Tyramine-Containing Poly(4-Nitrophenylacrylate) as Iodinatable Ligand Carrier in Biodistribution Analysis

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Purpose. Targeted label or drug delivery requires access to convenient carrier systems and methods for efficient ligand conjugation. The main purpose of this study is to design an iodinatable synthetic polymer, whose application *in vivo* in tumor-bearing mice is tested with several related carbohydrate ligands, namely ABH and Lewis blood group epitopes.

Methods. Tyramine and aminopropyl derivatives of the synthetic oligosaccharides were attached to poly(4-nitrophenylacrylate). Following iodination, the biodistribution of the sugar-free and the substituted polymers was determined in tumor-bearing mice. Flow cytofluorimetric analysis assessed tumor cell binding of further ligand types to human tumor cells *in vitro*.

Results. Quantitative ligand incorporation was achieved under mild conditions. Whereas the ligand-free poly[N-(2-hydroxyethyl)acrylamide] (MW 30 kDa) showed preferential accumulation in kidney, neoglycopolymers were found in substantial amounts in liver, kidney or spleen. The nature of the carbohydrate structure quantitatively influenced the distribution pattern. Tumor cell binding of blood group determinants and three further ligand types revealed non-uniform intensity in labeling and percentage of positive cells even in comparison between lines with identical histogenetic origin.

Conclusions. Carbohydrate-exposing poly[N-(2-hydroxyethyl)acrylamide] polymers with tyramine as an iodine acceptor distribute in mice with a profile which is quantitatively influenced by small structural variations of the ligand part. Further refinement of the ligand structure may increase the level of selectivity for organ and tumor accumulation.

KEY WORDS: radiopharmaceutical; imaging; targeting; neoglycoconjugate; lectin; tumor cell.

INTRODUCTION

The quest for efficacious delivery systems for imaging or drug targeting is continuously fueling research efforts to define smart combinations of a carrier and the selectivity-confering ligand. Water solubility, biocompatibility, stability under physiological conditions and ease in incorporating even delicate substituents are among the requirements for a potentially suitable polymer. Ideally, the ligand should direct the loaded carrier to a distinct organ/cell type with the precision of a postal code. On the organism level any degree of selectivity can translate into reduction of undesirable drug side effects and the generation of beneficial imaging qualities. Thus, careful and extensive monitoring of well-described receptor systems which mediate the arrest of a circulating conjugate is warranted.

Theoretical considerations advocate paying special attention to the coding capacity of oligosaccharides (1). The investigation of the recognitive interplay between cell lectins and ligand sites of glycoconjugates, which is expedited by the design of defined carrier-immobilized sugar structures, has already indicated promising routes for further evaluation (2–6). In addition to a peptide bond backbone like that of albumin, other custom-made polymers can offer suitable properties which encourage assaying the resulting neoglycoconjugates (7,8). We herein present a convenient synthetic procedure which produces ligand- and tyramine-substituted poly[N-(2-hydroxyethyl)acrylamide] for application in radioimaging. Due to the proven and the assumed intriguing physiological functions of blood group epitopes outlined elsewhere (9,10), we have selected the sugar structures presented in Fig. 1 for the initial *in vivo* testing of the iodinatable synthetic polymer. In view of potential diagnostic and therapeutic implications, biodistribution of the synthetic probes is determined in tumor-bearing mice. The properties of the ligand-bearing matrix are also evaluated *in vitro*. A panel of human tumor cell lines of different histogenetic origin is subjected to flow cytofluorimetric analysis to monitor the cell type-dependent extent of receptor expression. The versatility of the general synthetic approach is emphasized by additionally employing other types of ligand, i.e., the P-glycoprotein-reactive chemotherapeutical agent doxorubicin, the annexin-binding phosphorylcholine, and heparin, in the *in vitro* measurements.

MATERIALS AND METHODS

Synthesis of Probes

The carrier poly(4-nitrophenylacrylate) was prepared by azodiisobutyronitrile-initiated monomer polymerization in dry benzene and the aminopropyl derivatives of the tested blood group oligosaccharides, shown in Fig. 1, were synthesized, as described elsewhere (11–13). Four and nine tenths mg of the polymeric product (25.42 μmol monomer equivalent) were dissolved in 200 μl amine-free N,N-dimethylformamide and 100 μl N,N-dimethylformamide containing 5 μmol of the respective aminopropyl derivative of the oligosaccharide as well as 40 μl N,N-dimethylformamide containing 0.25 μmol tyramine hydrochloride (Sigma, Munich, FRG) and 20 μl triethylamine were successively added. The mixture was incubated for 24 h at 37°C. Following the addition of 40 μl ethanolamine to block residual reactive sites, the mixture was kept at 25°C for another 24 h period. The substituted poly[N-(2-hydroxyethyl)acrylamide] was separated from 4-nitrophenol and residual amine by gel filtration in acetonitrile/water (1:1) on a Sephadex LH-20 column (2 × 20 cm). Acetonitrile was evaporated in vacuo at 40°C; the remaining aqueous solution was frozen, and the resulting solid was lyophilized. The yield was approximately 90%. The extent of incorporation of sugar ligands and tyramine was determined by monosaccharide analysis after acid hydroly-

