Lectin-Mediated Drug Targeting: Preparation, Binding Characteristics, and Antiproliferative Activity of Wheat Germ Agglutinin Conjugated Doxorubicin on Caco-2 Cells

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Purpose. To investigate the usefulness of wheat germ agglutinin as a targeting carrier protein for an acid-labile chemotherapeutic prodrug directed against colon carcinoma cells *in vitro*.
Methods. Cis-aconityl-linked doxorubicin-wheat germ agglutinin was prepared by a two step procedure and the conjugate-binding capacity of target- and non-target cells was assayed by flow cytometry. The antiproliferative activity of the prodrug on Caco-2 and MOLT-4 cells was determined by the XTT- and BrdU-test and compared with that of the parent drug and the lectin alone.
Results. At pH 4.0, about 50% of the conjugated doxorubicin were released within 24 h from the water soluble prodrug exhibiting a conjugation number of 24 (mol doxorubicin/mol WGA). The prodrug-binding capacity of colon carcinoma cells exceeded that of human colonocytes and lymphoblastic MOLT-4 cells 4.5-fold. Additionally, the antiproliferative effect of the conjugate on Caco-2 cells was 39% as opposed to 5% in case of MOLT-4 cells. As the unmodified carrier protein inhibited or stimulated Caco-2 cell growth in a concentration-dependent manner, the cytostatic activity of the conjugate was determined at WGA concentrations without an effect on cell-proliferation. Considering 50% release of conjugated drug at the most, the prodrug yielded 160% of the cytostatic activity of free doxorubicin.
Conclusions. WGA-prodrug targeting offers new perspectives for site-specific, cytoinvading drug delivery in colon cancer chemotherapy.

KEY WORDS: wheat germ agglutinin; lectin; doxorubicin; Caco-2; drug targeting.

INTRODUCTION

The concept of drug targeting originates from the observation that many drugs are not selective with regard to their cellular sites of uptake or action. Especially in the field of cancer chemotherapy, action of drugs in non-target tissue causes unwanted side effects restricting successful therapy (1). Realization of targeted drug delivery was hampered by difficulties in finding appropriate carrier molecules, which interact specifically with the affected tissue. Due to the progress in biotechnology, monoclonal antibodies (2) and recombinant proteins (3) directed to specific sites were established as promising candidates for active targeting. Additionally, colloidal particles such as albumin- and polystyrene microspheres are used for passive compartimental delivery of drugs (4,5).

Lectins, playing an important role in biosignaling, cell/cell- and cell/matrix interactions are expected to be another group of molecules appropriate for targeting drugs. Lectins are known as proteins originally identified in plant extracts being capable of binding to certain oligosaccharide moieties (6). Taking advantage of this carbohydrate-specific interaction, the lectin-mediated drug targeting is proposed to be a promising approach towards site-specific drug delivery (7), since the glycosylation pattern of cells is altered upon malignant transformation (8).

Wheat germ agglutinin (WGA) is a dimeric, carbohydrate-free protein composed of two identical subunits exhibiting a molecular weight of 35 kDa. Each monomeric subunit contains two identical and independent binding sites for N-acetyl-D-glucosamine (9). As wheat germ forms part of the regular diet of man and contains about 300 mg WGA/kg wheat germ (10), peroral toxicity might be negligible. The WGA-binding capacity of Caco-2 cells was found to exceed 13 fold that of human colonocytes as observed by saturation analysis using the fluorescently labelled lectin (11).

To exploit this different lectin-binding capacity for targeting cytostatic agents to the tumor tissue, the objective of this work was the preparation and characterization of WGA-conjugated DOX via an acid labile cis-aconityl linkage. The *in vitro* release of doxorubicin (DOX) from the prodrug as well as the conjugate-binding capacity of colon carcinoma cells, human colonocytes and lymphocytes was investigated. Proliferation-tests of cell lines co-incubated with the conjugate offer an outlook on the potential of lectin-targeted drug delivery.

MATERIALS AND METHODS

Chemicals

Wheat germ agglutinin from *Triticum vulgare* and its fluorescein labeled analogue (molar ratio fluorescein/protein = 3.2) were purchased from Vector laboratories (Burlingame, U.S.A). Doxorubicin, cis-aconitic anhydride, N,N′-dicyclohexylcarbodiimide, ε-amino-n-caproic acid and N-hydroxysuccinimide were obtained from Sigma (St. Louis, MO, U.S.A.); all other chemicals were of analytical grade and purchased from Merck (Darmstadt, G).

Tissue culture reagents were from Biowhittaker (Workingham, U.K.), kits for XTT- (EZ4U) and BrdU-test were purchased from Biomedica (Vienna, Austria) and Boehringer Mannheim (Vienna, Austria), respectively.

