Published in final edited form as:

J Drug Deliv Sci Technol. 2013 ; 23(2): 111–118.

An inulin and doxorubicin conjugate for improving cancer therapy

C.A. Schoener¹, B. Carillo-Conde², H.N. Hutson², and N.A. Peppas^{1,2,3,*}

¹Department of Chemical Engineering, The University of Texas at Austin, 1 University Station, C0400, Austin, TX 78712, USA

²Department of Biomedical Engineering, The University of Texas at Austin, 1 University Station, C0400, Austin, TX 78712, USA

³College of Pharmacy, The University of Texas at Austin, 1 University Station, C0400, Austin, TX 78712, USA

Abstract

Chemotherapy is one of the primary treatment mechanisms for treating cancer. Current chemotherapy is systemically delivered and causes significant side effects; therefore the development of new chemotherapeutic agents or enhancing the effectiveness of current chemotherapeutic could prove vital to patients and cancer care. The purpose of this research was to develop a new conjugate composed of doxorubicin (chemotherapeutic) and inulin (polysaccharide chain) and evaluate its potential as a new therapeutic agent for cancer treatment. The synergistic effect of inulin conjugated to doxorubicin has allowed the same cytotoxic response to be maintained or improved at lower doses as compared to doxorubicin. Supporting results include cytotoxicity profiles, calf thymus DNA binding studies, confocal microscopy, and transport studies.

Keywords

Inulin; Doxorubicin; Drug resistance; Conjugate; Cytotoxicity

Mutations to a healthy cell's DNA eventually allows them to acquire the hallmarks of cancer: sustained angiogenesis, limitless replication potential, tissue invasion and metastasis, insensitivity to anti-growth signals, self-sufficiency in growth signals, and evading apoptosis [1, 2]. After surgery, adjuvant treatment with chemotherapy is routinely used because it is most effective after surgical removal due to decreased tumor burden, increased blood perfusion to the surgical site, and increased number of proliferating cells. Unfortunately, drug resistance cancer cells or cancer stem cells can resist the chemotherapeutic treatment and result in tumor reoccurrence [3–6]. Furthermore, increasing the dose of chemotherapy to overcome these resistant or cancer stem cells can result in severe side effects for the patient including bone loss, hair loss, and mucositis [7]. Therefore the development of new chemotherapeutic agents or enhancing the effectiveness of current chemotherapeutic could prove vital to patients and cancer care. The purpose of this research was to develop a new conjugate composed of doxorubicin (chemotherapeutic) and inulin (polysaccharide chain) and evaluate its potential as a new therapeutic agent for cancer treatment. Doxorubicin is one of today's most effective anthracycline antibiotics used for many solid tumors [8]. Doxorubicin stops cell proliferation and replication by intercalating between DNA

*Correspondence: peppas@che.utexas.edu.

nucleotides and inhibiting topoisomerase II which blocks DNA synthesis and transcription. Furthermore, anthracyclines are known to produce harmful free radicals upon metabolism [9]. Despite doxorubicin's great success, its greatest drawback is cardiotoxicity; thus, the maximum lifetime dose is not to exceed 500–600 mg/m² [10]. New delivery strategies have used liposomal and nanoparticle technology to deliver and target doxorubicin to the tumor of interest and reduce toxicity [11, 12]. However, direct modification to the doxorubicin molecule is limited and could provide new methods for enhancing the effectiveness of this chemotherapeutic while reducing cardiotoxicity.

Inulin is a naturally occurring polysaccharide in many plants such as onion, garlic, and chicory and consists of a mixture of oligomers and polymers containing 2 to 60 β, 2–1 linked D-fructose molecules (Figure 1) [13]. Inulin's average degree of polymerization is 12 and its short length gives rise to unique properties including fat reduction, dietary fiber, and prebiotic effects on colonic microflora [14]. Inulin is also FDA approved, non-toxic, and has demonstrated cancer prevention in animals [15]. Inulin has been conjugated to CoB₁₂ vitamin, noradrenaline, and cysteine and has shown the conjugate can increase drug stability to light, temperature, hydrolysis, and chemical agents [16]. However, these same inulin conjugates demonstrate very short serum half-life with rapid and complete urinary elimination. The efficacy and enhanced performance of inulin conjugates for cancer care must outweigh its insufficiency to avoid quick elimination by the urinary system to be successful. Besides conjugates, inulin has been extensively utilized as a matrix component for drug delivery [17–21]. The purpose of our project was to form a conjugate which could synergistically combine the effectiveness of doxorubicin with the positive attributes of inulin. Doxorubicin has been commonly used for the treatment of lymphomas, melanomas, and carcinomas, but has demonstrated reduced effectiveness for colon cancers. Doxorubicin possesses a primary amine for chemical attachment to inulin. By carboxymethylating inulin followed by sulfo-NHS and EDC chemistry, doxorubicin was conjugated to inulin (Figure 1). In this paper, the conjugate's cytotoxicity was tested against a variety of colon cancer cell models. The conjugate was investigated for its ability to bind to calf thymus DNA, localization in cell nuclei, and its transport properties across a co-culture representing the GI tract, assessed.