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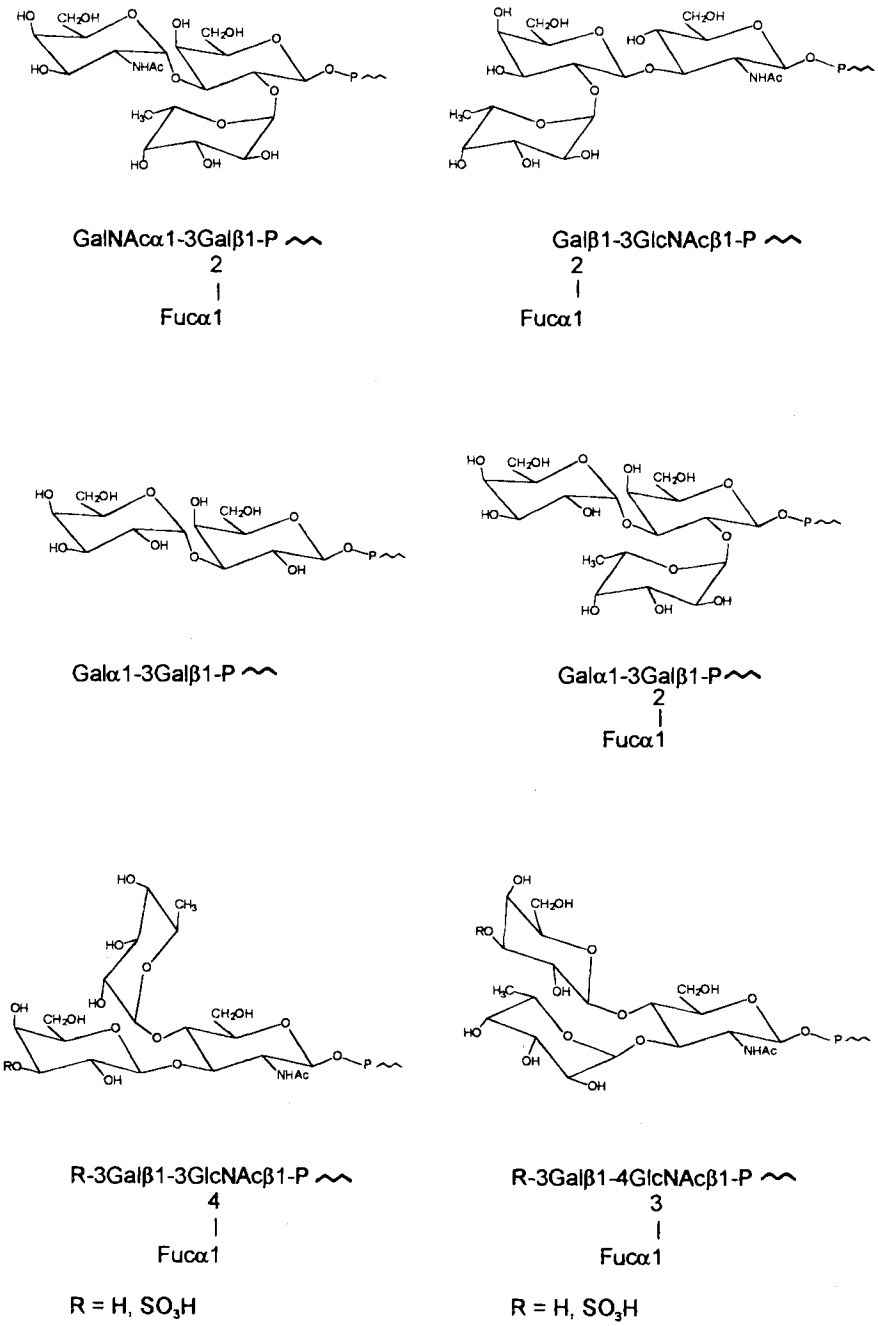

Fig. 1. Structural representation of the carbohydrate ligand part of the blood group determinant-carrying neoglycoconjugates, namely A-trisaccharide (a), H(type 1)-trisaccharide (b), B-disaccharide (c), B-trisaccharide (d), Le^a-trisaccharide (e) and Le^x-trisaccharide (f).

sis and by UV absorbance measurements. Doxorubicin was covalently attached to carbohydrate-free bovine serum albumin (BSA) with glutardialdehyde as a crosslinker, as described (14). Albumin-conjugated phosphorylcholine was obtained by diazonium coupling of the respective derivative, prepared from p-nitrophenylphosphorylcholine (Sigma, Munich, FRG), after catalytic reduction and reaction with $NaNO_2$ in 0.1 N HCl, as described (15).

The neoligandoproteins were biotinylated with biotinyl-N-hydroxysuccinimide ester, which was also employed in the labeling of the aminoalkyl derivative of heparin, originating from mild cyanogen bromide activation and incorporation of diaminohexane, as described (16). Neoglycopolymers were labeled by incorporating a spacered NH_2-terminating derivative of biotin, as given in detail previously (12).

