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Design of a multivalent galactoside ligand for selective targeting of HPMA copolymer–doxorubicin conjugates to human colon cancer cells

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Abstract

N-(2-hydroxypropyl)methacrylamide (HPMA)-based copolymers have been shown to be efficient carriers for anticancer drugs because of their versatile chemistry and good biocompatibility. As demonstrated with hepatocytes, targeting efficacy of anticancer drugs could be further improved when the drug (doxorubicin) was conjugated to HPMA copolymers with biorecognisable groups, such as simple carbohydrates. The present study was devised to learn whether the cluster (multivalent) construction of carbohydrate residues could improve the targeting capability of HPMA copolymer–doxorubicin (DOX) conjugates towards human colon adenocarcinoma cells. DOX was linked via a lysosomally degradable tetrapeptide sequence to HPMA copolymers bearing galactosamine (GalN), lactose (Lac), or multivalent galactose residues (TriGal) to produce targetable polymeric drug carriers. The effect of the type of sugar moiety and its three-dimensional cluster arrangement on biorecognition by three human colon-adenocarcinoma cell lines was studied. The role of galectin-3 in the biorecognition of HPMA copolymer conjugates was explored. Biorecognition of the targetable (glycoside-bearing) conjugates decreased their IC_{50} doses in comparison to the non-targetable (non-glycosylated) conjugates. The biorecognition of the TriGal-containing HPMA copolymer-doxorubicin conjugate by the cells was superior with concomitant decrease of its IC_{50} doses. It is suggested that the increased cytotoxicity of the glycosylated HPMA-copolymer–DOX conjugates toward human colon-adenocarcinoma cells was caused by their biorecognition and effective internalisation via receptor-mediated endocytosis. All three human colon adenocarcinoma cell lines tested, Colo-205, SW-480 and SW-620, expressed the galectin-3 protein and the galectin-3-specific RNA. However, contrary to expectation, Colo-205 cells did not express a detectable amount of galectin-3 on the cell surface. This suggests that the binding of the glycoside-bearing HPMA copolymer–DOX conjugates to the cells was mediated not only by galectin-3. We conclude that targeting of the anticancer agent, doxorubicin, using HPMA copolymer conjugates bearing multivalent galactoside residues can improve their cytotoxicity.

Keywords: HPMA copolymer; Adriamycin; Doxorubicin; Cluster glycosides; Drug targeting; Galectins; Glycoconjugate; Colon cancer

1. Introduction

Anticancer drugs bound to macromolecular carriers based on N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers containing monosaccharides have been tested in the context of targeted chemotherapy for cancer treatment [1,2]. HPMA copolymer–doxorubicin (DOX) conjugates containing fucosylamine moieties enhanced the survival time of mice bearing L1210 tumours in a dose- and route of administration dependent manner [3]. Biodistribution studies in mice revealed that incorporating galactosamine into side-chains of HPMA copolymer-DOX conjugates resulted in efficient liver targeting at low doses and receptor saturation at high doses [4]. HPMA copolymer-anticancer drug conjugates were found to be effective in a variety of animal cancer models when administered parenterally [5,6]. Pharmacokinetics and imaging studies have indicated the potential application of these drug delivery systems in the treatment of cancer [7].

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In previous studies, we reported on the targeting capabilities of naive (no drug) HPMA copolymer conjugates of galactosamine (GalN), lactose (Lac) and trivalent galactose (TriGal) to human colon adenocarcinoma and hepatocarcinoma cells [8,9]. The binding intensity was found to depend on the relative amount of saccharide moiety in the copolymer and on the three-dimensional arrangement of the galactose residues. Polymer conjugates bearing lactose moiety (P-Lac) and clusters of galactose residues (P-TriGal) displayed enhanced binding affinity towards hepatocytes when compared with (N-acylated) galactosamine-bearing conjugate (P-GalN) [9,10]. It was suggested that endocytosis, mediated by the asialoglycoprotein receptor (ASGP-R) of hepatocytes, was responsible for increased internalisation of the copolymers into the cells [11,12]. In another study, the binding affinity of the glycosylated HPMA–copolymer conjugates towards colon carcinoma cells was tested [8] and was found to be inferior when compared with hepatocytes. Furthermore, the rank order of the copolymer's binding was different: polymer conjugates bearing GalN and TriGal moieties displayed superior binding affinity towards colon cancer cells, followed by an enhanced internalisation into the cells [8].

The galectin family of carbohydrate receptors represents a promising target for drug delivery [13–15]. Individual members of the galectin family are expressed in neoplastic tissues [13,16], an observation which presents a fascinating opportunity to use carbohydrates as a targeting tool for the specific delivery of cytotoxic drugs in cancer therapy. With respect to colon biology, galectin-1 and galectin-3 have been most extensively studied. Two reports on transfected cells and tissue sections emphasise the potential relevance of galectin-3 for colon cancer metastasis [20,21]. However, the prototype of galectin-1 is encountered less frequently in colon cancer. Therefore, in this study, we first selected galectin-3 as a model receptor for targeting of glycoside-bearing drug carriers. Galectin-3 was previously shown to play a role in endocytosis of various glycoconjugates [22–24]. Thus, it attracted our attention in this context of lectin-mediated drug targeting, although contradictory reports on the nature of its expression in normal and neoplastic colonic epithelium have been published. While some reports describe the upregulation of galectin-3 in the progression of colorectal cancer [17–21,25,26], others proclaim the opposite [27,28]. These controversial data stimulated us to examine the galectin-3 protein levels and its specific RNA expression in three human colon adenocarcinoma lines. Recently, a comprehensive profile of all known human galectins in tumour cell lines from various sources has been published [29]. These data indicate that additional binding sites other than galectin-3 (e.g., galectin-1, -4, -7, or -8) could also be involved in the recognition process.