Conjugation of Doxorubicin to WGA

N-cis-aconityl-doxorubicin was prepared according to Yang and Reisfeld (12) with modifications. All reactions were carried out under protection from light and monitored by TLC on KGF_{254} using chloroform/methanol/water 9 + 9 + 1.8 or

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ABBREVIATIONS: ADH, adipic acid dihydrazide; BrdU, 5-bromo-2′-deoxyuridine; DOX, doxorubicin; EGF, epidermal growth factor; HEPES, 20mM HEPES/NaOH-buffer pH7.4, isotone; XTT, Sodium 3,3′-{1-[(phenylamino) carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate; TNBS, 2, 4, 6-trinitrobenzenesulfonic acid; WGA, wheat germ agglutinin.

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8 + 10 + 2.5 (v/v/v) as the mobile phase. DOX (1.79 μmol) was dissolved in 1.5 ml methanol and was stirred with an etheric solution of cis-aconitic anhydride (5.2 μmol) for 1 h at room temperature. After evaporation to 500 μl, the doxorubicin-cis-aconitate was activated by reacting with 7.2 μmol N,N′-dicyclohexylcarbodiimide and 7.2 μmol N-hydroxysuccinimide. The reaction mixture was stirred for 2 h at room temperature and overnight at 4°C. Dicyclohexylurea was precipitated by addition of 1 ml 3% aqueous sodium bicarbonate solution and collected by centrifugation at 7500 rpm for 5 min. The residual methanol was removed from the supernatant by evaporation. The doxorubicin-cis-aconitate-N-hydroxysuccinimide ester was conjugated to WGA by dropwise addition of a solution containing 0.033 μmol WGA in 500 μl 3% aqueous sodium bicarbonate pH 8.0. After incubation overnight at 4°C, the conjugate (DOX-WGA) was dialyzed against 20 mM HEPES/NaOH-buffer pH 7.4 at 4°C until no free drug was detected by spectrophotometric examination of the dialysis medium at 468nm (detection limit: 2 μg/ml DOX). Upon TLC DOX-WGA was the only fluorescent compound observed indicating no detectable free DOX.

Estimation of the Coupling Rate

The number of DOX molecules coupled per molecule of WGA was calculated from the absorption at 468 nm relatively to WGA in 20 mM HEPES-buffer pH 7.4 using DOX for calibration (U-3000 UV-VIS Spectrophotometer, Hitachi).

Determination of Amino-Residues on WGA

The total number of amino-groups of WGA susceptible to derivatization was determined by a modified trinitrobenzenesulphonic acid (TNBS)/adipic acid dihydrazide (ADH) test (13). Briefly, 200 μl of a solution containing 125–175μg WGA in 20 mM HEPES pH 7.4 were mixed with 200 μl 0.02% TNBS in saturated sodium tetraborate/distilled water (1 + 1, v/v) and incubated for 10 min. at 70°C. After addition of 100 μl 0.5 M aqueous ADH-solution and gently vortexing, the absorbance of trinitrobenzene adipic acid dihydrazone was read at 520 nm. The number of amino-residues was calculated from a calibration curve using ε-amino-n-caproic acid. The test allows quantification of 80–680 nmol amino residues/ml.

In Vitro Release of Conjugated Doxorubicin

In vitro release studies of DOX from the conjugate were performed at 37°C under protection from light. A small dialysis tubing (MW cut-off 12 kDa) containing 25 μl DOX-WGA corresponding to 7.5 μg DOX was placed in a small screw tube filled with 1.5 ml 0.1 M phosphate/citrate-buffer pH 4.0. To guarantee unimpeded diffusion of released DOX, air bubbles were carefully removed from the surface of the membrane. While stirring the dialysis medium, 500 μl aliquots were withdrawn in regular intervals and returned immediately to the vessel after reading the absorbance at 468 nm. The amount of DOX released from the conjugate was calculated from a calibration curve of DOX.

Cells and Culture Conditions

The human colon carcinoma cell line Caco-2 and the human lymphoblastic cell line MOLT-4 were obtained from the American Type Culture Collection (Rockville, ML, U.S.A.). Cells were grown in culture medium consisting of RPMI-1640 with 10% fetal calf serum, 4 mM L-glutamine and 75 μg/ml gentamycine in a humidified 5% CO_2/95% air atmosphere at 37°C and subcultured by trypsination.

Human colonocytes were obtained from normal tissue adjacent to resected colon carcinoma specimen. The tissue was dissociated by incubation in collagenase-solution (1000 U/ml medium) at 37°C for about 1 h until single cells were released as shown by light microscopy. Cells were repeatedly washed with PBS by centrifugation at 1300 rpm for 5 min. at 4°C. The pellet was resuspended in PBS and the preparation was analyzed using a Casy1 DT cell counter and analyser system (Schärfe, G). Thus the cell-preparation was composed of 1×10^6 colonocytes/ml (diameter 7–10 μm) and 17×10^6 cells/ml exhibiting diameters lower than 7 μm, mainly red blood cells as observed by light microscopy.