Radioiodination and Biodistribution Studies

The tyramine-harboring neoglycoconjugates were iodinated with ^{125}I-NaI by the chloramine-T method using limiting amounts of reagents, yielding a specific activity of 2.4 MBq/mg polymer (17). For the measurement of biodistribution in

tumor-bearing mice, 7 week-old ddY mice (Nihon Clea Co., Tokyo, Japan) received a subcutaneous inoculation with 2×10^7 Ehrlich ascites tumor cells, which had been suspended in sterilized phosphate-buffered saline, into the right rear leg. The iodinated neoglycopolymer was injected intravenously into the tail vein at a dose of about 6.0 KBq per animal seven days after tumor inoculation. The percentage of the injected dose per gram of wet tissue or ml of blood retained was calculated after the determination of the ^{125}I-radioactivity in a γ-counter for 3–4 animals per group, as defined by the type of injected polymer and the period between injection and sacrificing, namely 1 h, 6 h or 24 h.

Flow Cytofluorimetric Measurements

A selection of human tumor cell lines of different histogenetic origin was purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured, as recommended by the distributor. The pre-B cell line was kindly provided by Dr. B. Woermann (Goettingen, FRG) and the B-lymphoblastoid cell line Croco II was established and maintained *in vitro*, as given in detail elsewhere (18). The myeloma line RPMI 8226 and a T-lymphoblastoid line (CCRF-CEM) supplemented the array of lymphoid cell lines. The designations of the leukemia cell lines are as follows: a monocytic leukemia line (THP-1), an erythroleukemia line (K-562), two acute myelogenous leukemia lines (KG-1, KG-1a), and a promyelocytic line (HL-60). Cell lines from different types of carcinomas completed the panel of tumor cells: three mammary carcinoma lines (DU4475, ZR-75-30, T-47D), two prostate carcinoma lines (LNCaP, DU-145), three colon carcinoma lines (SW480, SW620, COLO 205), one ovarian carcinoma line (NIH:OVCAR-3) and one embryonic carcinoma line (Tera-2). Automated flow cytofluorimetric analysis of cells, which had been carefully washed to remove serum constituents, started with an incubation of aliquots from the cell suspension (4×10^5 cells per assay) with the labelled substance (either 50 μg biotinylated neoglycopolymer/heparin and 25 μg doxorubicin-BSA conjugate per ml of Dulbecco's phosphate-buffered saline solution containing 0.1% BSA or 25 μg phosphorylcholine-BSA conjugate per ml of 20 mM Hepes (pH 7.2) containing 5 mM $CaCl_2$ and 0.1% BSA) for 30 min at 4°C to reduce endocytic uptake. Streptavidin-R-phycoerythrin conjugate (1:40 dilution; Sigma, Munich, FRG) was used as an indicator for quantitation of specific probe binding on the surface of thoroughly washed cells by a FACScan instrument (Becton-Dickinson, Heidelberg, FRG) equipped with the software CONSORT 30.

RESULTS

The activated ester, namely poly(4-nitrophenylacrylate), ensures quantitatively and reproducibly incorporation of NH_2-group-presenting substances into the polymer. These process steps and a representation of part of the structure of the final product are schematically depicted in Fig. 2. Oligosaccharide ligand and tyramine conjugation with the carrier was adjusted to a final yield of 20 mol% saccharide and 1 mol% iodinatable moiety. The polymer displayed an average molecular weight of 30 kDa, as determined by gel filtration and ultrafiltration. The readily water-soluble product was iodinated by a standard procedure (specific activity of 2.4 MBq/mg polymer). To

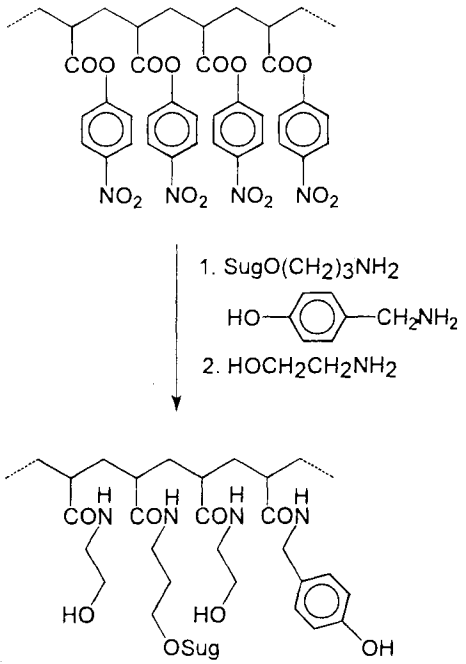

Fig. 2. Schematic representation of the production of the final carbohydrate ligand- and tyramine-bearing and fully ethanolamine-blocked polyacrylamide derivative, generated from the activated ester poly(4-nitrophenylacrylate) and incorporation of ligand and label.