Based on the mechanism of cellular uptake and the subcellular trafficking of the naive glycosylated HPMA copolymer conjugates [8–10], we hypothesised that HPMA copolymer–DOX conjugates could be targeted to galectin-3 and serve as a site-specific drug-delivery system. With this in mind, we designed three types of targetable HPMA copolymer–DOX conjugates, containing GalN, Lac, and TriGal. Our aims were to evaluate the effect of the structure and cluster construction of carbohydrate residues on the targeting capability of HPMA copolymer–DOX conjugates towards human colon adenocarcinoma cells. Cytotoxicity towards the colon cancer cells was compared with the cytotoxicity towards the hepatocellular carcinoma cell line HepG2. These cells are known to express ASGP-R on the cell surface, and thus have high affinity for clusters of galactose residues.

2. Materials and methods

2.1. Abbreviations

AIBN, 2,2′-azobisisobutyronitrile; AHT, (6-aminohexanamido)-tris-(hydroxymethyl)methane; ASGP-R, asialoglycoprotein receptor; DMSO, dimethyl sulphoxide; DOX, doxorubicin; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; galactosamine; GFLG, the lysosomally degradable glycyl-phenyalanyl-leucyl-glycine spacer; HPMA, N-(2-hydroxypropyl)methacrylamide; IC_{50} dose, drug concentration which inhibits 50% of cell growth; Lac, lactose; MA-GG-GalN, N-methacryloylglycylglycylgalactosamine; MA-Ph-Lac, 4-methacryloylamidophenyl-β-lactoside; MA-AHT-$(Gal)_3$, N-methacryloylaminohexyl-trisgalactoside; MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide); M_w, average molecular weight; P, HPMA copolymer backbone; P-GalN, galactosamine-containing HPMA copolymer; P-Lac, lactose-containing HPMA copolymer; P-TriGal, trivalent galactose-containing HPMA copolymer; P-(GFLG)-DOX, HPMA copolymer bound-DOX; RT-PCR, reverse transcription polymerase chain reaction; TriGal, trivalent galactose cluster construct.

2.2. Cell lines, culture media and antibodies

The human colon-adenocarcinoma cell lines: Colo-205, SW-480 and SW-620 and the human hepatocarcinoma cell line, $HepG_2$, were purchased from the American Type Culture Collection (ATCC, Rockville, MD). L-15 Leibovitz medium, RPMI-1640 medium and Dulbecco's phosphate-buffered saline (DPBS) were obtained from Sigma, St. Louis, MO. MEM-α medium and FBS (fetal bovine serum) were obtained from HyClone (Ogden, UT). Anti-galectin-3 monoclonal antibody was purchased from Research Diagnostics, Inc. (Flanders, NJ). Fluorescein goat anti-mouse IgG (H + L) conjugate was

obtained from Molecular Probes (Eugene, OR). Anti-mouse IgG, peroxidase-linked species-specific whole antibody (from donkey) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

SW-480 and SW-620 cells were cultured in L-15 Leibovitz medium. Colo-205 cells were cultured in RPMI-1640 medium. $HepG_2$ cells were cultured in MEM-α medium. All media were supplemented with 10% FBS and grown at 37 °C, under 5% CO_2 (v/v) in air.

2.3. Monomers

The monomers, N-methacryloylglycylglycylgalactosamine (MA-GG-GalN) [30], 4-methacryloylamidophenyl-β-lactoside (MA-Ph-Lac) [9], and N-methacryloyl-6-(aminohexanamido) - tris - (β - D - galactopyranosyloxymethyl)methane (MA-AHT-GalN) [9] and N-methacryloylglycylphenyalanylleucylglycyl-doxorubicin (MA-(GFLG)-DOX) [31,32] were synthesised as previously described.

2.4. Synthesis of the HPMA copolymer bound-DOX (denoted as P-(GFLG)-DOX)

P-(GFLG)-DOX was synthesised as described elsewhere in Ref. [33]. The conjugate was purified on a Sephadex LH 20 (Pharmacia) column using methanol containing 10% dimethyl sulphoxide (DMSO) and 1% CH_3COOH as the eluent. The P-(GFLG)-DOX contained 7.8 wt.% (1.8 mol%) of DOX.

2.5. Synthesis of the targetable HPMA copolymer–DOX conjugates (Fig. 1)

The polymer conjugates were synthesised by direct copolymerisation of the saccharide-containing monomers with HPMA and MA-GFLG-DOX. Radical precipitation copolymerisation was performed in acetone/DMSO mixture at 50 °C for 40 h using AIBN as the initiator as described elsewhere in Refs. [34,35]. The concentration of monomers, with respect to initiator and solvent, was at a ratio of 12.5: 1.0: 86.5 wt.%, respectively. The characteristics of the polymers and the methods of characterisation used are summarised in Table 1.