Binding of DOX-WGA to Human Colonocytes, Caco-2 and MOLT-4 Cells

Using a 96-well microtiter plate, 50 μl of cell-suspension (5×10^4 colonocytes or lymphoblasts) in PBS, 100 μl 20 mM HEPES pH 7.4 and 50 μl of a solution containing 3.6, 1.8 or 0.9 μg DOX-WGA in HEPES were incubated for 1 h, 2 h or 12 h at 4°C. Cells were spun down (1000 rpm, 5 min., 4°C) and 120 μl of the supernatant were discarded. After addition of 120 μl HEPES, the washing step was repeated in the same manner. Cells were resuspended in 1.8 ml Cell Pack and assayed by flow cytometry.

Negative controls were included in each experiment consisting of unlabeled cells for estimation of autofluorescence. Each concentration was tested in quadruplicate and repeated at least twice.

Flow Cytometry

Flow cytometric measurements were carried out on an Epics XL-MLC analytical flow cytometer (Coulter, FL, U.S.A). Labeled cells were analyzed using a forward versus side scatter gate for the inclusion of single cell populations and exclusion of debris and cell aggregates. Fluorescence was detected at 575 nm (10 nm bandwith) and the mean channel number of the logarithmic fluorescence intensities of individual peaks was used for further calculations. Amplification of the fluorescence signals was adjusted to put the autofluorescence signal of unlabeled cells in the first decade of the 4-decade log range. For each measurement 5000 cells were accumulated.

Confocal Microscopy

Cells were stained by incubation of 1000 μl cell-suspension (2×10^6/ml HEPES) with 100 μl solution of DOX-WGA (69 μg/ml HEPES) or fluorescein labeled WGA (100 μg/ml HEPES) for 1 h at 37°C. Cell were spun down (5 min., 1000 rpm), washed twice as described above but using 150 μl HEPES and mounted for microscopy. Confocal images of fluorescent labeled cells were obtained using a Zeiss Axiovert confocal microscope. Transmission light and fluorescence pictures were acquired at 40x magnification and intracellular DOX as well as fluorescein were detected by excitation at 488 nm and emission >515 nm.

Cell Proliferation Assays

Cell proliferation was determined by the XTT- and the BrdU-assay, which were performed following the manufacturers instructions using colorless supplemented RPMI 1640 medium for cell culture.

To estimate the influence of WGA and F-WGA on Caco-2 cell proliferation, 100 μl cell suspension (2×10^4 cells) and 75 μl of each of a dilution series of WGA and F-WGA (0, 0.06, 0.15, 0.2, 0.3, 0.4, 0.6, 1.5, 3, 6, and 30 μg) were incubated under tissue culture conditions for 3d followed by addition of 20 μl XTT-reagent solution. The absorbance of the formazan formed was determined at 450 nm (Anthos ELISA Reader 2001) versus a negative control consisting of medium and XTT-reagent solution.

The cytotoxic acivity of DOX-WGA was determined by the XTT-assay as described above, but using 10^4 MOLT-4- or Caco-2 cells/100 μl RPMI 1640 medium and 75 μl of each of a solution containing DOX (0.15 or 0.10 μg), WGA (0.45 or 0.32 μg) or DOX-WGA (0.57 or 0.41 μg), respectively.

Moreover, cell proliferation of Caco-2 cells pretreated with DOX-WGA was investigated using a colorimetric immunoassay, the BrdU-test. 75 μl of each of a solution containing WGA (0.6, 0.43, 0.30 μg), DOX (0.15, 0.11, 0.075 μg) or DOX-WGA (0.57, 0.41, 0.285 μg) in HEPES were added to the well of a microtiter plate containing 100 μl cell suspension (5×10^3 Caco-2 cells). After incubation under tissue culture conditions for 96 h 20 μl BrdU-labeling solution were added followed by another 14 h incubation. The supernatant was discarded and upon addition of 200 μl FixDenat-solution the cells were fixed and the DNA was denatured. The supernatant was removed 30 min. later and 100 μl of BrdU-antibody-peroxidase was added. After 90 min. of incubation, cells were washed three times with 200 μl washing buffer and 100 μl substrate were added. After 10 min, the enzyme activity was stopped by addition of 25 μl 1M sulfuric acid and the absorbance was measured at 450 nm versus the blank prepared as above but omitting the cells.

All assays were performed in quadruplicate and repeated at least twice. Additionally, positive controls were included in each experiment by omission the substances under investigation.

RESULTS

Preparation and Characterization of Cis-Aconityl-Linked Doxorubicin-WGA (DOX-WGA)

To use WGA as a carrier protein for targeted prodrug delivery of DOX, the cytostatic agent was linked covalently to the protein by a two step procedure. First, the drug was converted quantitatively to the corresponding carboxylic-acid derivative by reacting the free amino-residue of daunosamine with cis-aconitic anhydride ($Rf_{product} = 0.21$, $Rf_{DOX} = 0.14$; chloroform/methanol/water, 9 + 9 + 1.8 (v/v/v). In contrast to the literature (12), the reaction yielded N-cis aconityl DOX only in anhydrous medium. Despite of formation of both, the α- and the β-monoamide isomer only one spot was observed upon TLC.