determine its properties *in vivo*, a constant amount of each polymer preparation was injected intravenously into the tail vein of solid Ehrlich tumor-bearing mice. Besides monitoring the retained percentage of the injected dose per ml of blood or per g of tissue in various organs, accumulation of radioactivity was also measured in the tumor, which had been implanted by tumor cell inoculation seven days prior to the injection of the iodinated test substance. It is obvious that the carbohydrate ligand-free carrier showed a distinct preference for kidney tissue in mice (Tables I–III). The amount of radioactivity was not constant; it decreased from 1 h to 24 h after the injection by more than 50%. The neoglycoconjugates differed from the ethanolamine-blocked polymer (unsubstituted poly[N-(2-hydroxyethyl)acrylamide]) in the exchange of 20 mol% ethanolamine by the carbohydrate ligand. As already emphasized in the introduction, the structure of the oligosaccharide is the only parameter, having been altered within the panel of the tested neoglycoconjugates, to delineate ligand structure-dependent organ retention.

Minimal structural changes of the ligand involving one saccharide moiety can indeed influence the organ content, as shown in the comparison of the ABH-blood group determinants. Accumulation of the H(type1)-trisaccharide-bearing neoglycoconjugates in liver was markedly less than that of the carriers with A- or B-determinants, whereas the relation for retention in kidneys was inverse (Tables I–III). Besides these two organs, only spleen contained a share of injected dose that exceeded 1% per gram of tissue. As likewise observed for kidney, the introduction of a sulfate group into the two Le-determinants effected increases in the content of radioactivity for spleen. Conversely, the relative amount of these neoglycopolymers in the liver decreased when compared to the non-sulfated Le-

Table I. Biodistribution of ^{125}I-neoglycoconjugates (% Injected Dose/g Tissue) in Ehrlich Solid Tumor-Bearing Mice After 1 h[a]

PAA-derivatives / Tissues	PAA-Tyr	H (type 1)-PAA-Tyr	A (tri)-PAA-Tyr	B (di)-PAA -Tyr	B (tri)-PAA-Tyr	Le^a-PAA-Tyr	HSO_3 -Le^a-PAA-Tyr	Le^x-PAA-Tyr	HSO_3-Le^x-PAA-Tyr
Blood	2.29 ± 0.06	2.56 ± 0.10	0.43 ± 0.01	0.40 ± 0.02	0.64 ± 0.02	0.45 ± 0.03	0.71 ± 0.04	0.73 ± 0.03	1.63 ± 0.11
Liver	5.05 ± 0.25	7.87 ± 0.25	22.40 ± 0.86	31.65 ± 1.71	29.12 ± 0.92	30.88 ± 1.21	11.32 ± 0.63	31.57 ± 0.94	14.68 ± 1.20
Kidneys	55.75 ± 2.52	5.58 ± 0.39	0.36 ± 0.04	0.67 ± 0.02	2.29 ± 0.38	0.68 ± 0.12	43.84 ± 3.50	0.93 ± 0.09	37.53 ± 4.46
Spleen	1.86 ± 0.09	2.02 ± 0.16	0.78 ± 0.14	0.37 ± 0.05	0.64 ± 0.02	1.51 ± 0.21	5.27 ± 0.10	1.09 ± 0.02	4.72 ± 0.46
Heart	0.40 ± 0.06	0.54 ± 0.03	0.13 ± 0.02	0.22 ± 0.03	0.28 ± 0.02	0.28 ± 0.07	0.45 ± 0.05	0.29 ± 0.01	0.62 ± 0.03
Lung	0.63 ± 0.05	0.48 ± 0.01	0.26 ± 0.03	0.39 ± 0.07	0.34 ± 0.02	0.31 ± 0.04	0.53 ± 0.09	0.42 ± 0.04	0.57 ± 0.03
Thymus	0.48 ± 0.02	0.39 ± 0.08	0.09 ± 0.02	0.22 ± 0.03	0.30 ± 0.04	0.26 ± 0.06	0.29 ± 0.03	0.31 ± 0.04	0.47 ± 0.06
Pancreas	0.51 ± 0.08	0.22 ± 0.01	0.07 ± 0.01	0.14 ± 0.01	0.18 ± 0.01	0.22 ± 0.02	0.41 ± 0.06	0.30 ± 0.02	0.52 ± 0.05
Muscle	0.23 ± 0.01	0.14 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.04	0.07 ± 0.00	0.16 ± 0.02	0.11 ± 0.02	0.25 ± 0.02
Vertebrae	0.61 ± 0.04	0.45 ± 0.02	0.09 ± 0.03	0.11 ± 0.00	0.18 ± 0.01	0.18 ± 0.01	1.39 ± 0.12	0.22 ± 0.01	1.09 ± 0.08
Brain	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
Tumor	0.91 ± 0.03	0.71 ± 0.06	0.22 ± 0.03	0.26 ± 0.02	0.48 ± 0.02	0.34 ± 0.05	0.60 ± 0.05	0.54 ± 0.02	0.83 ± 0.03

[a] Each value represents the mean ± SEM for three or four animals and an injected dose of 2–2.5 μg (≈6.0 KBq per animal).

