating in vacuo, the residue was washed with ether (3 × 10 mL) and extracted with 1 M ammonium hydroxide (3 × 15 mL). Insoluble triphenylphosphine and triphenylphosphine oxide were removed by filtration, and the filtrate was washed with toluene (3 × 10 mL) and ether (3 × 15 mL). Concentration of the aqueous phase in vacuo resulted in a pale green solid which was extracted with boiling ethanol (2 × 20 mL). The solution was filtered hot then evaporated to dryness to yield **4a** (0.230 g, 51%). The product had identical NMR, IR, and R_f to the 5′-amino compound prepared by the hydrogenation procedure.

5-Fluoro-5′-amino-2′,5′-dideoxyuridine (4b). (i) Via Hydrogenation. Activated charcoal (20 mg) was added to a solution of the 5-fluoro-5′-azido derivative **3b** (188 mg, 0.693 mmol) in aqueous ethanol (5 mL). The mixture was warmed to 40–50 °C, shaken, and filtered through a Celite pad to remove the charcoal. The filtrate was hydrogenated (2 h) in the presence of 10% palladium on charcoal catalyst (20 mg). After completion of the

reaction (single spot TLC, R_f 0.46, system 1), the mixture was concentrated and centrifuged (1500*g*) for 20 min, the supernatant was filtered through a Celite pad, and the filtrate was centrifuged once more (1500*g*) for 10 min. Removal of water and recrystallization (ethanol–water–ether) afforded **4b** as pale green crystals (161 mg, 85%): mp 194.5–198 °C (lit.[21] mp 199–200 °C); λ_{max} (PBS) 266 nm (log ϵ 3.79), ν_{max} (KBr)/cm^{-1} 3500 (OH) and 1730 (C=O); *m/z* (FAB) 246 ($M^+ + 1$, 35), 217 (31), 181 (32), 126 (22), 91 (100); δ (D_2O) 7.63 (1 H, d, J = 6 Hz, 6-H), 6.18 (1 H, td, J = 6 and 1.5 Hz, 1′-H), 4.40 (1 H, q, J = 4.5 Hz, 3′-H), 4.03 (1 H, m, 4′-H), 3.27 (2 H, d, J = 4.5 Hz, 5′-H_2), 2.37 (2 H, t, J = 6 Hz, 2′-H_2). Anal. ($C_9H_{12}FN_3O_4 \cdot 1.5H_2O$) C, H, N.

(ii) Via the Triphenylphosphine Method. Via the procedure outlined for **4a** the 5-fluoro-5′-amino nucleoside **4b** was afforded as a pale green solid in 61% yield and was identified as authentic material by TLC, NMR, and IR.

Methyl *N*-[1,2,5-Trideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]succinamate (5a). A solution of methyl succinyl chloride (171 mg, 1.14 mmol) in dry tetrahydrofuran (THF) (3 mL) was added dropwise to a solution of 5′-amine **4a** (258 mg, 1.14 mmol) and triethylamine (116 mg, 1.15 mmol) in dry DMF (10 mL). The reaction mixture was left overnight at 4 °C, and a further lot of triethylamine (11 mg, 0.11 mmol) and acid chloride (16 mg, 0.11 mmol) was added. After 1 h, solvent was removed under reduced pressure at 45 °C and the residue was subjected to flash chromatography on a silica activity III column with chloroform–methanol (95:5) as eluant. Appropriate fractions were pooled, solvent was removed, and the residue recrystallized from methanol–ether furnishing the title compound **5a** as colorless crystals (142 mg, 36%): mp 151–153 °C; λ_{max} (PBS) 262 (log ϵ 4.08); ν_{max} (KBr)/cm^{-1} 3460 (OH), 1690 (C=O); *m/z* (FAB) 342 ($M^+ + 1$, 62), 230 (sugar$^+$, 31), 115 (87), 81 (100); δ [$(CD_3)_2SO$] 10.93 (1 H, br s, D_2O exchangeable, 3-NH), 7.85 (1 H, m, D_2O exchangeable, 5′-NH), 7.67 (1 H, d, J = 7.5 Hz, 6-H), 6.15 (1 H, m, t, J = 7.5 Hz, 1′-H), 5.57 (1 H, d, J = 7.5 Hz, 5-H), 5.07 (1 H, d, J = 4.5 Hz, D_2O exchangeable, 3′-OH), 4.18 (1 H, m, 3′-H), 3.80 (1 H, m, 4′-H), 3.60 (3 H, s, O_2CCH_3), 3.38 (2 H, t, collapses to doublet with D_2O, J = 6 Hz, 5′-H), 2.47 (4 H, m, $O_2CCH_2CH_2$), 2.13 (2 H, dd, J = 6 and 4.5 Hz, 2′-H_2). Anal. ($C_{14}H_{19}N_3O_7$) C, H, N.

Pyridine may be used as an alternative solvent to DMF/THF.