P-(GFLG-DOX)-GalN (A) was prepared by copolymerisation of 20 mol% MA-GG-GalN (0.0232 g), 3 mol% MA-GFLG-DOX (0.0063 g) and HPMA (0.0205 g) in acetone/DMSO. The product was purified by precipitation to diethyl ether followed by separation of free DOX by chromatography on Sephadex G-25M column in water (with 30% acetonitrile). The conjugate was isolated after dialysis and freeze-dried.

P-(GFLG-DOX)-Lac (B) was prepared by copolymerising 20 mol% MA-Ph-Lac (0.0322 g) and 3 mol% MA-GFLG-DOX (0.0063 g) with HPMA (0.0205 g) in a similar procedure as described for P-(GFLG-DOX)-GalN above.

P-(GFLG-DOX)-TriGal (C) was prepared by copolymerising 20 mol% MA-AHT-TriGal (0.0500 g) and 3 mol% MA-GFLG-DOX (0.0063 g) with HPMA (0.0205 g) as described for P-(GFLG-DOX)-GalN above.

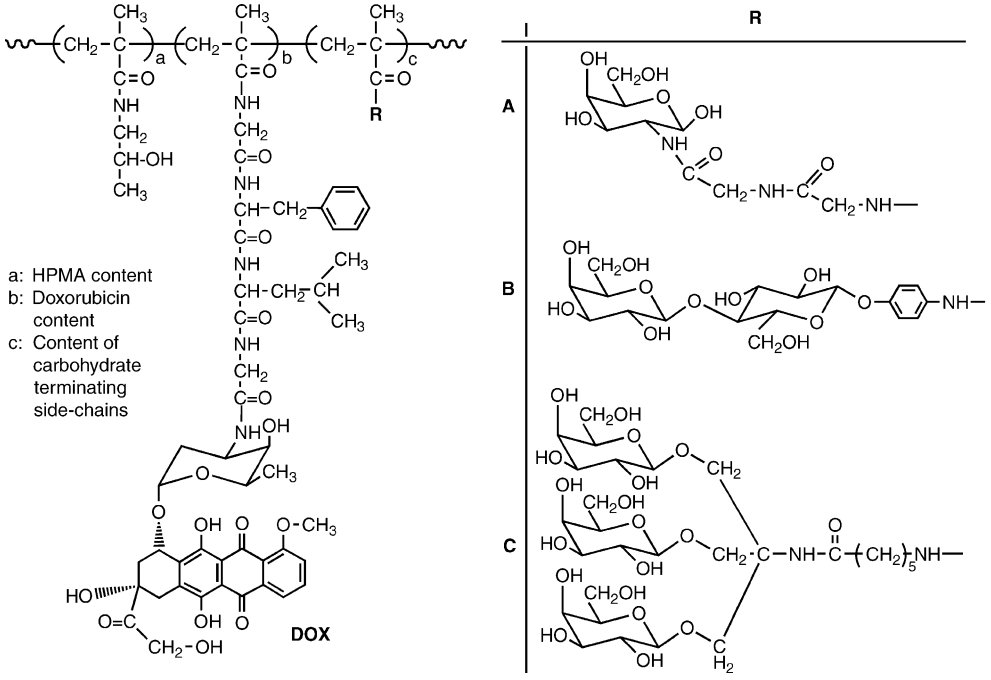

Fig. 1. Structure of the saccharide containing HPMA-copolymer–DOX conjugates and the corresponding pendant saccharide moieties (R-H): (a) glycylglycine-galactosamine; (b) 4-aminophenyl-lactose; (c) 6-(hexanamino)-tris-((β-D-galactopyranosyl)-oxymethyl)methane.

Table 1
Chemical characteristics of the HPMA copolymer-doxorubicin conjugates

HPMA copolymer conjugate	Mol% DOX[a]	Mol% saccharide moiety[b,c]	Approx. M_w of conjugate (kDa)[d]
P-(GFLG)-DOX[e]	1.8	–	23
P-(GFLG-DOX)-GalN[f]	1.5	16	46
P-(GFLG-DOX)-Lac[g]	1.4	21	20–32
P-(GFLG-DOX)-TriGal[h]	2.1	15	20–32

[a] The content of DOX was determined spectrophotometrically using ϵ 11 000 M^{-1} cm^{-1} (DOX, H_2O).
[b] The content of GalN was determined by colorimetric analysis (Morgan-Elson assay).
[c] The content of lactose and the trivalent galactose was determined by colorimetric phenol-sulphuric acid assay, after hydrolytic cleavage of the DOX quinone structure at pH 9.
[d] The average molecular weight (M_w) of the polymers was estimated by size-exclusion chromatography using Superose 12 column, fast performance liquid chromatography (FPLC) system, 30% CH_3CN/PBS buffer pH 7.3, calibrated with poly(HPMA) fractions.
[e] P-(GFLG)-DOX, HPMA copolymer–DOX conjugate.
[f] P-(GFLG-DOX)-GalN, galactosamine-containing HPMA copolymer–DOX conjugate.
[g] P-(GFLG-DOX)-Lac, lactose-containing HPMA copolymer–DOX conjugate.
[h] P-(GFLG-DOX)-TriGal, trivalent galactose-containing HPMA copolymer–DOX conjugate.