Table II. Biodistribution of ^{125}I-neoglycoconjugates (% Injected Dose/g Tissue) in Ehrlich Solid Tumor-Bearing Mice After 6 h[a]

PAA-derivatives / Tissues	PAA-Tyr	H (type 1)-PAA-Tyr	A (tri)-PAA-Tyr	B (di)-PAA-Tyr	B (tri)-PAA-Tyr	Le^a-PAA-Tyr	HSO_3-Le^a-PAA-Tyr	Le^x-PAA-Tyr	HSO_3-Le^x-PAA-Tyr
Blood	0.32 ± 0.04	0.89 ± 0.08	0.13 ± 0.02	0.22 ± 0.02	0.16 ± 0.03	0.40 ± 0.13	0.29 ± 0.02	0.44 ± 0.06	0.37 ± 0.07
Liver	2.04 ± 0.09	13.20 ± 0.39	18.30 ± 1.45	35.47 ± 2.91	19.96 ± 1.58	31.16 ± 3.26	16.30 ± 0.93	25.10 ± 1.56	9.77 ± 0.05
Kidneys	38.24 ± 2.89	7.27 ± 0.89	0.29 ± 0.06	0.67 ± 0.05	1.67 ± 0.16	0.89 ± 0.06	41.35 ± 3.02	0.67 ± 0.12	20.48 ± 2.73
Spleen	0.84 ± 0.09	2.87 ± 0.58	0.45 ± 0.12	0.20 ± 0.02	0.29 ± 0.03	0.27 ± 0.10	6.58 ± 0.60	0.70 ± 0.09	2.92 ± 0.06
Heart	0.17 ± 0.02	0.56 ± 0.07	0.14 ± 0.02	0.15 ± 0.02	0.15 ± 0.01	0.25 ± 0.05	0.48 ± 0.08	0.24 ± 0.02	0.43 ± 0.06
Lung	0.17 ± 0.02	0.40 ± 0.02	0.15 ± 0.03	0.20 ± 0.02	0.15 ± 0.02	0.32 ± 0.10	0.58 ± 0.02	0.32 ± 0.03	0.39 ± 0.07
Thymus	0.20 ± 0.03	0.37 ± 0.03	0.08 ± 0.04	0.13 ± 0.01	0.12 ± 0.02	0.26 ± 0.07	0.36 ± 0.11	0.29 ± 0.03	0.30 ± 0.06
Pancreas	0.17 ± 0.03	0.35 ± 0.03	0.06 ± 0.00	0.11 ± 0.01	0.10 ± 0.00	0.32 ± 0.08	0.38 ± 0.04	0.22 ± 0.02	0.38 ± 0.06
Muscle	0.06 ± 0.00	0.16 ± 0.02	0.03 ± 0.004	0.04 ± 0.00	0.04 ± 0.00	0.07 ± 0.01	0.13 ± 0.01	0.06 ± 0.01	0.13 ± 0.02
Vertebrae	0.22 ± 0.03	0.52 ± 0.06	0.06 ± 0.017	0.08 ± 0.02	0.08 ± 0.00	0.23 ± 0.04	0.27 ± 0.08	0.13 ± 0.01	0.73 ± 0.02
Brain	0.01 ± 0.00	0.23 ± 0.00	0.01 ± 0.002	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Tumor	0.32 ± 0.04	0.84 ± 0.06	0.11 ± 0.03	0.18 ± 0.02	0.21 ± 0.01	0.35 ± 0.04	0.48 ± 0.03	0.34 ± 0.05	0.53 ± 0.05

[a] Each value represents the mean ± SEM for three or four animals and an injected dose of 2–2.5 μg (≈6.0 KBq per animal).

Table III. Biodistribution of ^{125}I-neoglycoconjugates (% Injected Dose/g Tissue) in Ehrlich Solid Tumor-Bearing Mice After 24 h[a]