Methyl *N*-[1,2,5-Trideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]succinamate (5b). 5-Fluoro 5′-amine **4b** (189 mg, 0.77 mmol) and triethylamine (78 mg, 0.77 mmol) in DMF (10 mL) was reacted with methyl succinyl chloride (116 mg, 0.77 mmol) in THF (2 mL). After 1.5 h further aliquots of both acid chloride (12 mg, 0.08 mmol) and triethylamine (8 mg, 0.08 mmol) were added. After 30 min the reaction was complete (TLC, R_f 0.22, system 1). The residue of the reaction mixture was flash chromatographed on a silica activity III column [chloroform–methanol as eluant (95:5, then 90:10)] as described previously. Recrystallization from methanol–hexane gave **5b** (131 mg, 47%): mp 130–133 °C; λ_{max} (0.01 M HCl) 267 nm (log ϵ 3.92); λ_{max} (PBS) 267 nm (log ϵ 3.89), λ_{max} (0.01 M NaOH) 266 nm (log ϵ 3.80); ν_{max} (KBr)/cm^{-1} 3480 (OH), 1690 (C=O); *m/z* (FAB) 360 [$M^+ + 1$, 100], 230 (sugar$^+$, 80), 181 (60), 149 (48), 131 (34) and 115 (99); δ [$(CD_3)_2SO$–D_2O] 7.93 (1 H, d, J = 7.5 Hz, 6-H), 6.20 (1 H, td, J = 6 and 1.5 Hz, 1′-H), 4.35 (1 H, m, 3′-H), 3.98 (1 H, m, 4′-H), 3.67 (3 H, s, CO_2CH_3), 2.58 (4 H, m, $O_2CCH_2CH_2$), 2.32 (2 H, t, J = 6 Hz, 2′-H_2). Anal. ($C_{14}H_{18}FN_3O_7$) C, H, N.

Ethyl *N*-[1,2,5-Trideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]succinamate (6b). Use of 5′-amine **4b** (197 mg, 0.87 mmol) and ethyl succinyl chloride (143 mg, 0.87 mmol) in the procedure described for **5a** gave ethyl ester **6b** after purification by flash chromatography on a silica activity III column using chloroform–ethanol (92:8) as eluant. The product was recrystallized from ethanol–ether (104 mg, 32%): mp 148–150.5 °C; ν_{max} (KBr)/cm^{-1} 3460 (OH), 1690 (C=O); *m/z* (FAB) 374 [$M^+ + 1$, 18], 244 (47), 226 (32), 129 (100), 101 (100), 81 (99); δ [$(CD_3)_2SO$] 11.02 (1 H, br s, D_2O exchangeable, 3-NH), 8.20 (1 H, d, J = 6 Hz, 6-H), 7.90 (1 H, m, D_2O exchangeable, 5′-NH), 6.38 (1 H, td, J = 6 and 1.5 Hz, 1′-H), 5.01 (1 H, br s, D_2O exchangeable, 3′-OH), 4.02 (3 H, m, 4′-H and $CH_3CH_2O_2C$), 3.49 (2 H, m, collapses to a doublet with D_2O, J = 6 Hz, 5′-H_2), 2.50 (4 H, m, $O_2CCH_2CH_2$), 2.20 (2 H, m, 2′-H_2), 1.18 (3 H, t, J = 7 Hz, $CH_3CH_2O_2C$). Anal. ($C_{15}H_{20}FN_3O_7$) C, H, N.

***N*-[1,2,5-Trideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]succinamic Acid (7a).** To a solution of the methyl ester **5a** (45 mg, 0.13 mmol) in 50% aqueous methanol (10 mL) was added 1 M sodium hydroxide (260 μL, 0.26 mmol). After 2 h, when the product gave a yellow color with bromocresol green spray (R_f 0.64, system 2), the solution was concentrated to about 3 mL and passed down a zerolit 325 (H^+ form) ion-exchange column (1 × 5 cm) to obtain **7a** as a glass solid which was washed with a little cold ethyl acetate and dried in vacuo, 45 °C, over P_2O_5 for 48 h (38 mg, 89): λ_{max} (PBS) 261 nm (log ϵ 4.03); *m/z* (FAB) 328 [$M^+ + 1$, 16], 216 (sugar$^+$, 7), 207 (31), 131 (23), 115 (100), 91 (32); δ [$(CD_3)_2SO$/$(CD_3)_2CO$] 10.65 (1 H, br s, D_2O exchangeable, 3-NH), 7.68 (2 H, m, on D_2O, exchange collapses to doublet, 1 H, J = 7.5 Hz, 6-H and 5′-NH), 6.20 (1 H, t, J = 6 Hz, 1′-H), 6.38 (1 H, br s, D_2O exchangeable, 3′-OH), 5.63 (1 H, d, J = 7.5 Hz, 5-H), 4.28 (1 H, m, 3′-H), 3.37 (1 H, m, 4′-H), 3.43 (2 H, t, J = 6 Hz, collapses to a doublet with D_2O, 5′-H_2), 2.52 (4 H, m, $O_2CCH_2CH_2$), 2.20 (2 H, dd, J = 6 and 4.5 Hz, 2′-H_2).