2.6. Analysis of galectin-3 gene expression in human colon carcinoma cells

2.6.1. RNA preparation and cDNA synthesis by reverse transcription

Total cellular RNA from the human colon adenocarcinoma cell lines was isolated using an RNeasy kit (Qiagen) and a QIAshredder micro-spin homogeniser (Qiagen). First-standard cDNA was synthesized by Ready-To-Go You-Prime First-standard beads (Pharmacia) according to the manufacturer's instructions with 5 μg of total cellular RNA (from 1×10^6 cells) and 100 ng of random hexadeoxynucleotide primer (Pharmacia). After synthesis, the reaction mixture was diluted 1:3 with water and immediately subjected to polymerase chain reaction (PCR).

2.7. Analysis of Mac-2 gene expression by reverse transcriptase (RT)-PCR

A combination of reverse transcription (RT) and polymerase chain reaction (PCR) was used for the analysis of *Mac-2* gene expression. The β_2 microglobulin (β_2m) mRNA was used as an internal standard during analysis [33]. *Mac-2*-specific sequences were amplified by using the sense-strand primer GGCCACTGATTGTGCCTTAT and the antisense-strand primer CTGTCTTTCTTCCCTTCCCC. Amplimers used for the amplification of β_2m were the sense-strand primer ACCCCCACTGAAAAAGATGA and the antisense-strand primer ATCTTCAAACCTCCATGATG.

PCR was carried out using an Air Thermocycler (Idaho Technology) with the diluted first-stranded reaction mixture, 1 unit of Taq Polymerase (GibcoBRL), and 0.5 μM of specific primers in a final volume of 50 μl. For the *Mac-2* gene, each cycle of PCR included 30 sec of denaturation at 94 °C, 1 min of annealing at 56 °C, and 2 min of elongation at 72 °C, for 30 cycles. Agarose gel electrophoresis was used for the separation of PCR products. MetaPhor agarose (FMC Bio products) at a concentration of 4% w/v in 0.5X TBE buffer (0.0445 M Tris/Borate, 0.001m ethylene diamine tetra acetic acid (EDTA), pH 8.3 (Research Organics Inc.) was selected for the separation. PCR products were separated by submarine electrophoresis using 5–10 V/cm voltage. The gels were stained with ethidium bromide and photographed with a Polaroid camera. To calculate the size and the amount of PCR products, a Low DNA Mass Ladder (GibcoBRL) was used. *Mac-2* gene expression was estimated as the ratio between the amounts of PCR product (area under the curve in the gel scan) corresponding to the mRNA of the *Mac-2* genes and those of the internal standard (β_2m).

2.8. Galectin-3 extraction and Western blotting analysis

1×10^6 cells were suspended using 0.25% trypsin (1×) solution and collected by centrifugation. The cells were rinsed twice with 2 ml cold PBS, re-suspended in 1 ml lysis buffer (25 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton-X-100, 1 mM phenyl methyl sulphonyl fluoride (PMSF), 10 mg/ml leupeptin, 10 mg/ml aprotinin), and incubated on ice for 30 min. The lysates were then cleared by centrifugation for 10 min at 14 000 rotations per minute (rpm). Equal aliquots of protein homogenate were resolved by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After a blocking step with 5% dry milk solution, the blots were incubated for 1 h at room temperature with mouse anti-galectin-3 monoclonal antibody (dilution 1:500) which recognises the 31-kilodalton protein. After washing with phosphate-buffered saline- 0.05% tween-20 (PBST) the blots were incubated with donkey anti-mouse IgG-horse radish peroxidase (HRP)-linked (dilution 1:5000). The membrane was rinsed again twice with PBST, and developed with enhanced chemilumi-

nescence Western-blotting detection reagent. The blots were visualised by exposure to autoradiography film [36]. The NIH Image Ver 1.61 program was used to quantify the bands on the blots.

2.9. Flow cytometry analysis

Colo-205, SW-480 and SW-620 cell monolayers were trypsinised to produce cell suspensions. 1×10^6 cells were suspended in PBS (pH 7.5) containing 1% bovine serum albumin (BSA). The cells were incubated with anti-galectin-3 (dilution 1:500) in PBS (pH 7.5) containing 1% BSA, for 1 h at 4 °C. Control cells were incubated in PBS (pH 7.5) containing 1% BSA under the same conditions. After incubation, cells were collected, rinsed twice with cold PBS, and incubated for another 30 min with the fluorescein–goat anti-mouse IgG conjugate (dilution 1:1000) in PBS (pH 7.5) containing 1% BSA. Cells were collected, rinsed twice with cold PBS, and cell-associated fluorescence was determined immediately using flow cytometry (excitation at 485 nm, emission at 525 nm).

2.10. Fluorescence microscopy analysis of the anti-galectin-3 binding to fixed colon adenocarcinoma cells

Colo-205, SW-480 and SW-620 cells were seeded on microscope cover slips and grown in a 6-well plate. After 24 h, the medium was removed and cells were incubated for 1 h at 4 °C with anti-galectin-3 (dilution 1:100) in PBS (pH 7.5) containing 1% BSA. Control cells were incubated in PBS pH 7.5 containing 1% BSA under the same conditions. After incubation, cells were washed twice with cold PBS, and incubated for another 30 min at 4 °C with the fluorescein–goat anti-mouse IgG conjugate (dilution 1:1000) in PBS (pH 7.5) containing 1% BSA. The cells were rinsed 3× with PBS and then fixed with 3%-paraformaldehyde. The differences between the binding patterns of the anti-galectin-3 antibody for the different adenocarcinoma cell lines were examined using fluorescence microscopy.