To avoid cross-linking of WGA by preactivation of the protein, the N-cis-aconitate-DOX was reacted with dicyclohexylcarbodiimide/N-hydroxysuccinimide to form an active ester intermediate prior to coupling (14). As indicated by TLC (chloroform/methanol/water 8 + 10 + 2.5 (v/v/v), the N-cis-aconityl-derivative ($R_f = 0.24$) was converted to the corresponding N-hydroxysuccinimide ester ($R_f = 0.88$) to an extent of about 75%. Involving the active ester of either the β- or γ-carboxyl-residue of cis-aconitate-doxorubicin, the drug was conjugated to accessible amino-residues of WGA forming an amide-bond. UV-difference spectroscopy of the purified conjugate confirmed covalent attachment of DOX to the carrier protein resulting in a conjugation number of about 24 moles DOX/mol WGA. As WGA represents a mixture of isolectins containing 24 ± 1.53 amino groups (mean ± SD, n = 6) as determined by the TNBS/ADH-test, the coupling reaction yielded densely coated lectin, but still being freely water-soluble.

As the cis-aconityl spacer was reported to be a pH-sensitive linkage (15) for intralysosomal release of the conjugated drug, the *in vitro* release profile of conjugated DOX at pH 4.0 was determined spectrophotometrically using a dialysis tubing for exclusion of the conjugate. Within 24 h of incubation at pH 4.0, 46 ± 7% of conjugated DOX were released from WGA; but upon prolonged incubation, the release rate was not increased markedly, since exposure of DOX-WGA to acidic milieu for 168 h resulted in release of 51 ± 6% conjugated DOX. As indicated by the release profile, half of the available drug was released after 65 min. (Figure 1).

Binding of DOX-WGA to Caco-2 Cells, Human Colonocytes and Lymphoblasts

In order to estimate the conjugate-binding capacity of target- and non-target cells, increasing amounts of DOX-WGA were allowed to interact with a fixed number of cells of different origin and assayed by flow cytometry. This technique allows quantification of cell-bound fluorescence intensity only (16). Caco-2 cells were used as a representative for malignant transformed cells, whereas human colonocytes and the human lymphoblastic MOLT-4 cells were included as a model for unaffected - and stem cells, respectively.

In comparison to the autofluorescence of the cells (0.6 ± 0.1), the mean cell-bound fluorescence intensity of Caco-2 cells increased markedly upon addition of DOX-WGA yielding 11.8 ± 0.7 − 37.9 ± 0.1 relative fluorescence intensity concurrent

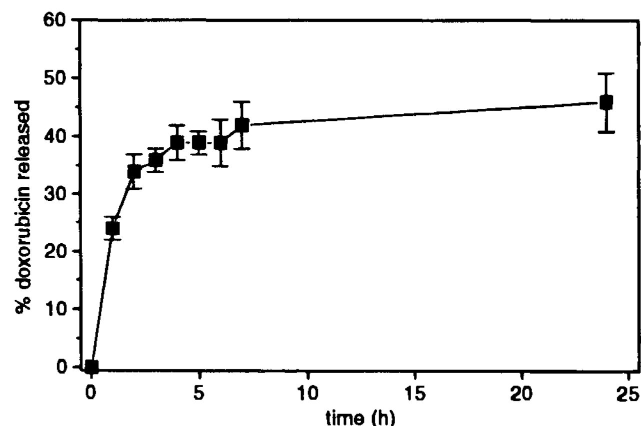

Fig. 1. In vitro release profile of DOX from DOX-WGA in 0.1M citrate/phosphate buffer pH 4.0 at 37°C (n = 3, mean ± S.D.).

with increasing concentration of the conjugate. In contrast, binding of increasing amounts of DOX-WGA to non-target cells resulted in rather low and slightly increasing cell-bound fluorescence intensity ranging from 2.4 ± 0.05 to 8.5 ± 1.0 (human colonocytes) and 2.5 ± 0.07 to 9.3 ± 0.1 (MOLT-4), respectively. As the mean cell-bound fluorescence intensity increased upon addition of increasing amounts of DOX-WGA, binding to the cell lines under investigation might be attributed to a specific interaction (Figure 2).

Since the preparation of human colonocytes contained 94% cells of different kind, mainly erythrocytes, conjugate-binding was investigated by adjusting the forward versus side scatter gate to record the cell population <7μm exclusively. As the mean relative fluorescence intensity of 0.3 covered the range of the autofluorescence of these cells, DOX-WGA-binding to erythrocytes might be neglected.

In general, the DOX-WGA-binding capacity of Caco-2 cells was 4.6 ± 0.3 fold higher than that of human colonocytes. The amount of DOX-WGA bound to MOLT-4 cells was in the same order of magnitude like that of human colonocytes being only 10 ± 3% higher.