***N*-[1,2,5-Trideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]succinamic Acid (7b).** Fluorinated 5′-succinamic acid **7b** was prepared in 92% yield from the methyl ester **5b** or the ethyl ester **6b** using the foregoing procedure: λ_{max} (0.01 M HCl) 268 nm (log ϵ 3.98); λ_{max} (PBS) 266 nm (log ϵ 3.92); λ_{max} (0.01 M NaOH) 264 nm (log ϵ 3.84); ν_{max} (Nujol)/cm^{-1} 3000–2400 (COOH), 1720, 1680 (C=O); *m/z* (FAB) 346 [$M^+ + 1$, 29], 288 (11), 216 (sugar$^+$, 24), 131 (24), 115 (100); δ [$(CD_3)_2CO/D_2O$] 8.18 (1 H, d, J = 6 Hz, 6-H), 6.20 (1 H, td, J = 6 and 1.5 Hz, 1′-H), 4.31 (1 H, q, J = 4.5 Hz, 3′-H), 3.95 (1 H, q, J = 4.5 Hz, 4′-H), 2.58 (4 H, m, $O_2CCH_2CH_2CO$), 2.23 (2 H, t, J = 4.5 Hz, 2′-H_2).

***N*-[1,2,5-Trideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]maleamic Acid (8a).** 5′-Amine **4a** (159 mg, 0.709 mmol) was dissolved in warm pyridine (15 mL), the solution was then cooled to 0–10 °C, and maleic anhydride (76 mg, 0.779 mmol) was added with stirring. The mixture was stirred at room temperature overnight and concentrated, and the last traces of pyridine were removed by azeotroping with a little water. The resultant residue was dissolved in water (2 mL), and pyridinium cations were removed by a Zerolit 325 (H^+ form) column (1 × 4 cm). The solution was left to stand at 4 °C for several hours to allow for complete crystallization of the target compound. The product (169 mg, 73%) was collected by suction and recrystallized from water: mp 214–216.5 °C; ν_{max} (KBr)/cm^{-1} 3400 (OH), 2900–2200 (COOH), 1710 and 1670 (C=O); *m/z* (FAB) 326 [$M^+ + 1$, 20], 271 (18), 255 (48), 199 (70), and 115 (100); δ (D_2O/NaOD) 7.62 (1 H, d, J = 9 Hz, 6-H), 6.30 (2 H, m, 1′-H and O_2CCH=), 5.97 (1 H, d, J = 12 Hz, =CHCONH), 5.85 (1 H, d, J = 9 Hz, 5-H), 4.33 (1 H, m, 3′-H), 3.95 (1 H, m, 4′-H), 3.57 (2 H, m, 5′-H_2), 2.30 (2 H, t, J = 6 Hz, 2′-H_2). Anal. ($C_{13}H_{15}N_3O_7$) C, H, N.

***N*-[1,2,5-Trideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]maleamic Acid (8b).** 5-Fluoro 5′-amine **4b** (74 mg, 0.33 mmol) was reacted with maleic anhydride (32 mg, 0.33 mmol) in pyridine (10 mL) by the above procedure. After 4 h, the solvent was removed under reduced pressure, and the residue was dissolved in a minimum amount of distilled water. The solution was carefully acidified with dilute hydrochloric acid and the mixture allowed to stand at 4 °C for 30 min. The precipitated product was collected by filtration, washed with cold water and ethanol, and dried in vacuo over P_2O_5 at 50 °C for 4 h, yielding the title compound **8b** as a pale brown powder (71 mg, 54%): mp 208–210 °C; λ_{max} (PBS) 264 nm (log ϵ 3.94); λ_{max} (sodium carbonate buffer) 260 nm (log ϵ 3.88); ν_{max} (KBr)/cm^{-1} 3460 (OH), 2900–2400 (COOH), 1700 (C=O); *m/z* (FAB) 344 [$M^+ + 1$, 77], 274 (65), 232 (99), 216 (85), 214 (sugar$^+$, 50), 59 (100); δ (D_2O/NaOD) 7.57 (1 H, d, J = 6 Hz, 6-H), 6.30 (2 H, d, J = 12 Hz, O_2CCH=), 6.18 (1 H, td, J = 6 and 1.5 Hz, 1′-H), 5.88 (1 H, d, J = 12 Hz, =CHCONH), 4.30 (q, J = 4.5 Hz, 3′-H), 3.95 (1 H, q, J = 4.5 Hz, 4′-H), 3.48 (2 H, d, J = 6 Hz, 5′-H_2), 2.23 (2 H, t, J = 6 Hz, 2′-H_2). Anal. ($C_{13}H_{14}FN_3O_7$) C, H, N.

N-Butylmaleamic Acid. *n*-Butylamine (487 mg, 6.66 mmol) in methylene chloride (4 mL) was added dropwise to a stirred suspension of maleic anhydride (344 mg, 5.54 mmol) in methylene chloride (10 mL) at 0–10 °C. After 2 h the solvent was removed and the residue recrystallized (ethyl acetate–hexane) to give *N*-butylmaleamic acid (760 mg, 74%): δ ($CDCl_3$) 8.00 (1 H, m, D_2O exchangeable, NH), 6.47 (1 H, d, J = 12 Hz, $CHCO_2H$), 6.17 (1 H, d, J = 12 Hz, $CHCONH$), 3.33 (2 H, q, J = 6 Hz, $NHCH_2$), 1.13–1.77 (4 H, m, $(CH_2)_2CH_3$), 0.92 (3 H, t, CH_3).