2.11. Cytotoxicity assay

All concentrations of targetable P-(GFLG)-DOX are expressed in DOX equivalents. All solutions were sterilised by filtering through a 0.2 μm membrane filter.

The cytotoxicity of free-DOX, non-targetable DOX conjugate and the three targetable DOX conjugates toward human colon adenocarcinoma cell lines, Colo-205, SW-480, SW-620, and human hepatocarcinoma $HepG_2$ cells was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide) (MTT) assay [37]. The results of the cytotoxicity assay were used to calculate the IC_{50} dose (drug concentration causing 50% growth inhibition) relative to a control of non-treated cells [38]. Cells were seeded into 96-well microtitre plates at a density of 10 000 cells per well. Twenty-four hours after plating, the medium was removed and 12 different concentrations of sterile products (free-DOX, targetable, or non-targetable HPMA copolymer–DOX conjugates) in fresh media were added, followed by 72 h of incubation at 37 °C, under 5% CO_2 (v/v) in air. Cell survival assay was performed by discarding the medium followed by the addition of 100 μl of fresh medium and 25 μl of 5 mg/ml MTT solution in DPBS to each well. Plates were incubated under cell culture conditions for 3 h. Formazan crystals were dissolved overnight in 50% (v/v) dimethylformamide in water containing 20% (w/v) SDS. The absorbance of each sample was measured at 570 nm with a background correction at 630 nm.

2.12. Statistics

IC_{50} data obtained were analysed using descriptive statistics, single factor analysis of variance (ANOVA) by Experimental Data Analysis Toll Pack from Microsoft Office-97 and a program written by Dr. V. Pozharov for non-linear regression and least squares analysis. The difference between the variants was considered significant when $P<0.05$.

3. Results

To verify the hypothesis that galectins may function as receptors for saccharide-containing drug carriers, the expression of galectin-3 in three human-colon adenocarcinoma cell lines was investigated. Western-blotting analysis showed the expression of the 31-kDa galectin-3 protein in all three human colon-adenocarcinoma cell lines tested. The expression of the galectin-3 was significantly higher in SW-480 and SW-620 than in the Colo-205 cell line (Fig. 2a). The relative intensity values of the Colo-205, SW-480 and SW-620 bands on the blots, as determined by the NIH imaging program, were 56: 96: 99, respectively. This was accompanied by a parallel difference in the relative abundance of galectin-3-specific RNA as determined by RT-PCR analysis (Fig. 2b). The analysis shows the corresponding differences in 271-bp galectin-3 RNA product in the three colon-adenocarcinoma cell lines. The mean relative intensities of the RT-PCR product bands derived from the *Mac-2* gene encoding the galectin-3 protein were 88: 124:100 for Colo-205, SW-480 and SW-620 cells, respectively.

Cell surface presentation of galectin-3, as determined by flow cytometry analysis, also revealed that SW-480 cells expressed higher levels of membranous galectin-3 than SW-620 cells (a 2.6-fold difference in the fluorescent intensity) (Fig. 3). However, the expression of

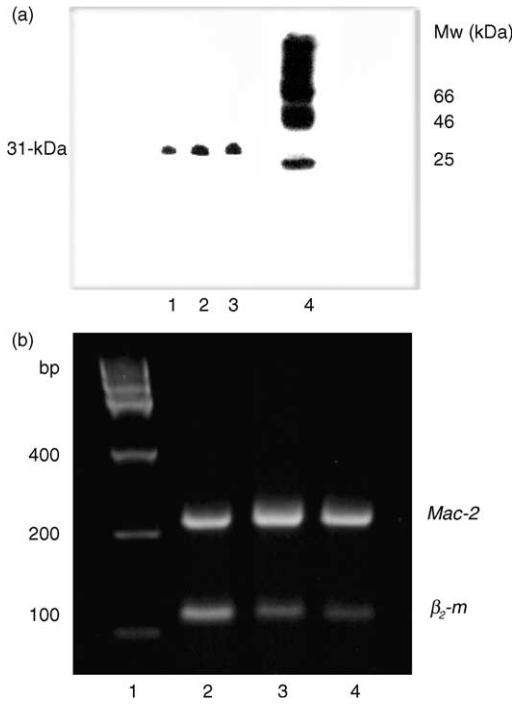

Fig. 2. (a) Expression of galectin-3 in human colon adenocarcinoma cells as determined by Western blotting analysis. Equal amounts of homogenate protein were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and subjected to Western-blotting analysis, as described in Materials and methods. Panels show typical images. 1—Colo-205 cells; 2—SW-480 cells; 3—SW-620 cells; 4—Molecular weight marker. (b) Expression of *Mac-2* gene encoding galectin-3 protein as determined by RT-PCR. Equal amounts of total RNA were subjected to RT-PCR using primers specific for the *Mac-2* gene. RT-PCR products were separated by agarose gel electrophoresis and stained by ethidium bromide. Panels show typical images. 1—DNA ladder; 2—Colo-205 cells; 3—SW-480 cells; 4—SW-620 cells.

galectin-3 could not be detected on the surface of Colo-205 cells by flow cytometry. Fluorescent images of the anti-galectin-3 antibody binding to the fixed colon-adenocarcinoma cells confirmed these observations (Fig. 4).