I. MATERIALS AND METHODS

1. Materials

Inulin from dahlia tubers, solid sodium hydroxide 20–40 mesh beads (NaOH), chloroacetic acid 99+ % ACS grade, phenolphthalein, phenol, Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), fibronectin, and calf thymus DNA (ctDNA) were purchased from Sigma-Aldrich (St. Louis, United States). Glacial acetic acid, anhydrous methanol, nitric acid, acetone, 1N hydrochloric acid, 10× phosphate buffer solution, sulfuric acid, ethanol, paraformaldehyde and ProLong Gold Antifade Reagent with DAPI were purchased from Fisher Scientific (Fair Lawn, United States). N-Hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were from ThermoScientific (Rockford, United States). Fetal bovine serum (FBS) and trypsin with EDTA were obtained from Hyclone (South Plainfield, United States). 1× phosphate buffered saline (PBS) without calcium or magnesium along with penicillin and streptomycin were from MediaTech (Manassas, United States). Hanks' Balanced Salt Solution was from SAFC Biosciences (Lenexa, United States). The cell proliferation MTS assay was purchased from Promega (Madison, United States). Doxorubicin was purchased from Selleck Chemicals (Houston, United States). Caco-2 and SW620 cells were obtained from American Type Culture Collection (ATCC, Rockwell, United States) and HT29-MTX cells were a gift from Dr. Thecla Lesuffleur, Inserm, Paris, France. Double distilled water was used in all studies.

2. General cell culture

Caco-2 and HT29-MTX cells were maintained in DMEM containing 4.5 g/L glucose, 1 % non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin, 2.7 g/L sodium bicarbonate, and 10 % by volume of heat-inactivated FBS. SW620 cells were maintained in the same conditions as the cells above except RPMI replaced DMEM. All cells were grown in T-75 vented flasks (Corning, Corning, United States) in a 5 % CO₂ and 95 % humidity environment maintained at 37 °C.

For plating 96-well plates (Nunc, Rochester, United States), a fibronectin coating was incubated for 1 h at a concentration of 1 µg/cm². The fibronectin was removed and the cells immediately plated. Caco-2 cells were seeded at a density of 2.0×10^3 cells/cm², HT29-MTX seeded at a density of 3.0×10^4 cells/cm², and SW620 seeded at 1.5×10^4 cells/cm². Cells were incubated for 48 h before testing. For transport studies, all experiments used co-cultures of Caco-2 and HT29-MTX cells plated on 12-well Costar Transwell plates (Corning, Corning, United States) with a 0.4 µm porous membrane. After subculturing, cells were counted and mixed together in a 1:1 ratio before seeding onto the Transwell plate at a density of 1.0×10^5 cells/cm² and cultured for 21–24 days with media replaced every other day.

3. Carboxymethylation of inulin

The first step to conjugate doxorubicin to inulin was to carboxymethylate the inulin (CMI) [22]. Inulin molecular weight was determined by gel permeation chromatography (Waters, Milford, United States) and used to calculate the proper number of monomeric units for the carboxymethylation reaction. Next, 3.4 g of inulin (~20 mmol of monomeric units) was dissolved in 25 mL of cold, distilled water. Then 6.4 g of solid NaOH (160 mmol) and was added and dissolved followed by 7.5 g of solid chloroacetic acid. The temperature was increased to 60 °C for 2 h followed by neutralization of the reaction mixture with glacial acetic acid. The product was precipitated in methanol, filtered, washed twice with absolute methanol, and dried in a vacuum oven (25 mmHg) at 60 °C for 24 h. The resulting product was dialyzed in water using 500–1,000 molecular weight cut off dialysis bags (Spectra/Por, Rancho Dominguez, United States) for 24 h with the water changed twice in the 24 h period. The final solution was freeze dried (Labconco Freezone 4.0, Kansas City, United States).