Succinimido N-Butylmaleamate. Dicyclohexylcarbodiimide (68 mg, 0.33 mmol) was added to a solution of *N*-butylmaleamic acid (51 mg, 0.298 mmol) and *N*-hydroxysuccinimide (28 mg, 0.337 mmol) in dry dioxane (5 mL). After 2 h dicyclohexylurea was removed by filtration, and the filtrate was evaporated to dryness. The residue was extracted with cold dry acetone (3 × 5 mL), filtered to remove any undissolved material, and concentrated under reduced pressure. The residue was recrystallized (chloroform-hexane) at low temperature to afford the title compound as colorless crystals (38 mg, 48%): δ [$(CD_3)_2CO$] 7.09 (1 H, m, D_2O exchangeable, N*H*), 6.62 (1 H, d, J = 12 Hz, $CHCO_2$), 6.13 (1 H, d, J = 12 Hz, $CHCONH$), 3.13 (2 H, m, $NHCH_2$), 2.78 (4 H, s, $CO(CH_2)CO$), 1.05–1.71 (4 H, m, $(CH_2)_2CH_3$), 0.91 (3 H, t, CH_3).

N-Butyl-N'-[1,2,5-trideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]maleamide (9a). To a stirred solution of 5'-amine **4a** (50 mg, 0.205 mmol) in a dry DMF–dioxane (1:1) mixture (20 mL) was added succinimido *N*-butylmaleamate (82 mg, 0.306 mmol). After 2 h, the solvent was removed, and the residue was washed with a little cold water and dissolved in a minimum of acetone; this solution was allowed to stand at 4 °C for 2 h prior to filtering. Acetone was removed, and the residue was recrystallized from acetone–hexane to obtain **9a** (24 mg, 31%): *m/z* (FAB) 381 [M^+ + 1, 10], 300 (29), 273 (31), 269 (sugar$^+$, 11), 220 (50), and 91 (100); δ [$(CD_3)_2CO/D_2O$] 7.80 (1 H, d, J = 7.5 Hz, 6-H), 6.40 (2 H, s, $CH{=}CH$), 6.22 (1 H, t, J = 6 Hz, 1'-H), 5.80 (1 H, d, J = 9 Hz, 6-H), 4.10 (1 H, m, 3'-H), 3.62 (1 H, m, 4'-H), 3.50 (2 H, d, J = 6 Hz, 5'-H_2), 3.27 (2 H, m, CH_2NHCO), 2.37 (2 H, t, J = 6 Hz, 2'-H_2), 1.10–1.67 (4 H, m, $H_3CCH_2CH_2$), 0.93 (3 H, m, CH_3). An alternative procedure for preparation of **9a** involved reaction of equimolar quantities of 5'-amine **4a** and *N*-butylmaleamic acid in the presence of *N*-hydroxysuccinimide, using 1.2–1.5 molar equiv of either carbodiimide DCCI (DMF) or ECDI (dioxane–water). The title compound was afforded in 32–36% crude yield.

N-Butyl-N'-[1,2,5-trideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranos-5-yl]maleamide (9b). To a stirred solution of 5'-maleamic acid **8b** (30 mg, 87.8 μmol) in a dry DMF–dioxane (1.5:1) mixture (3.5 mL) at 5 °C was added triethylamine (9.1 mg, 90 μmol), followed, after a 5 min interval, by isobutyl chloroformate (11.9 mg, 87 μmol). After 15 min *N*-butylamine (6.4 mg, 87 μmol) was introduced, and the mixture was left overnight at room temperature. After filtering, the filtrate was evaporated under reduced pressure and the oily residue on trituration with water yielded a white solid which was collected and washed with a little cold dilute hydrochloric acid and water. Flash chromatography on a silica gel activity III column [chloroform–methanol (95:5 and 0.4% acetic acid) as eluant] afforded the title compound **9b** as a pale brown powder (10 mg, 29%) (TLC R_f 0.43, system 3 and 0.32, system 4): mp 232–234 °C; λ_{max} (PBS) 263 nm (log ε 3.78); λ_{max} (sodium carbonate buffer) 261–262 nm (log ε 3.71); ν_{max} (KBr)/cm^{-1} 3280 (OH), 1730 and 1670 (C=O); *m/z* (FAB) 399 [M^+ + 1, 19], 293 (14), 237 (60), 170 (86), 154 (98), 106 (100), 91 (99), 81 (98); δ [$(CD_3)_2CO/(CD_3)_2SO$] 11.20, 9.90, and 9.05 (3 × 1 H, m, D_2O exchangeable peaks, N'H, NH, and 3-NH), 7.95 (1 H, d, J = 7.5 Hz, 6-H), 6.20 (1 H, td, J = 6 and 1.5 Hz, 1'-H), 6.25 (2 H, s, $CH{=}CH$), 5.02 (1 H, s, D_2O exchangeable, 3'-OH), 3.90 (1 H, m, 4'-H), 3.52 (2 H, m, CH_2NHCO), 2.98 (2 H, m, 5'-H_2), 2.17 (2 H, dd, J = 6 and 4.5 Hz, 2'-H_2), 1.10–1.90 (4 H, m, $H_3CCH_2CH_2$), and 0.85 (3 H, m, CH_3). Anal. ($C_{17}H_{23}FN_4O_3$) C, H, N.