Not only binding to the Caco-2 membrane, but also internalization of DOX-WGA was observed by confocal laser scanning microscopy of viable Caco-2 cells preincubated with the conjugate (Figure 3). After 1 h of incubation at 37°C, the fluorescent DOX-WGA-conjugate accumulated near the nuclear membrane of the dividing Caco-2 cell. In contrast, when Caco-2 cells were stained with fluorescein labeled WGA under the same conditions, the lectin was distributed over the cytoplasm with some intensely stained granular regions indicative for vesicular accumulation. Thus, cell-binding of the conjugate is followed by uptake of the prodrug, but the intracellular distribution pattern being dependent on the substance used for conjugation.

Antiproliferative Effects von DOX-WGA on Tumor Cells and Lymphoblasts

The effect of WGA, F-WGA, DOX and DOX-WGA on tumor cell growth was investigated by the XTT-test. In this assay, the tetrazolium salt XTT is metabolically reduced by

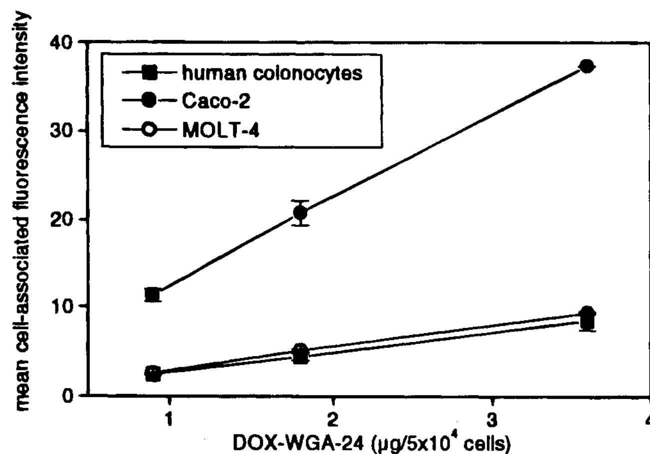

Fig. 2. Cell-bound fluorescence intensity of DOX-WGA incubated with human colonocytes, MOLT-4 and Caco-2 cells for 2h at 4°C after subtraction of the autofluorescence of cells (n = 8, mean ± S.D.).

viable cells yielding the soluble, highly colored formazan indicative for cell proliferation.

Although WGA inhibited proliferation of Caco-2 cells in a dose-dependent manner exhibiting 50% inhibition of cell growth (IC_{50}) at 537 ± 40 ng WGA/2 × 10^4 Caco-2 cells as calculated from a sigmoidal plot according to Boltzman, WGA-concentrations <60 ng/well not markedly influenced cell growth (Figure 4). In contrast, labeling of WGA using fluorescein isothiocyanate altered the inhibitory activity of the lectin resulting in the IC_{50} = 594 ± 84 ng F-WGA/2 × 10^4 Caco-2 cells. Moreover, upon addition of lower amounts than 200 ± 40 ng F-WGA, the labeled lectin stimulated proliferation of Caco-2 cells.

Considering the influence of the unmodified carrier protein on the inhibitory activity of DOX-WGA, the concentrations of WGA and DOX applied to the XTT-test were equivalent to the conjugate in order to provide comparability of the data obtained. Whereas WGA inhibited Caco-2 cell growth to an extent of 40.3 ± 14.6% (at 450 ng WGA/10^4 cells) and 6.7 ± 3.3% (at 320 ng WGA/10^4 cells), the cytostatic activity of DOX-WGA on Caco-2 cells increased concurrent with decreasing WGA-concentrations by 14.3 ± 8.3% and 34.0 ± 6.1%, respectively (Figure 5A). On the other hand, equivalent amounts of DOX-WGA yielded 63% or 46% of the antiproliferative effect of DOX.

Compared to Caco-2 cells, equal amounts of the substances under investigation exerted less influence on the viability of lymphoblastic MOLT-4 cells (Figure 5B). Whereas DOX inhibited cell growth to an extent of about 13 ± 5.2%, the inhibitory activity of DOX-WGA on MOLT-4 cells was 9.4 ± 3.2% (at 450 ng/10^4 cells) and 5.0 ± 2.6% (at 320 ng/10^4 cells), respectively.

In contrast to the XTT-test, the influence of carrier protein alone on Caco-2 cell proliferation was quite different as determined by quantification of the DNA content by the BrdU-test. As the concentration of the carrier protein decreased from 600 to 300 ng WGA/5000 cells, initially no considerable inhibitory effect (4.6 ± 2.3%) was observed, but this effect was converted to increasing stimulation of Caco-2 cell-growth (Figure 6). On the other hand, in comparison to DOX-WGA equivalent amounts of the free drug exhibited high cytostatic activity resulting in an growth inhibition of 95.2 ± 2.1% on average. Upon preincubation of Caco-2 cells with DOX-WGA, the antiproliferative activity of equivalent amounts of DOX decreased by 20 ± 5.6%.