PAA-derivatives / Tissues	PAA-Tyr	H (type 1)-PAA-Tyr	A (tri)-PAA-Tyr	B (di)-PAA-Tyr	B (tri)-PAA-Tyr	Le^a-PAA-Tyr	HSO_3-Le^a-PAA-Tyr	Le^x-PAA-Tyr	HSO_3-Le^x-PAA-Tyr
Blood	0.01 ± 0.01	0.18 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.08 ± 0.01	0.16 ± 0.01	0.07 ± 0.00
Liver	3.85 ± 0.17	11.46 ± 0.66	30.00 ± 1.21	30.46 ± 0.97	29.55 ± 2.77	31.95 ± 1.28	13.76 ± 0.77	23.82 ± 1.80	12.57 ± 0.67
Kidneys	21.40 ± 1.73	6.05 ± 0.40	0.16 ± 0.02	0.40 ± 0.02	1.09 ± 0.09	0.31 ± 0.02	31.73 ± 3.14	0.41 ± 0.05	20.08 ± 2.60
Spleen	1.60 ± 0.13	2.83 ± 0.28	0.83 ± 0.06	0.22 ± 0.01	0.57 ± 0.10	1.31 ± 0.17	5.36 ± 0.38	1.08 ± 0.21	4.24 ± 0.34
Heart	0.12 ± 0.01	0.37 ± 0.03	0.12 ± 0.01	0.07 ± 0.00	0.18 ± 0.03	0.01 ± 0.00	0.34 ± 0.04	0.13 ± 0.02	0.30 ± 0.02
Lung	0.10 ± 0.01	0.24 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.17 ± 0.01	0.08 ± 0.01	0.37 ± 0.06	0.15 ± 0.01	0.28 ± 0.02
Thymus	0.15 ± 0.03	0.48 ± 0.06	0.10 ± 0.01	0.09 ± 0.00	0.12 ± 0.00	0.05 ± 0.02	0.19 ± 0.02	0.32 ± 0.06	0.19 ± 0.03
Pancreas	0.14 ± 0.00	0.20 ± 0.002	0.08 ± 0.01	0.74 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.26 ± 0.03	0.16 ± 0.02	0.33 ± 0.03
Muscle	0.05 ± 0.01	0.16 ± 0.01	0.04 ± 0.006	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.08 ± 0.01	0.06 ± 0.05	0.10 ± 0.01
Vertebrae	0.34 ± 0.02	0.47 ± 0.03	0.06 ± 0.008	0.03 ± 0.00	0.08 ± 0.01	0.16 ± 0.01	1.07 ± 0.07	0.11 ± 0.01	1.00 ± 0.04
Brain	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.002	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00
Tumor	0.24 ± 0.03	0.70 ± 0.04	0.16 ± 0.016	0.07 ± 0.00	0.21 ± 0.03	0.13 ± 0.01	0.22 ± 0.02	0.32 ± 0.06	0.34 ± 0.06

[a] Each value represents the mean ± SEM for three or four animals and an injected dose of 2–2.5 μg (≈6.0 KBq per animal).

epitopes. It is interesting to note that the organ levels often appeared to be quantitatively maintained over the 24 h period. The differences in the biodistribution patterns of the A- and B-trisaccharides underscored that a slight structural difference will have a detectable impact on organ arrest, pointing to underlying recognitive interactions. Similarly, a change at the penultimate sugar unit, i.e., fucose introdution into the B-disaccharide, evoked measurably different responses in kidney uptake (Tables I–III). Among the tested probes, the H(type1)-trisaccharide-containing neoglycoconjugates yielded the relatively highest extent of tumor retention with 0.7% of injected dose per g tumor after 24 h. This result prompted a need to determine the binding of ABH-blood group oligosaccharide-exposing neoglycoconjugates to cultured human tumor cells of different histogenetic origin.

Binding was detected by flow cytofluorimetric analysis for human lymphoma, leukemia and carcinoma cells (Table IV; Fig. 3a). The observed differences between either lines from different tumor types for aliquots of the same probe or the three probes with different carbohydrate ligand part for aliquots of the same cell line batch were quantitative without exception. Notably, individual lines from the same type of epithelial tumor, i.e., breast, prostate or colon carcinoma, revealed non-uniform binding properties, expressed as percentage of positive cells and median fluorescence (Table IV). Besides sugar moieties as targeting unit for a labeled carrier, other structural elements may be useful. To illustrate the feasibility of employing structurally completely different compounds for conceptually identical studies, we measured tumor cell binding with a carrier-immobilized chemotherapeutical agent, i.e., doxorubicin, and a probe with affinity to phospholipid-binding proteins such as annexins. Flow cytometric analysis led to results which reinforced dependence on the nature of the cell type (Table V, Fig. 3b, c). This aspect was further graphically illustrated for labeled heparin, whose application required no polymer conjugation. The median fluorescence after exposure to the proteoglycan ranged from 5.4 for a colon carcinoma cell line to 426.1 for an ovarian carcinoma cell line and the percentage of positive cells from 5.7 to 83.7 (Table V, Fig. 3d).

DISCUSSION

The itinerary of derivatized carbohydrate ligands between injection and excretion, which is guided by presence of tissue lectins, offers the possibility for diagnostic and therapeutic exploitation. Convenient labeling has been rendered feasible for carrier-free oligosaccharides by tagging with fluorescent, radioactive or iodinatable groups (19–22). Systematic variations in the structure of N-linked complex-type oligosaccharides encompassing the number of antennae, their stepwise size reduction down to the trimannoside core region and the presence of core fucosylation entailed ensuing alterations in the biodistribution profile (20,23). Whereas the presence or absence of core fucosylation did not influence the uptake of the agalacto derivative of a biantennary neoglycopeptide into salivary gland tissue, its presence slowed the elimination rate of a biantennary carbohydrate unit with lacdiNAc-termini from liver, leading to decreased accumulation in the small intestine (20,23). These studies also convincingly documented that a derivative formation with acetyltyrosine or tyrosinamide will not compromise the targeting capacity of the sugar ligands. A similar conclusion has been reached on the basis

Table IV. Flow Cytofluorimetric Analysis of Binding of Carrier-Immobilized A, B and H (Type 1) Blood Group Trisaccharides to Human Tumor Cell Lines of Diverse Histogenetic Origin[a]