1,2-Dideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronic Acid (10a). Oxygen gas was bubbled through a stirred, heated solution (70–80 °C) of 2'-deoxyuridine **1a** (0.5 g, 2.19 mmol), sodium hydrogen carbonate (184 mg, 2.19 mmol), and reduced platinum(IV) oxide, Adams' catalyst[27] (300 μg) in water (60 mL). After 3 h, a further aliquot of catalyst (1–5 mg) was added, and the reaction was allowed to continue a further 33 h at 70 °C. The mixture was purged with nitrogen gas, allowed to stand for 1 h at room temperature, and then filtered through a Celite pad. The filtrate was concentrated to about 30 mL before passing twice through an Amberlite IR ion exchange resin (H^+ form) 1- × 5-cm column. Removal of solvent under reduced pressure at 50 °C resulted in a pale yellow solid which was recrystallized from water, affording target compound **10a** as colorless crystals. Traces of water were removed by azeotroping with ethanol, and the crystals were dried in vacuo over P_2O_5, 45 °C for 24 h (254 mg, 48%): mp 218–222 °C (lit.[25] mp 220.5–222 °C); ν_{max} (KBr)/cm^{-1} 3570 (OH) and 3000–2400 (COOH); δ [$(CD_3)_2CO/D_2O$] 8.37 (1 H, d, J = 7.5 Hz, 6-H), 6.47 (1 H, dd, J = 7.5 and 6 Hz, 1'-H), 5.82 (1 H, d, J = 7.5 Hz, 5-H), 4.67 (1 H, m, 3'-H), 4.50 (1 H, s, 4'-H), 2.13–2.57 (2 H, m, 2'-H_2). Anal. ($C_9H_{10}N_2O_6 \cdot 0.5H_2O$) C, H, N.

1,2-Dideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronic Acid (10b). Freshly reduced platinum(IV) oxide catalyst (50 mg) was added to a heated (60–70 °C) mixture of FdUR **1b** (500 mg, 2.03 mmol) and sodium hydrogen carbonate (65 mg, 0.77 mmol) in water (70 mL). Oxygen was bubbled through the vigorously stirred solution for 2 h prior to addition of a second aliquot of catalyst (125 mg), and the reaction continued for a further 23 h. The mixture was allowed to cool to room temperature and centrifuged (1300*g*, 20 min), the supernatant was filtered through a Celite pad, and the filtrate was centrifuged once more (1300*g*, 10 min). The solution was concentrated to 20 mL and then stirred with Dowex 50-W X-4 (H^+ form) cation exchange resin (3 g) for 50 min. After filtering, the solvent was removed in vacuo to yield a white solid which was recrystallized (water) and dried as described for **10a** to furnish **10b** as colorless crystals (370 mg, 66%): mp 216–219 °C (lit.[43] mp 218–221 °C); λ_{max} (PBS) 268 nm (log ε 3.88); ν_{max} (KBr)/cm^{-1} 3430 (OH), 3000–2400 (COOH), and 1680 (C=O); *m/z* (FAB) 278 [$M^+ + H_2O$, 37], 261 [M^+ + 1, 100], 181 (60), 131 (sugar$^+$, 96), and 113 (43); δ [$(CD_3)_2CO$] 10.30 (1 H, br s, D_2O exchangeable, NH), 8.50 (1 H, d, J = 6 Hz, 6-H), 6.40 (1 H, td, J = 6 and 1.5 Hz, 6-H), 4.63 (1 H, d, J = 6 Hz, 3'-H), 4.47 (1 H, s, 4'-H), 1.98–2.47 (2 H, m, 2'-H_2). Anal. ($C_9H_9FN_2O_6 \cdot H_2O$) C, H, N.

Ethyl 4-[1,2-Dideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronamido]benzoate (11a). To a mixture of ethyl 4-aminobenzoate (104 mg, 0.63 mmol) and DCCI (155 mg, 0.759 mmol) in dioxane (5 mL) was added a solution of ribofuronamidobenzoic acid **10a** in water (5 mL). Dicyclohexylurea that precipitated after 2 min was removed by filtration and washed with water–dioxane (1:1). The filtrate and washings were combined and allowed to stand overnight. The solution was concentrated, and the residue flash chromatographed on a silica gel 60 column (activity I) using chloroform-ethanol (10:1) as eluant. The product (R_f 0.42, system 1; 0.37, system 5) was obtained as a colorless powder (140 mg, 52%) and recrystallized (ethanol) and dried (in vacuo over P_2O_5, 4 h): mp 224–227.5 °C; λ_{max} (PBS) 265 nm (log ε 4.56); ν_{max} (KBr)/cm^{-1} 3450 (OH), 1710 and 1660 (C=O); *m/z* (FAB) 390 [M^+ + 1, 20], 306 (19), 278 (sugar$^+$, 29), 253 (81), 192 (100); δ [$(CD_3)_2CO/D_2O$] 7.92 (3 H, d, J = 7.5 Hz, 6-H and Ar-H_2), 7.65 (2 H, d, J = 7.5 Hz, Ar-H_2), 6.15 (1 H, dd, J = 6 and 3 Hz, 1'-H), 5.70 (1 H, d, J = 7.5 Hz, 5-H), 4.57 (1 H, m, 3'-H), 4.40 (1 H, d, J = 1.5 Hz, 4'-H), 4.28 (2 H, q, J = 7.5 Hz, OCH_2CH_3), 2.33 (2 H, m, 2'-H_2), and 1.30 (3 H, t, CH_3CH_2O). Anal. ($C_{18}H_{19}N_3O_7$) C, H, N.