DISCUSSION

In order to derive advantage from the quite different WGA-binding capacity of Caco-2 cells and human colonocytes for targeting drugs to colon carcinoma cells *in vitro* (11), DOX was covalently coupled to WGA via an acid-sensitive cis-aconitic linkage by a two-step mechanism (12). Since WGA represents a mixture of four isolectins (17), for estimation of the coupling efficiency the number of amino-residues on WGA was determined to be 24 ± 1.4 by combining the TNBS-test with ADH. Coupling was confirmed qualitatively and quantitatively by UV/VIS-difference spectroscopy and yielded a conjugate free of detectable non-covalently linked drug containing 24 moles DOX/mol WGA. Despite derivatization of nearly all accessible amino-residues of WGA, water solubility of DOX-WGA was retained.

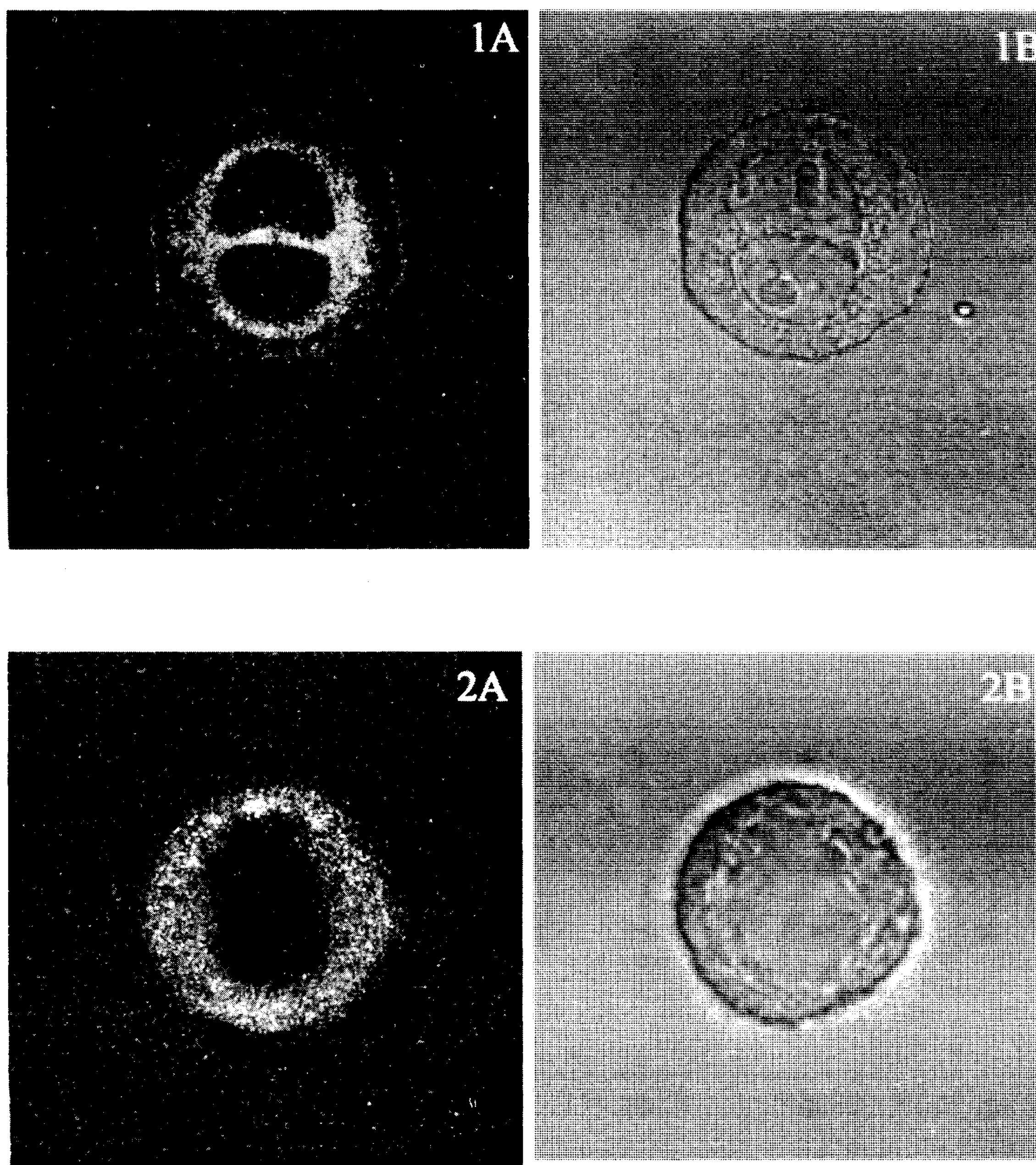

Fig. 3. Confocal fluorescence (A) and transmission light (B) microscopic images of Caco-2 cells incubated with DOX-WGA (1) and fluorescein labelled WGA (2). The cell diameter refers to about 20 μm.