	A (tri)-PAA (50 μg/ml)		B (tri)-PAA (50 μg/ml)		H (type 1)-PAA (50 μg/ml)	
Type of cell line	% of positive cells	Median fluorescence	% of positive cells	Median fluorescence	% of positive cells	Median fluorescence
BLIN-1	16.8	4.0	23.0	4.1	38.0	7.6
Croco II	12.8	4.7	13.9	5.1	16.7	6.0
RPMI 8226	28.5	23.5	23.7	23.4	32.2	31.7
CCRF-CEM	15.1	8.5	15.4	9.1	38.3	12.2
THP-1	22.2	9.3	26.6	11.5	28.5	12.8
K-562	18.2	6.4	21.6	6.8	27.9	8.1
KG-1	13.3	5.7	6.2	5.5	6.2	6.0
KG-1a	17.8	4.1	21.3	3.9	17.7	5.2
HL-60	7.8	5.8	11.7	6.6	8.6	10.4
DU4475	12.7	23.9	14.5	23.6	21.5	29.7
ZR-75-30	13.6	12.0	10.8	10.5	12.9	12.9
T-47D	28.0	6.4	13.0	11.9	20.0	8.1
LNCaP	12.5	8.5	19.4	9.7	23.4	10.5
DU-145	25.3	18.6	29.1	21.4	24.2	18.0
SW480	16.2	5.7	19.1	6.3	30.7	8.9
SW620	17.1	5.5	19.5	6.0	34.9	10.2
COLO 205	7.4	6.7	7.0	5.8	7.6	6.7
NIH:OVCAR-3	24.8	16.9	21.7	15.1	26.6	17.5
Tera-2	9.6	10.4	12.0	12.6	3.1	12.5

[a] The median fluorescence of the binding of the fluorescent probe in the absence of biotinylated blood group substance (background binding) varied in the range from 3.0 (BLIN-1) to 17.8 (DU4475).

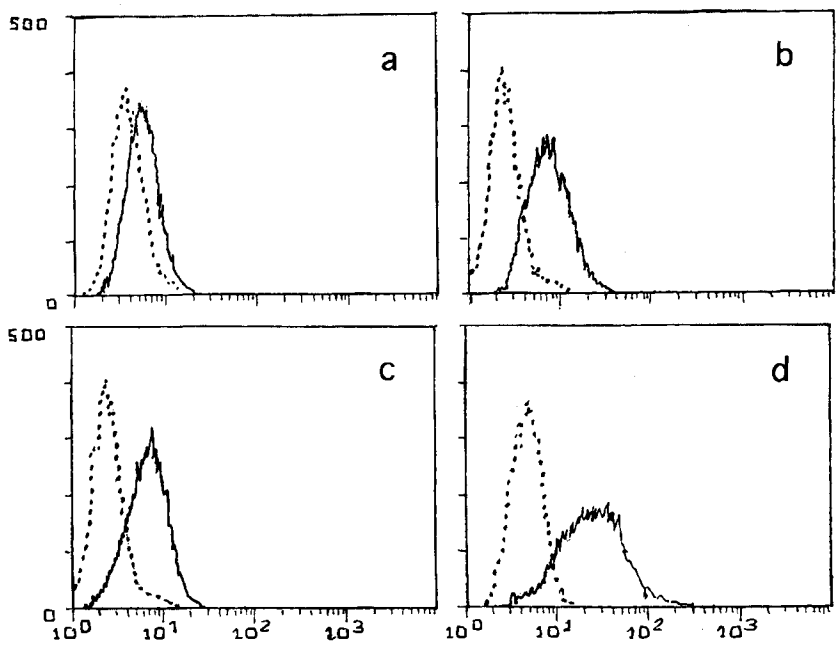

Fig. 3. Flow cytofluorimetric analysis of tumor cell binding of biotinylated markers, namely H(type 1)-exposing neoglycoconjugate (a), doxorubicin-albumin conjugate (b), phosphorylcholine-albumin conjugate (c) and heparin (d), to cells of the human T-lymphoblastoid line CCRF-CEM (a), the human pre-B line BLIN-1 (b, c) and the erythroleukemia line K-562 (d) (solid line: staining in the presence of biotinylated substance; dashed line: control experiment).

Table V. Flow Cytofluorimetric Analysis of Binding of Serum Albumin-Immobilized Doxorubicin and Phosphorylcholine as Well as Heparin to Human Tumor Cell Lines of Diverse Histogenetic Origin[a]