Ethyl 4-[1,2-Dideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronamido]benzoate (11b). To a stirred solution of ribofuronamidobenzoic acid **10b** (100 mg, 0.384 mmol) in water (4 mL) was added ethyl 4-aminobenzoate (69.7 mg, 0.422 mmol) in THF (4 mL). Water-soluble carbodiimide ECDI (80.9 mg, 0.422 mmol) was introduced and the reaction left overnight. The mixture was concentrated to about 2 mL and the solution allowed to stand for several hours. The white solid was collected by suction and washed with a little water and ether to give the title compound **11b** (single spot TLC R_f 0.16 system 1; 0.66, system 5). The product was dried in vacuo at 45 °C for 4 h (112 mg, 72%). A small sample was recrystallized from ethanol–ether: mp 259–260 °C; λ_{max} (PBS) 267 nm (log ε 4.28), λ_{max} (sodium carbonate buffer) 269 nm (log ε 4.18); ν_{max} (KBr)/cm^{-1} 3570 (OH) and 1690 (C=O); *m/z* (FAB) 408 [M^+ + 1, 31], 306 (20), 276 (65), 232 (84), 149 (85), 136 (100), 278 (sugar$^+$,

20); δ [$(CD_3)_2SO$] 11.80, 10.57 (2 × 1 H, 2 × br s, D_2O exchangeable 3-NH and NHCO), 8.62 (1 H, d, J = 6 Hz, 6-H), 7.90 (2 H, d, J = 7.5 Hz, Ar-H_2), 7.70 (2 H, d, J = 7.5 Hz, Ar-H_2), 6.30 (1 H, td, J = 6 and 1.5 Hz, 1'-H), 5.72 (1 H, d, J = 4.5 Hz, D_2O exchangeable, 3'-OH), 4.43 (2 H, m, 3'-H and 4'-H), 4.23 (2 H, q, J = 7.5 Hz, H_3CCH_2O), 2.18 (2 H, m, 2'-H_2), 1.27 (3 H, t, CH_3CH_2O). Anal. Found: C, 52.33; H, 4.62; N, 9.90. $C_{18}H_{18}FN_3O_7$ requires: C, 53.07; H, 4.45; N, 10.32.

Methyl 4-[1,2-Dideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronamido]benzoate (12b). Reaction of methyl 4-aminobenzoate (25 mg, 0.165 mmol) with ribofuronamidobenzoic acid **10b** in the presence of ECDI (37 mg, 0.193 mmol) using a dioxane–water (1:1) solvent system by the above procedure afforded **12b** as a colorless powder (50 mg, 79%): ν_{max} (KBr)/cm^{-1} 3270 (OH), 1680 (C=O); δ [$(CD_3)_2SO$/$(CD_3)_2CO$] 11.47, 10.42 (2 × 1 H, 2 × br s, D_2O exchangeable, 3-NH and N*H*CO), 8.68 (1 H, d, J = 7.5 Hz, 6-H), 7.93 (2 H, d, J = 7.5 Hz, Ar-H_2), 7.73 (2 H, d, J = 7.5 Hz, Ar-H_2), 6.40 (1 H, td, J = 6 and 1.5 Hz, 1'-H), 5.48 (1 H, d, D_2O exchangeable, J = 4.5 Hz, 3'-OH), 4.53 (2 H, m, 3'-H and 4'-H), 3.8 (3 H, s, OCH_3), 2.27 (2 H, dd, J = 6 and 4.5 Hz, 2'-H).

4-[1,2-Dideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronamido]benzoic Acid (13a). To the ethyl ester **11b** (26 mg, 66 μmol) in 50% aqueous ethanol, were added two aliquots of 1 M sodium hydroxide (2 × 76 μmol), the second after an interval of 1 h. After a further 2 h, sodium ions were exchanged on a zerolit 325 (H^+) column (1 × 5 cm), and water was removed under reduced pressure. The residue was washed with cold water, ethanol, and finally ether before drying in vacuo over P_2O_5 for 8 h, affording the title compound **13a** (19 mg, 80%): mp 271 °C dec; λ_{max} (PBS) 263 nm (log ε 4.39), ν_{max} (KBr)/cm^{-1} 3860 (OH), 3300–2400 (COOH), 1710, 1660 (C=O); m/z (FAB) 362 [M^+ + 1, 19], 274 (53) (sugar$^+$, 9), 181 (66), and 149 (100); δ [$(CD_3)_2CO/D_2O$] 7.83 (3 H, m, Ar-H_2 and 6-H), 7.47 (2 H, d, J = 7.5 Hz, Ar-H_2), 6.25 (1 H, dd, J = 7.5 and 6 Hz, 1'-H), 5.72 (1 H, d, J = 7.5 Hz, 5-H), 4.6 (1 H, m, 3'-H), 4.39 (1 H, d, J = 4.5 Hz, 4'-H), 2.33 (2 H, m, 2'-H_2).