In accordance with the literature (15), the cis-aconityl linkage between the drug and WGA was pH-sensitive being stable at pH 7.0 and exhibited a half life of 1 h at pH 4.0. But only 50% of the drug content were released from DOX-WGA within 168 h as opposed to almost 100% release of antibody-conjugated daunomycin at pH 8.0 (14). In our work N-cis-aconityl-doxorubicin was activated in aprotic milieu probably yielding equal amounts of the N-hydroxy-succinimide ester of both, the β- and the γ-carboxyl. Since one free cis-carboxylic function is a prerequisite for pH-sensitivity of the linkage (15), the release of only half of the DOX conjugated might be attributed to formation of an amide bond between WGA and the β-carboxyl of the α-monoamide isomer of N-cis-aconityl-doxorubicin.

As confirmed by confocal laser scanning microscopy of F-WGA and DOX-WGA, the N-acetylglucosamine specific lectin retained its bioadhesive and cytoinvasive properties upon conjugation of the cytostatic agent. Due to acid-sensitivity of the linkage, intralysosomal release of conjugated DOX is expected when DOX-WGA reaches the acidic milieu of the lysosomal compartment. As a consequence of derivatisation, the intracellular distribution pattern of WGA was altered resulting in accumulation of DOX-WGA near the nuclear membrane of Caco-2 cells.

Binding-specificity of the conjugate was assessed by flow cytometric determination of cell-bound fluorescence intensity derived from DOX-WGA. Independent from time of exposure, DOX-WGA exhibited dose-dependent binding to target- and

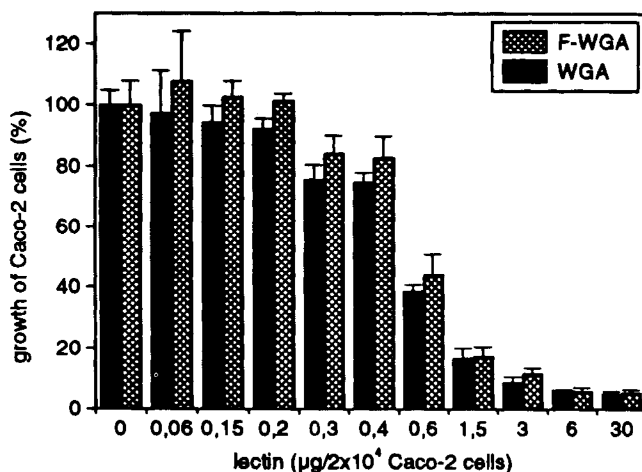

Fig. 4. Effect of F-WGA and WGA on Caco-2 cell growth as determined by the XTT-test (n = 8, mean ± S.D.).

non-target cells, but the extent being quite different. On average, the binding capacity of the colon carcinoma cells Caco-2 exceeded that of human colonocytes and lymphoblastic MOLT-4 cells 4.5 fold. Additionally binding of DOX-WGA to human erythrocytes was not detected. These results indicate for high

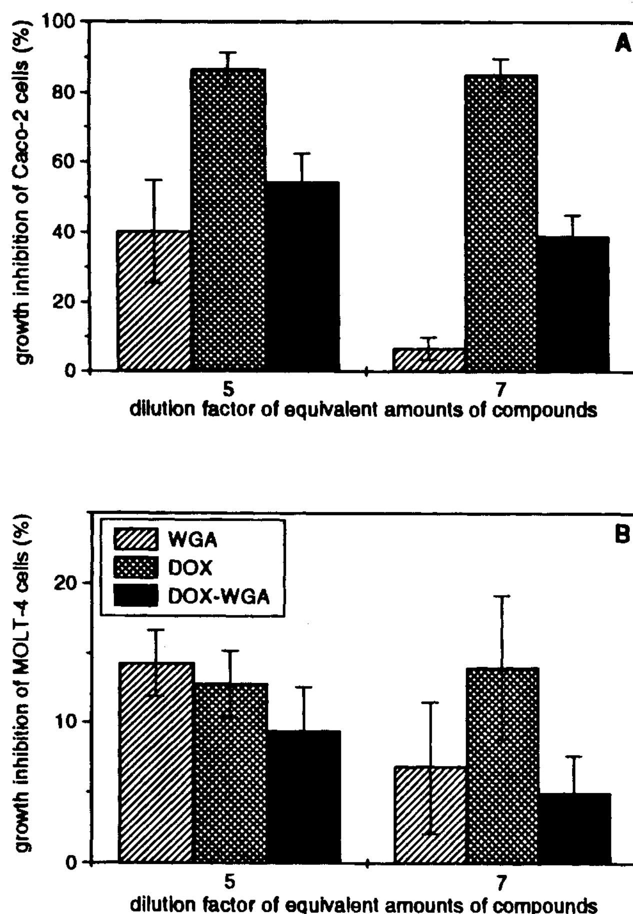

Fig. 5. Antiproliferative effects of WGA, DOX-WGA and DOX at equivalent concentrations on Caco-2 (A) and MOLT-4 cells (B) as determined by the XTT-test (n = 8, mean ± S.D.).