The cell cytotoxicity, measured by calculating the IC_{50} doses of the targetable and non-targetable HPMA copolymer–DOX conjugate in the three colon cancer cell lines, is shown in Table 2. The Table also shows the IC_{50} values of free-DOX in all three cell lines and the IC_{50} values measured in the hepatocarcinoma cells (for the purpose of comparison, as they were previously shown to bind efficiently to the three targetable HPMA copolymer– DOX conjugates [9], as well as the P-(GFLG)-DOX-containing GalN [7]). The HPMA homopolymer was tested with SW-480 cells and did not exert any cytotoxic activity, excluding the possibility of intrinsic growth inhibition (data not shown).

As expected, the lowest IC_{50} dose was calculated for the free-DOX and was found to be at least 10^3 times lower than the IC_{50} doses of the non-targetable conjugate P-(GFLG)-DOX. In SW-480, Colo-205 and in the control $HepG_2$ cells, the IC_{50} dose of the targetable HPMA copolymer–DOX conjugates was lower than the IC_{50} dose of non-targetable P-(GFLG)-DOX, suggesting that the targetable conjugates are more toxic to the cells. The cytotoxicity of the trivalent copolymer P-(GFLG-DOX)-TriGal was significantly higher than the monovalent product P-(GFLG-DOX)-GalN in the SW-480 and SW-620 cell lines only (cytotoxicity was at least 10 times higher for the clustered trivalent product). Table 2 also shows that, for Colo-205 and $HepG_2$, P-(GFLG-DOX)-GalN and P-(GFLG-DOX)-TriGal were more cytotoxic than P-(GFLG-DOX)-Lac.

4. Discussion

Non-specific toxicity of anticancer drugs is one of the major obstacles restricting their efficacy in cancer treatment. To overcome this difficulty and achieve selective chemotherapy, a drug may be directed to the tumour site by a selective drug delivery. The local increase of drug concentration allows doses to be lowered, which in turn, leads to a reduction in non-specific toxicity (reviewed in [39]). In previous reports, we demonstrated the targeting of native (without drug) HPMA copolymer conjugates of galactose (P-GalN), lactose (P-Lac) and trivalent galactose (P-TriGal) to human colon-adenocarcinoma and hepatocarcinoma cells. Their binding intensity was found to depend on the content of the

Table 2
IC_{50} doses (μM) in human colon adenocarcinoma cells and human hepatocarcinoma cells incubated with free DOX, HPMA copolymer–DOX conjugate and three types of targetable (glycoside bearing) HPMA copolymer–DOX conjugates

Sample	Colo-205	SW-480	SW-620	$HepG_2$
Free DOX	0.024±0.002	0.008±0.001	0.053±0.005	0.008±0.001
P(GFLG)-DOX	78.7±3.9	193±8.8	68±4.5	15.8±1.4
P(GFLG-DOX)-GalN	7.7±0.7	65±2.3	52.7±3.9	3.1±0.7
P(GFLG-DOX)-Lac	27.5±2.4	27.5±1.3	32.7±3.1	10.8±1.0
P(GFLG-DOX)-TriGal	9.7±0.6	3.8±0.2	5.1±0.2	2.7±0.2

Cells were exposed to 12 different concentrations of DOX (or the DOX equivalent in the case of the polymers) to determine the concentration necessary to inhibit the growth of 50% (relative to non-treated) cells (IC_{50} doses). The results shown are the mean values of the IC_{50} doses±standard deviation (S.D.), $n=4$.

saccharide moiety and on the three-dimensional arrangement of the galactose residues. We found that the increase in binding intensity, resulting from the increased sugar density, leveled off at around 20 mol% of sugar [8,9], a value which was apparently sufficient to fully engage the available binding sites on the cell surface.

The aim of this study was to verify the hypothesis that carbohydrate-mediated drug targeting, based on specific recognition by endogenous lectins, can lead to improved drug efficacy towards human colon adenocarcinoma. Targetable HPMA copolymer–DOX conjugates decorated with different saccharide groups were prepared (Table 1) and their cytotoxic activity towards human colon-adenocarcinoma cells was assessed. Their cytotoxicity was compared with that of a non-targetable HPMA copolymer–DOX conjugate and that of free-DOX. The glycoside ligands were selected for their ability to engage the galectin-3 expressed on colon-adenocarcinoma cells.

Using an RT-PCR analysis, we demonstrated the presence of the galectin-3-specific RNA in the whole-cell extract of all three human colon-adenocarcinoma cell