To determine the degree of substitution (DS), CMI was converted into its free acid form by adding 1 g to 14 mL of the acid reagent composed of anhydrous methanol and 70 % (v/v) nitric acid (10:1) in an Erlenmeyer flask and mixing for 3 h. The precipitated product was filtered by vacuum and washed several times with methanol until the filtrate reached neutrality as determined by pH paper. The free acid version of CMI was dried in a vacuum oven (25 mmHg) at 60 °C for 24 h. Then 0.2 g of the free acid form of CMI was dissolved in 20 mL of water. To this solution was added 10 µL of 2 % (w/v) phenolphthalein in ethanol and 5 mL of 0.1 N NaOH. Using a burette, the exact amount of 0.1 N HCl was measured that turned the solution clear. The degree of substitution was determined by using equation 1 and 2. Equation 1 was used to determine the millequivalents of –COOH groups per gram of CMI and defined as “A”:

$$A = ([\text{mL of NaOH} \times N_{\text{NaOH}}] - [\text{mL of HCl} \times N_{\text{HCl}}]) / \text{g of CMI} \quad \text{Eq. 1}$$

Using “A” as defined above, the DS, defined as the average number of sodium carboxymethyl groups per anhydrofructose units, could be determined by Equation 2:

$$\text{DS} = (0.162 \times A) / (1 - [0.08 \times A]) \quad \text{Eq. 2}$$

where 0.162 is derived from the molecular weight of the anhydrofructose unit of inulin and 0.08 from the net increase in the weight of the fructose unit for each carboxymethyl group substituted [22].

4. Amine-linked inulin-doxorubicin conjugate

Sulfo-NHS and EDC was used to prepare amine-reactive esters of the carboxylate groups located on the CMI to react with the primary amine of the doxorubicin. First, 10 mg of CMI were dissolved in 500 μL of distilled water. Then 1:12 and 1:10 molar equivalence of CMI to sulfo-NHS and CMI to EDC, respectively, were added to the CMI dissolved in water. After 1 h of mixing, doxorubicin was added to the solution at a 1:1 molar equivalence and mixed for 2 h. The inulin-doxorubicin conjugate was dialyzed in a 2,000 molecular weight cut off cassettes (Thermo Scientific) for two days to remove unreacted doxorubicin, CMI, sulfo-NHS, and EDC. The final purified solution was placed on the freeze dryer for three days.

5. Amount of doxorubicin present in the inulin-doxorubicin conjugate

The degree of substitution of the CMI limits the amount of doxorubicin which can react and chemically bond to the inulin backbone. To determine the amount of doxorubicin present in the inulin-doxorubicin conjugates, a doxorubicin calibration curve was formed and compared to an inulin-doxorubicin sample using fluorescent measurements from a UV/Vis spectrometer (Biotek Synergy-HT, Winooski, United States) operating at 485 excitation and 590 emission wavelengths. The average of three different concentrations of the inulin-doxorubicin conjugate in water determined the amount of doxorubicin present in the inulin-doxorubicin conjugate.

6. Amount of inulin present in the inulin-doxorubicin conjugate

A colorimetric assay for reducing sugars contained in inulin was completed to determine the presence of inulin in the conjugate. Inulin-doxorubicin conjugate samples were dissolved in water and compared to a calibration curve of CMI (unconjugated) dissolved in water. To 250 μL samples of the inulin-doxorubicin conjugate and calibration standards were added 150 μL of sulfuric acid and 30 μL of 5 % (v/v) phenol in water. The solutions were incubated at 90 °C and shaken at 600 rpm for 30 min to form the colorimetric assay. Sample absorbance at 490 nm was determined using the UV/Vis spectrometer. A doxorubicin sample at a concentration level equal to the amount of doxorubicin present in the inulin-conjugate sample, EDC, and sulfo-NHS were also run and used to subtract out background from UV/Vis reading.

7. Cytotoxicity of inulin-doxorubicin conjugate

The combination of inulin and doxorubicin should elicit unique properties for improving efficacy against colon cancer. To assess this hypothesis, doxorubicin and the inulin-doxorubicin conjugate were tested for toxicity against SW620, HT29-MTX, and Caco-2 cell lines. First, cell lines were exposed to just doxorubicin to determine IC_{50} values and if general trends matched previous published results. After 96-well plates of SW620, HT29-MTX, and Caco-2 cells were seeded and grown for 48 h, the cell media was removed, rinsed once with 1 $\times$ PBS, and then exposed to concentrations of doxorubicin dissolved in DMEM or RPMI ranging from 54.3 to 0.005 $\mu\text{g}/\text{mL}$ for 4 h. The doxorubicin was removed from the cells, rinsed three times with 1 $\times$ PBS, and finally incubated with fresh media. After 96 h, the media was removed and the MTS assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was used to determine cell viability.