4-[1,2-Dideoxy-1β-(5-fluoro-1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronamido]benzoic Acid (13b). Hydrolysis of ethyl (**11b**) or methyl (**12b**) esters by addition of 2 molar equiv of sodium hydroxide gave the title acid **13b** in 80% yield. The product (R_f 0.58, system 5) was isolated by acidification of the reaction mixture and purified by recrystallization from water: mp 259–263 °C; λ_{max} (PBS) 262 nm (log ε 4.07), λ_{max} (sodium carbonate buffer) 266 nm (log ε 4.27); ν_{max} (KBr)/cm^{-1} 3520 (OH), 3400–3000 (COOH), 1690 (C=O); m/z (FAB) 380 [M^+ + 1, 16], 250 (sugar$^+$, 12), 181 (12), 164 (18), and 55 (100); δ [$(CD_3)_2CO/(CD_3)_2SO$] 10.32 (1 H, br s, D_2O exchangeable, 3-NH), 8.67 (1 H, d, J = 7.5 Hz, 6-H), 7.92 (2 H, d, J = 7.5 Hz, Ar-H_2), 7.90 (1 H, br s, D_2O exchangeable, N*H*CO), 7.73 (2 H, d, J = 7.5 Hz, Ar-H_2), 6.40 (1 H, td, J = 6 and 1.5 Hz, 1'-H), 4.55 (2 H, m, 3'-H and 4'-H), 2.32 (2 H, dd, J = 6 and 4.5 Hz, 2'-H_2). Anal. ($C_{16}H_{14}FN_3O_7 \cdot 2.5H_2O$) C, H, N.

4-[1,2-Dideoxy-1β-(1,2,3,4-tetrahydro-2,4-dioxopyrimidin-1-yl)-D-ribofuranuronamido]benzamide (15a). Carbodiimide ECDI (59 mg, 0.307 mmol) was added to a mixture of ribofuronamidobenzoic acid **10a** (40 mg, 0.154 mmol) and 4-aminobenzamide (20.9 mg, 0.154 mmol) in water (10 mL). After 2 h, reaction mixture was concentrated to about 2 mL and left overnight. The separated white solid was filtered, washed with cold dilute hydrochloric acid, water, and ether, recrystallized (methanol–ether), and dried in vacuo over P_2O_5 for 2 days to give colorless crystals (25 mg, 60%): mp 267–269 °C; λ_{max} (PBS) 265 nm (log ε 4.35), λ_{max} (sodium carbonate buffer) 268 nm (log ε 4.34); ν_{max} (KBr)/cm^{-1} 3270 (OH), 1680 (C=O); m/z (FAB) 379 [M^+ + 1, 9], 349 (6), 291 (106), 219 (12), 202 (52), 10 (100); δ [$(CD_3)_2CO/(CD_3)_2SO$] 11.82, 10.48 (2 × 1 H, 2 × br s, D_2O exchangeable, 3-NH and N*H*CO), 8.70 (1 H, d, J = 7.5 Hz, 6-H), 7.88 (2 H, d, J = 7.5 Hz, Ar-H_2), 7.70 (2 H, d, J = 7.5 Hz, Ar-H_2), 7.15 (2 H, br s, D_2O exchangeable, CONH_2), 6.37 (1 H, td, J = 6 and 1.5 Hz, 1'-H), 5.70 (1 H, d, J = 4.5 Hz, D_2O exchangeable, 3'-OH), 4.50 (2 H, m, 3'-H and 4'-H), 2.25 (2 H, dd, J = 6 and 4.5 Hz, 2'-H_2). Anal. ($C_{16}H_{15}FN_4O_5 \cdot 0.5H_2O$).

Methods. Cell Lines and Antibodies. Monoclonal antibody 791T/36 was prepared from cultured supernatants of hybridoma 791T/36 clone 3 by affinity chromatography on protein A-sepharose and purified as previously described.[7,8] HSA was obtained from Sigma Chemical Co. as a lyophilized crystalline powder (96–99%) and used without further purification. Osteogenic sarcoma cell line 791T expressed approximately 6 × 10^5 antigens per cell and the T-24 bladder carcinoma about 3–5% of this level. These cell lines were cultured in monolayers in Eagles minimum essential medium supplemented with 10% newborn calf serum and passaged routinely after detachment with trypsin (0.25%) and EDTA (0.5%). Cytotoxicity of FdUR derivatives, HSA–derivatives, and antibody–HSA–derivatives were determined by inhibition of [^{75}Se]selenomethionine uptake in a microplate assay against both 791T and T-24 cells. Although measuring protein synthesis, this assay correlates well with cell death as measured by trypan blue exclusion.[33] Retention of antibody binding activity to antigen positive 791T cells was estimated using flow cytofluorimetry by a direct competition assay between conjugate and fluorescent labeled antibody in comparison with unlabeled antibody.[32]

Conjugate Purification and Analysis. Hydroxylapatite, gel filtration, and high-pressure liquid chromatography were used to purify and analyse conjugates. Hydroxylapatite chromatography was carried out using Bio-gel HT matrix packed into 1.2- × 5-cm columns equilibrated with 0.01 M sodium phosphate buffer pH 6.8 and eluted with a sodium phosphate buffer gradient of 0.01–0.5 M at 95 mL/h. Gel filtration chromatography was performed on 1.6- × 90- or 2.5- × 90-cm columns packed with either Sephacryl S-200 or S-300 or Fractogel HW55s matrices. Columns were eluted with phosphate buffered saline at 2–3 mL/cm^2 per h. Samples were filtered or centrifuged to remove particulates before application in less than 2% of column volume. Columns were calibrated using thyroglobulin, ferritin, antibody, aldolase, HSA, ovalbumin, and cytochrome C as molecular weight markers. HPLC utilized a TSK G3000SW 60-cm column eluted with 0.1 M phosphate buffer pH 7.2 containing 0.2 M potassium chloride at a flow rate of 0.15–0.2 mL/min. Myosin, antibody, β-galactosidase, phosphorylase b, BSA, ovalbumin, and carbonic anhydrase were employed as calibration standards. Further analysis of conjugates for purity and molecular weight were carried out using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 5–15% acrylamide gradient gels run under reducing conditions. Gels were run on an LKB 2050 midget gel apparatus and calibrated with the same standards as for HPLC.