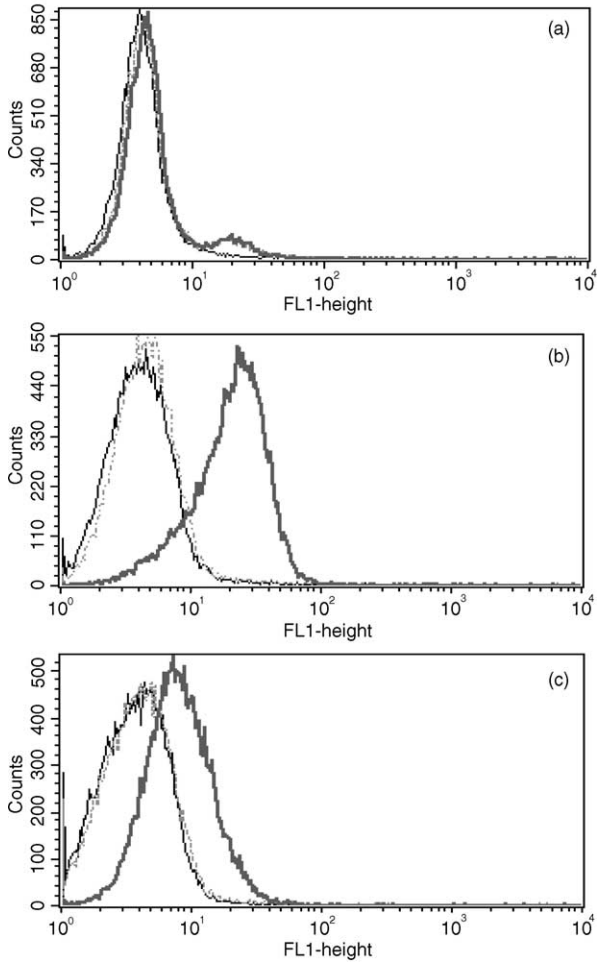

Fig. 3. Detection of cell membrane-associated galectin-3. Flow cytometry histograms of cell surface staining of colon adenocarcinoma cells using anti-galectin-3 (gray bold line, right). (a) Colo-205 cells; (b) SW-480; (c) SW-620. Dotted lines represent the control without the incubation step with the primary antibody, as described in Materials and methods.

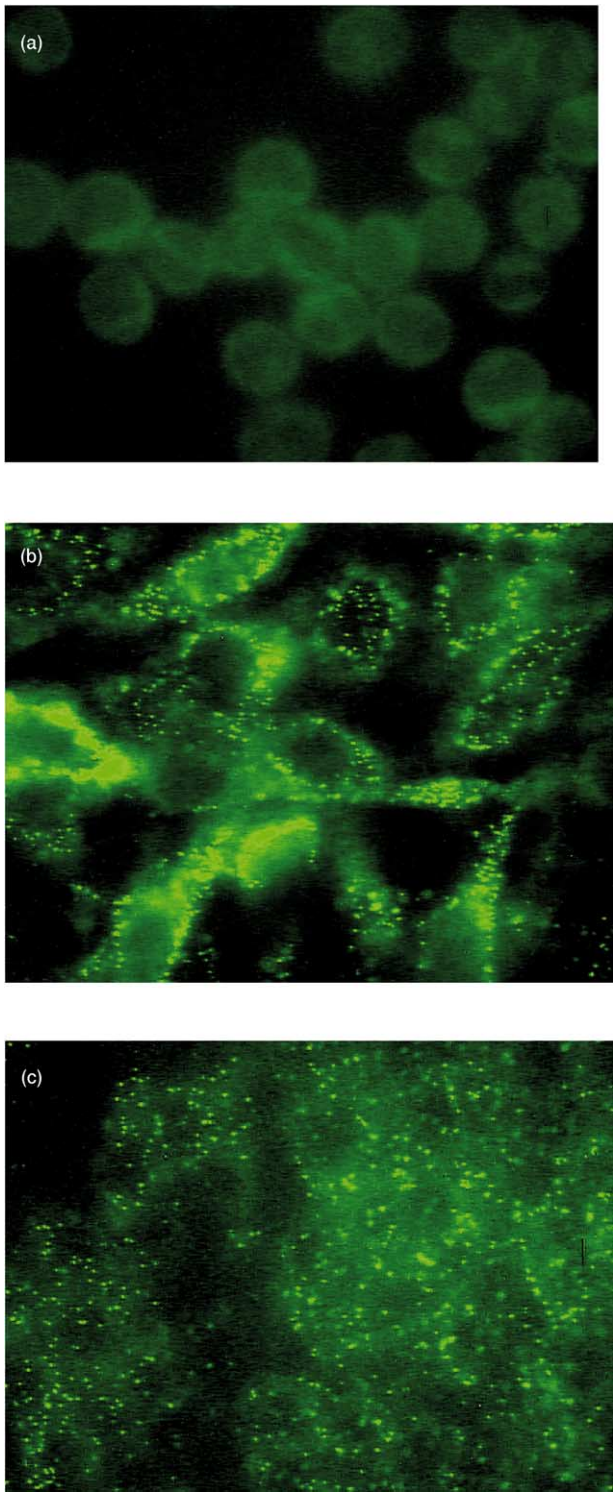

Fig. 4. Cell surface galectin-3 presentation in colon cancer cell lines as determined by fluorescence microscopy. Galectin-3 was determined using anti-galectin-3. (a) Colo-205 cells; (b) SW-480; (c) SW-620.

lines examined (Fig. 2b). Galectin-3 gene (*Mac-2*) expression appeared to be independent of the cells' differentiation state, since little difference in the galectin-3 expression was observed among the different cell lines (SW-480 is derived from a primary tumour, while SW-620 is derived from a metastatic lesion). Colo-205 cells (isolated from the ascite fluids) exhibited the lowest levels of *Mac-2* gene expression compared with SW-480 and SW-620 cells. These observations are consistent with the relative abundance of the 31-kDa galectin-3 protein as determined by the Western-blotting analysis (Fig. 2a).