The cytotoxicity of the inulin-doxorubicin conjugate versus doxorubicin was also completed in the same manner except the concentration of doxorubicin present in the inulin-doxorubicin conjugate was compared to the same concentrations of doxorubicin. The concentrations of doxorubicin in the inulin-doxorubicin conjugate and doxorubicin were 5.43, 2.715, 0.543, and 0.135 $\mu\text{g/mL}$. SW620, HT29-MTX, and Caco-2 cell lines were used. These studies were also completed within the same 96-well plate to eliminate any variability associated with separate plate analysis.

8. Cell free binding to ctDNA

Cell free binding experiments utilized fluorescent intensity to determine the binding capability of doxorubicin versus the inulin-doxorubicin conjugate. The chromophore site of the doxorubicin backbone is responsible for the majority of binding and the intercalation of DNA. As doxorubicin begins to bond and intercalate with DNA, fluorescent intensity is decreased [23, 24]. A 10 $\mu\text{g/mL}$ stock solution of doxorubicin and inulin-doxorubicin conjugate were made using 1 $\times$ PBS. Calf thymus DNA (ctDNA) standards were dissolved in 1 $\times$ PBS in ranging from 50 to 5 $\mu\text{g/mL}$. Stock solutions and standards were allowed to mix for 24 h before testing. The binding of ctDNA to doxorubicin was completed by adding 100 μL of doxorubicin or inulin-doxorubicin conjugate with 100 μL of one of the ctDNA concentrations. After 24 h of mixing and binding, the fluorescent intensity of the samples was determined using the UV/VIS spectrometer operating at 485 nm excitation and 590 nm emission. Fluorescent quenching was computed using Equation 3:

$$\text{Fluorescent quenching} = F/F_0 \quad \text{Eq. 3}$$

where F_0 is the fluorescent intensity of 100 μL of the doxorubicin or inulin-doxorubicin conjugate diluted with 100 μL of 1 $\times$ PBS.

9. Confocal imaging

Confocal imaging was completed to illustrate the localization of doxorubicin and the inulin-doxorubicin conjugate into the nucleus of SW620 cell line. LabTEK 8-well chamber slides (Nunc, Rochester, United States) were coated with fibronectin at a concentration of 1 $\mu\text{g/cm}^2$ and then seeded with SW620 cells at a seeding density of 1.5×10^4 cell/ cm^2 . Cells were incubated for 48 h at 37 $^\circ\text{C}$ and 5 % CO_2 .

In these studies, the concentration of doxorubicin present in the inulin-doxorubicin conjugate was compared to the same concentrations of doxorubicin. Doxorubicin and the inulin-doxorubicin conjugate were dissolved in 1 $\times$ PBS to a doxorubicin concentration level of 0.27 $\mu\text{g/mL}$. The cell line was exposed to this concentration for 4 h, then rinsed three times with 1 $\times$ PBS, and then fixed with 3.7 % (w/v) paraformaldehyde in water for 30 min. The cells were rinsed three times with 1 $\times$ PBS to remove the paraformaldehyde solution, air dried, and then stained with the Prolong Gold Antifade Reagent with DAPI overnight. The slides were covered with a #1 cover slip and imaged using a confocal microscope (Leica SP2 AOBS, Wetzlar, Germany).

10. Transport studies

Caco-2 and HT29-MTX cell lines can be used as colon cancer cell lines, but these cells can also be used for assessing transport properties. Caco-2 cells exhibit brush borders and form tight junctions while HT29-MTX secrete mucus and form tight junctions. The co-culture of these two cell lines provides an excellent GI tract model to help determine transport properties of a variety of therapeutic agents [25,26]. Transepithelial electrical resistance (TEER) measurements monitored the development of cell monolayers by measuring the resistance on the apical and basolateral side of Transwell plates using a chopstick electrode

and EVOM epithelial volt-ohm meter (World Precision Instruments, Sarasota, United States).