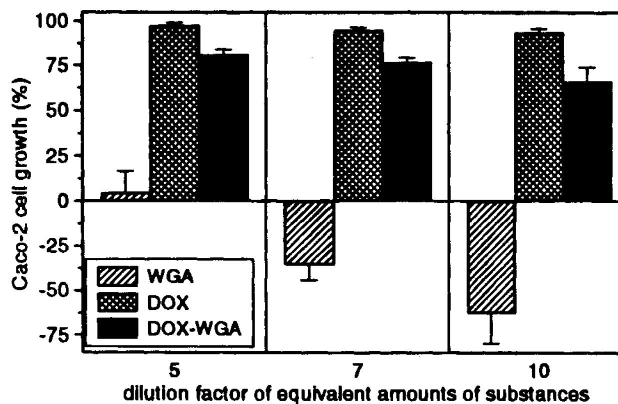

Fig. 6. Effect of equivalent amounts of WGA, DOX-WGA and DOX on Caco-2 cell growth as determined by the BrdU-assay (n = 8, mean ± S.D.).

target specificity of the conjugate. Considering the high extent of coupling, the decreasing ratio of binding to Caco-2 cells versus human colonocytes from 13:1 (F-WGA, 11) to 4:1 (DOX-WGA) might be related to densely derivatization of the lectin by the hydrophobic anticancer drug as observed for immunoconjugates (18).

When the effects of WGA and F-WGA on mitosis were investigated by determination of the mitochondrial dehydrogenase activity, in accordance with Ryder *et al.* (19) reversing effects on Caco-2 cell growth were observed: at comparable concentrations, the native lectin exhibited dose-dependent inhibition of Caco-2 cell growth, but upon conjugation of fluorescein this inhibitory effect converted to stimulation at concentrations <188 ng F-WGA/2 × 10^4 cells. As observed by Kawamoto *et al.* (20), epidermal growth factor (EGF) inhibited proliferation of A431 epidermoid carcinoma cells in the nM-range, but had a mitogenic effect in the pM-range. As the tyrosine kinase activity of EGF-receptor was activated by WGA to a similar extent as the enhancement induced by epidermal growth factor (21), this effect might be due to EGF-receptor binding of WGA.

However, contribution of the carrier protein of DOX-WGA to the antiproliferative effect of the conjugate on Caco-2 cells was rather low since the inhibitory activity of equivalent amounts of WGA and DOX-WGA was 6.7 ± 3.3% and 39 ± 6.1%, respectively. Thus the cytostatic activity of DOX-WGA mainly derives from the conjugated drug yielding about 60% of the cytostatic activity of free DOX. When the XTT-assay was performed with lymphoblastic MOLT-4 cells as a model for non-target cells, cell growth was inhibited by DOX-WGA in a dose dependent manner to an extent of 35% at the best as compared to free DOX (100%). This decreasing antiproliferative activity on non-target cells of equivalent amounts of DOX might be attributed to targeted delivery of the anticancer agent upon conjugation to WGA.

Since unaffected growth of Caco-2 cells preincubated with equivalent amounts of the carrier protein is required for clear estimation of the antiproliferative activity of DOX-WGA, BrdU-incorporation during DNA-synthesis was determined corresponding to the number of dividing cells. At equivalent concentrations of WGA and DOX as compared to DOX-WGA, the carrier protein alone inhibited slightly or stimulated Caco-2 cell growth and the free drug exhibited 95% growth inhibition

on average. The antiproliferative effect of conjugated DOX was 78% at the mean. Considering only 50% release of bound DOX *in vitro*, the cytostatic effect of DOX-WGA might be equal or quite higher than that of the free drug due to site-specific efficacy of conjugated DOX.

CONCLUSIONS

Following on from this work we consider WGA-prodrug targeting as a promising approach towards site-specific delivery of anticancer drugs to colon carcinoma cells. Fulfilling the main requirements for targeted systems such as specificity of binding, low toxic effects on unaffected cells and high antiproliferative effects on target cells, WGA mediates not only N-acetyl-glucosamine specific cytoadhesion, but also cytoinvasion of the conjugated drug possibly involving the EGF-receptor. Considering intracellular accumulation of WGA-conjugates, the acid-sensitive cis-aconityl linkage provides for release of the conjugated drug not before reaching the lysosomal compartment of the target cell. The gastrointestinal stability of WGA might enable enteric coated formulations of DOX-WGA to be peroral administered offering more convenient cytostatic therapy. Additionally, the receptor carbohydrate-mediated endocytosis induced by the lectin provides WGA as an appealing candidate for receptor-targeting vectors to deliver cDNAs into cells in gene therapy (22).

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