Among the endogenous lectins, those presented at the cell surface are of major interest with regard to drug targeting. To confirm the presence of galectin-3 on the cell surface of the three types of colon cancer cells, the surface binding of anti-galectin-3 antibody was examined. The levels of galectin-3 on the cell surface of colon adenocarcinoma lines, as determined by flow cytometry, demonstrated a different pattern for galectin-3 expression, when compared with the Western blotting and RT-PCR results. The flow cytometry analysis revealed that Colo-205 cells did not express a detectable amount of galectin-3 on the cell surface (Fig. 3). SW-480 cells expressed 2-fold higher surface levels of galectin-3 compared with SW-620 cells. To exclude the possibility that trypsinisation of cells prior to the flow cytometry analysis destroyed part of the cell surface-bound galectin-3, binding analysis was also performed using cell monolayers. Fluorescent images of the anti-galectin-3 antibody binding to fixed colon-adenocarcinoma cells verified the above observations (Fig. 4). From these results, one may speculate that galectin-3 is present only in the cytoplasm or the nucleus of Colo-205 cells, while distributed both in the cytoplasm/nucleus and on the cell surface of SW-480 and SW-620 cells. Nevertheless, in a previous report Ohannesian and colleagues [23] have identified the expression of galectin-3 on the cell surface of Colo-205 cells by FACS analysis, indicating its presence and possible involvement in the recognition process.

The galectin-3 results are partially consistent with our previous findings on the biorecognition properties of the naive targetable HPMA copolymer. It was found that the binding intensity of the naive copolymers to SW-480 cells was similar to Colo-205 cells, and 2-fold higher than of SW-620 cells [8]. Assuming that binding sites for the galactoside-bearing HPMA copolymers are similar to the binding sites of the anti-galectin-3, it is likely that the binding of the glycoconjugates to Colo-205 cells was mediated by a β-galactose binding protein other than galectin-3 (possibly galectin-1,-4,-7 or-8) [29]. It is possible that these galectins may also be involved in the recognition of the glycoside-bearing HPMA copolymer–DOX conjugates by SW-480 and SW-620 cells. Further studies on the involvement of other members of the galectin family may help to clarify their role in the observed recognition.

The lower IC_{50} doses of the targetable HPMA copolymer–DOX conjugates (compared with the non-targetable conjugates) (Table 2), provide additional evidence for their specific binding properties and their therapeutic potential. As expected, the lowest IC_{50} doses were those for the free-DOX. They averaged at least three orders of magnitude less than the IC_{50} doses of the HPMA copolymer–DOX conjugates. Decorating the P-(GFLG)-DOX with multivalent galactoside moiety increased the polymer cytotoxicity. The cytotoxicity of the P-(GFLG-DOX)-TriGal was at least 10-times higher than the non-glycosylated P-(GFLG)-DOX product in all three colon adenocarcinoma cells. These results point out the different modes by which the free and the polymer-bound drug enter cells. The former rapidly diffuses into all cells resulting in low IC_{50} values, whereas for polymer-bound drugs entry was limited to endocytosis, a time-consuming process [34]. Therefore, the IC_{50} doses of the polymer-bound drugs were considerably higher than of free drug. Comparison of the free DOX and P-(GFLG)-DOX cytotoxicities, based on intracellular (cell-associated) drug concentrations in ovarian carcinoma cells, revealed that the cytotoxicity of P-(GFLG)-DOX conjugate was at least equal to the cytotoxicity of free-DOX [33]. After reaching the lysosomal compartment, DOX was cleaved from both conjugates [39], and diffused via the cytoplasm into the cell nucleus [34]. In Colo-205 and SW-480 cells, the addition of biorecognition (saccharide) groups to P-(GFLG)-DOX decreased the IC_{50} doses substantially, due to its internalisation by receptor-mediated endocytosis, compared with the non-targetable conjugate, that was internalised by fluid phase pinocytosis, a much slower process. Given that the cytotoxicity of the glycoconjugates depends directly on the concentration of the drug inside the cell, and provided that the concentration of drug inside the cells depends on the extent of its cellular uptake, the more active copolymer is that one possessing the highest extent of endocytosis. Since fluid-phase pinocytosis is most probably responsible for the internalisation of the non-targetable DOX conjugate, the interaction of free-DOX with nuclear DNA may be less likely than in the case of the targetable conjugate [40].

In a previous study [8], we found that the binding intensity of P-Lac was weaker than that of P-GalN and P-TriGal in the three human colon-adenocarcinoma cells. The cytotoxicity of the targetable HPMA copolymer-DOX conjugates reported here verified this observation. Although the differences in the cytotoxicity of the targetable HPMA copolymer-DOX conjugates do not allow firmer conclusions, in all cases biorecognition of P-(GFLG-DOX)-TriGal was superior to P-(GFLG-DOX)-Lac. The results (Table 2) suggest that the extent of endocytosis of the targetable HPMA copolymer–

DOX conjugates into the cells was significantly higher than the non-targetable ones (less significantly for SW-620 cells), probably due to biorecognition by endogenous lectin and to lysosomotropism [34].

It is concluded that the use of directing tools for targeting doxorubicin into colon cancer cells is effective and has an interesting potential in localised cancer therapy. By using different types and constructs of sugar moieties, the cytotoxicity of P-(GFLG)-DOX can be manipulated. As shown in our study, a possible cause for the successful targeting of the glycosylated conjugates was a specific recognition process mediated by endogenous lectins.

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