Coupling of Derivatives to HSA. Three methods were used in these studies, a two-step carbodiimide reaction, activated ester, and mixed anhydride methods. All three reactions were carried out using 5–20 mg derivative.

(i) Two-Step Carbodiimide Method. ECDI (5 mol/mol of derivative) was added to a solution of derivative (5 mg/mL) in 12.5 mM phosphate buffer solution at pH 5.0–5.5. The pH was maintained using 0.01 M HCl for 1–2 min before addition of HSA (5 mg/mL in phosphate buffered saline) and a rapid readjustment to pH 6.4. Approximately 250 mol of derivative were used per mole of protein. The pH was maintained at 6.4 for 90 min and then left to react overnight in the dark.

(ii) Activated Ester Method. Derivative was dissolved in dry DMF (25–40 mg/mL) and preactivated by reaction with a slight excess of DCCI for 90 min. The mixture was then reacted with a slight excess of *N*-hydroxysuccinimide for a further 90 min. Following microcentrifugation, the crude product was added directly to HSA (5 mg/mL, 1 mol of HSA/150–250 mol of derivative) in phosphate buffered saline pH 8. The pH was maintained for 1 h and then left to react overnight in the dark.

(iii) Mixed Anhydride Method. The derivative was dissolved in dry DMF (20–40 mg/mL) containing 1 equiv of triethylamine and cooled in an ice bath. Isobutyl chloroformate (1.2 equiv) was introduced and reacted for 15 min before addition of a further 0.8 equiv. After 20 min a solution of HSA (5 mg/mL, 1 mol of HSA/150–250 mol of derivative) in 0.1 M buffer pH 8 was added and the solution allowed to reach room temperature. The pH was maintained for 30 min, followed by a further 7-h reaction in the dark.

Workup of HSA–Derivative Preparations. The reaction mixtures were first desalted through PD-10 columns (disposable G-25 columns, Pharmacia) and then fractionated by one or two passes on a gel filtration column to ensure removal of unwanted higher molecular weight albumin aggregates. Samples were

concentrated either by precipitation at low pH, ultrafiltration, or visking tubing and Aquacide II.

Coupling of HSA-Derivative to Antibody. The method used for coupling is essentially as described previously for antibody-HSA-methotrexate conjugates.[8] Briefly, antibody was iodoacetylated with *N*-(iodoacetyl)succinimide and HSA-derivative was reduced with dithiothreitol at low pH to give a free sulfhydryl group. These two components were then reacted to give a thioether-linked conjugate. Iodoacetylation and reduction reactions were carried out for 1 h and products separated from reactants by desalting. Equimolar quantities of antibody and HSA-derivative were reacted overnight in the dark at pH 8.5 after concentrating to a viscous solution by positive pressure ultrafiltration. Products were separated by gel filtration chromatography, and conjugates corresponding to a molecular weight 210 000–230 000 g/mol were pooled. Conjugates were concentrated, sterilized by passage through a 0.22-μm filter, and stored at 4 °C. Molecular size and purity of conjugates was estimated by SDS-PAGE and HPLC.

Yields and Purity of Conjugates. 5′-Succinamic acid conjugate H1.11 was produced with an efficiency of reaction of 6.0% with respect to drug derivative, and after purification by gel filtration yielded 40% of monomer conjugate with respect to input HSA (λ_{max} (PBS) 268 nm). 5′-Maleamic acid conjugate H2.8 had a reaction efficiency of 4.7% with respect to drug and a final yield of 38% monomer (λ_{max} (PBS) 263 nm). The reaction efficiency for ribofuranuronamido HSA conjugate H3.2 was 6.0% with a yield of 30% monomer product (λ_{max} (PBS) 267 nm).

Antibody conjugate AH1.11 yielded 12.6% of conjugate as a percentage of input antibody (10.7% monomer, 1.9% higher molecular weight material) after final cleanup. No HSA dimer was detectable in the conjugate, but the preparation contained 21% unconjugated antibody. Only a single conjugate band and antibody was visible on SDS-PAGE.

Maleamic acid conjugate yielded 16.7% monomer conjugate after the final refractionation by gel filtration chromatography. The purity was similar to that of the succinamic acid conjugate except that it contained 34% contaminating free antibody.

Ribofuranuronamido conjugate which was purified by a precipitation technique yielded 23% of conjugate with respect to input antibody and contained less than 10% free antibody. SDS-PAGE analysis showed two conjugate bands at 227 000–238 000 and 290 000–305 000 molecular weight. The latter is presumed to be (HSA-drug)$_2$-Ab. A small amount of HSA dimer was also present in this conjugate.

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