	Doxorubicin-BSA (25 μg/ml)		Phosphorylcholine-BSA (25 μg/ml)		Heparin (50 μg/ml)	
Type of cell line	% of positive cells	Median fluorescence	% of positive cells	Median fluorescence	% of positive cells	Median fluorescence
BLIN-1	76.5	17.3	70.7	8.7	53.7	20.5
Croco II	31.5	13.1	17.6	8.6	54.7	54.9
RPMI 8226	16.5	54.6	26.9	22.6	55.4	279.6
CCRF-CEM	32.0	14.0	11.0	8.3	57.5	45.7
THP-1	40.2	37.1	21.2	9.2	50.7	30.8
K-562	25.0	14.4	16.4	8.8	83.7	62.3
KG-1	17.1	13.9	14.4	7.6	26.0	45.2
KG-1a	29.0	9.7	28.6	4.8	53.1	18.2
HL-60	47.2	43.2	9.9	9.7	38.7	96.3
DU4475	20.2	95.3	22.5	33.9	22.9	130.3
ZR-75-30	24.1	104.6	12.3	10.3	36.9	88.1
T-47D	15.5	107.0	12.4	20.1	25.7	96.9
LNCaP	39.8	50.9	14.2	11.7	49.9	181.6
DU-145	41.6	185.3	41.9	38.8	60.3	322.1
SW480	26.3	11.3	24.8	8.0	48.1	58.8
SW620	34.0	12.3	23.4	7.7	58.4	59.7
COLO 205	16.0	26.3	19.2	12.0	5.7	5.4
NIH:OVCAR-3	20.3	39.3	29.8	26.1	54.7	426.1
Tera-2	16.0	57.2	25.3	32.1	47.8	166.3

[a] The median fluorescence of the binding of the fluorescent probe in the absence of biotinylated substance (background binding) varied in the range from 3.0 (BLIN-1) to 17.8 (DU4475).

of extensive experience with neoglycoproteins (2–6). Moreover, their preparation nets an important additional profit.

Conjugation of these routing devices to a polymeric backbone establishing a neoglycoconjugate can be followed by an advantageous cluster effect, conferring increased avidity for tissue lectin binding (7,24). As shown for interaction between neoglycoproteins or neoglycoenzymes with receptors on cell surfaces *in vitro* or in tissue sections, the carbohydrate ligands maintain their inherent selectivity to respective tissue lectins, unless the coupling yield exceeds critical limits (3,7,24,25). Taking advantage of the potential of (neo)glycopolymers to co-transport another substance such as a therapeutic agent, lectin-mediated endocytosis can find a place beyond basic science. Preferentially, respective studies have focused on the C-type lectins of hepatocytes and macrophages which serve in importing the conjugate into the lysosomal compartment (2–6,25).

Due to the natural physiological barriers which limit access of a circulating marker to parenchymal or tumor cells at various sites and the presence of abundant and potentially interfering serum glycoproteins it is an indispensable step to perform biodistribution studies following the chemical design. Respective studies with bovine serum albumin as a carrier have emphasized the importance of the ligand density and the type of linker connecting the ligand derivative to albumin for organ accumulation already on the level of the monosaccharide (26). As a substitute for the protein, biocompatible synthetic polymers can reduce the immunological response to the delivery compound, which has to fulfill several prerequisites for meaningful testing. The final soluble product should be stable under physiological conditions. Its generation should be reproducible with high yield, even when working with delicate ligands. As an alternative to the copolymerization of acrylamide and olefinic group-containing carbohydrate ligands as well as label-introducing derivatives, e.g., N-2-propenyl-(5-dimethylamino)-1-naphthalene sulfonamide (27,28), we herein show the suitability of incorporating tyramine into poly(4-nitrophenylacrylate) in addition to the aminopropyl derivatives of custom-made oligosaccharides. The lack of biodegradibility of such a backbone results in prolonged organ retention of polymer-bound radioactivity, as shown for p-vinylphenyl derivatives as monomeric building blocks for polymeric carriers (29). The marked difference between the profiles of accumulation of the carbohydrate ligand-free radioiodinated polymer and the array of neoglycopolymers can be judged as a measure of the confered selectivity, as also seen in the comparison between the different conjugates. Relative to already tested A- and B-trisaccharide-bearing neoglycoproteins retention of the respective neoglycopolymers in liver is more pronounced. Evidently, the sulfation of Le-epitopes reduces this parameter, which is primarily attributable to the presence of the hepatocyte lectin and to a lesser degree to expression of the fucose-specific Kupffer cell receptor (30,31). The fact that the hepatic receptors in mice and rats exhibit different discriminatory potency for biantennary Le^x-containing oligosaccharides and their non-fucosylated counterparts cautions against premature generalizations with clinical perspective. In view of the documented heterogeneity of marker expression in cell line populations by the FACScan measurements it is likewise appropriate to be sparing with the appraisal of tumor cell targeting. It should also not be overlooked that the actual extent of receptor presence can be modulated significantly by the individual organ environment, shown, e.g., histochemically for neoglycoprotein-binding sites (lectins) in the case of 3LL carcinomas at different sites (32). These unavoidable obstacles notwithstanding, the visualization of at least subpopulations within heterogeneous tumor entities can be of diagnostic value. The described *in vitro* experiments substantiate that the basis to introduce neoligandoconjugates to systematic *in vivo* studies beyond assaying tailor-made oligosaccharides is established. Combined chemical synthesis and *in vivo* monitoring will thus be essential in eventually assessing the extent of clinical applicability of iodinated ligand-bearing poly(4-nitrophenylacrylate).

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