The amount of doxorubicin and inulin-conjugate transported across a cell monolayer was determined in the following manner: co-cultures of Caco-2 and HT29-MTX cells in 12-well Transwell plates were grown for 21–24 days or until TEER values leveled indicating tight junction formation and cell monolayer confluence. Then cell media was removed from the apical and basolateral side and washed three times with pre-warmed HBSS and finally replaced with HBSS on both sides of the chamber. The cells were allowed to equilibrate for 1 h, the apical HBSS removed, and 25 $\mu\text{g/mL}$ of doxorubicin or inulin-doxorubicin conjugates in HBSS added.

After adding the doxorubicin or inulin-doxorubicin conjugates, 100 μL samples were taken from each basolateral well at 0, 0.5, 1, 2, and 3 h time points. Samples were replaced with 100 μL of HBSS warmed to 37 $^{\circ}\text{C}$ to maintain sink conditions. Doxorubicin or inulin-doxorubicin conjugate transport across the cell monolayer was determined by UV/VIS spectrometry operating at 485 nm excitation and 590 nm emission.

The apparent drug permeability coefficient, P_{app} , was calculated using Equation 4:

$$P_{\text{app}} = (dQ/dt) \cdot (1/[A \cdot C_0]) \quad \text{Eq. 4}$$

Here, dQ/dt is the steady-state flux of doxorubicin or inulin-doxorubicin conjugate across the cell monolayer. This value was calculated from the slope of doxorubicin or inulin-doxorubicin conjugate transported to the basolateral chamber versus time. Here, the term A is the surface area of the membrane (1 cm^2) and C_0 is the initial concentration of doxorubicin or inulin-doxorubicin added to the apical chamber.

II. RESULTS AND DISCUSSION

1. Synthesis and characterization of inulin-doxorubicin conjugate

Doxorubicin has two major functional groups in its structure: a primary amine group in a sugar moiety and a primary hydroxyl group in the aliphatic chain ring. Both of them can be utilized for the conjugation; however, conjugation through the primary amine present in the sugar moiety allows for maintaining the quinone ring intact for drug activity since conjugation is performed far from the activity site of the drug and mild-chemistry, compared to other conjugation approaches, is applied to achieve inulin conjugation. Besides this, the selected conjugation strategy will form a linkage that is expected to be more readily cleavable under physiological conditions, which is beneficial for drug release. With this in mind, an inulin-doxorubicin conjugate was formed by first placing carboxylic acids on the inulin backbone followed by linkage of the doxorubicin through its primary amine using sulfo-NHS and EDC chemistry.

The first step to the inulin-doxorubicin conjugate was to carboxymethylate the inulin. Inulin number average molecular weight was determined to be 1925 g/mol with a degree of polymerization (DP) of 11. With a DP of 11, inulin still possessed enough monomeric repeat units to conjugate to doxorubicin and still remained small enough to elicit different properties as compared to other longer polysaccharides such as dextran or pullulan. The CMI was titrated against 0.1 N HCl and the degree of substitution, defined as the average number of sodium carboxymethyl groups per anhydrofructose unit, was determined to be 0.9.

Semi-stable amine-reactive NHS-esters were first formed by dissolving and mixing CMI, sulfo-NHS, and EDC. Doxorubicin dissolved in water was added to the amine reactive esters and mixed form the inulin-doxorubicin conjugate. The resulting conjugate was dialyzed for two days to remove unreacted components and freeze dried.

The amount of doxorubicin present in the inulin-doxorubicin conjugate was determined by dissolving the inulin-doxorubicin conjugate in water at different concentrations. The fluorescent values of these samples were compared against a calibration curve of doxorubicin standards. The amount of doxorubicin present was determined to be 0.462 ± 3.3 μg per mg of inulin-doxorubicin conjugate.

The amount of inulin present in the inulin-doxorubicin conjugate was determined by a colorimetric assay for reducing sugars. Inulin-doxorubicin conjugates dissolved in water at concentrations of 0.5 mg/mL, 0.25 mg/mL, and 0.1 mg/mL, had the sugars reduced using sulfuric acid and a 5 % (v/v) phenol in water. The amount of sugar reduced was determined relative to standard curves of CMI (unconjugated) which also underwent the sugar reducing protocol. Doxorubicin's structure contains an amine sugar; thus, a doxorubicin sample at the same concentration that doxorubicin would be present in each inulin-doxorubicin conjugate sample was carried through. The amount of inulin present was determined to be 0.926 mg per mg of inulin-doxorubicin conjugate. These values indicated that inulin makes up the majority of the molecular weight of the inulin-doxorubicin conjugate and is supported by the evidence indicating a small amount of doxorubicin present in the inulin-doxorubicin conjugate.

2. Cytotoxicity of inulin-doxorubicin conjugate

The inulin-doxorubicin conjugate and doxorubicin was tested for its toxicity using SW620, HT29-MTX, and Caco-2 colon cancer cell lines. For all experiments cell lines were exposed to doxorubicin or the conjugate for 4 h, washed with $1\times$ PBS, allowed to growth for 96 h in fresh cell media, and then assayed using the MTS reagent.

The IC_{50} data for the three cell lines treated with varying concentrations of doxorubicin are presented in Figure 2. Caco-2, HT29-MTX, and SW620 demonstrated different sensitivities to doxorubicin. Caco-2 and HT29-MTX cell lines demonstrated 2–3 times more resistance to doxorubicin as compared to SW620, with Caco-2 demonstrating the greatest resistance. Concentrations of 25 $\mu\text{g}/\text{mL}$ and 2.7 $\mu\text{g}/\text{mL}$ corresponded to IC_{50} values for HT29-MTX and SW620 cell lines, respectively, while a concentration of 54.3 $\mu\text{g}/\text{mL}$ only reduced Caco-2 cells 56 %. These results are in close agreement with previous research investigating the effectiveness of doxorubicin against these same cell lines [27–29].

Cytotoxicity studies, comparing the inulin-conjugate versus doxorubicin, were completed on SW620 cells and extended to Caco-2 and HT29-MTX cell lines since these last two cell lines showed to be more resistant to doxorubicin. In these studies the concentration of doxorubicin present in the inulin-doxorubicin conjugate was compared to the same concentrations of doxorubicin and ranged from 5.4 $\mu\text{g}/\text{mL}$ to 0.135 $\mu\text{g}/\text{mL}$. In all cell lines, the inulin-doxorubicin conjugate was more toxic than doxorubicin (Figure 3). For SW620 cells, as concentration increased the degree of cytotoxicity increased for both doxorubicin and the inulin-doxorubicin conjugate. The inulin conjugate induced approximately 70 % more toxicity than doxorubicin for concentrations up to 2.7 $\mu\text{g}/\text{mL}$. For Caco-2 cells, toxicity was minimized until a concentration of 0.135 $\mu\text{g}/\text{mL}$ was reached at which point the inulin-doxorubicin conjugate induced approximately 60 % more toxicity for the remaining higher concentrations. The HT29-MTX cells experienced minor toxicity for doxorubicin, but for the inulin-doxorubicin conjugate experienced 76 % increase in toxicity for concentrations higher than 0.54 $\mu\text{g}/\text{mL}$. CMI (unconjugated) was investigated for any toxic effects against

the SW620 cells and determined to be non-toxic (data not shown). The increased cytotoxicity observed with the inulin conjugate might be a consequence of cell apoptosis associated with increased intracellular doxorubicin delivery via receptor-mediated endocytosis induced by the non-specific interaction of inulin with carbohydrate receptors present on cell membranes. These observations are consistent with other drug conjugates that involved other saccharides such as dextran and galactose [30].

3. Cell free binding to ctDNA

Stock solutions of doxorubicin and the inulin-doxorubicin conjugate were combined with increasing concentrations of ctDNA, mixed overnight, and imaged with the UV/VIS spectrometer to measure fluorescent intensity to determine cell-free binding to ctDNA. Fluorescence quenching indicated that doxorubicin and the inulin-doxorubicin conjugate were bound to ctDNA. As shown in Figure 4, the inulin-doxorubicin conjugate exhibited a higher degree of fluorescent quenching as compared to doxorubicin.

Similar studies utilizing dextran-doxorubicin conjugates with varying molecular weights was completed and demonstrated that the degree of ctDNA binding increased with polymer molecular weight [23]. Contrary to our results, doxorubicin demonstrated the highest degree of binding to ctDNA. It has been reported that the major binding of doxorubicin to DNA occurs with the chromophore site with the amino sugar extending into the minor groove of the DNA to form a hydrogen bond [31]. Since this inulin-doxorubicin conjugate is bonded through the primary amine on the amino sugar of doxorubicin, its ability to hydrogen bond with DNA would be limited by steric hindrance and should reduce overall binding to DNA. However, our results indicate the highest degree of ctDNA binding was the inulin-doxorubicin conjugate. Thus, the unique properties of inulin have aided in the binding process to ctDNA and could be contributive to the observed increased cytotoxic response as compared to doxorubicin.

4. Confocal imaging

SW620 was exposed to doxorubicin and inulin-doxorubicin-conjugate for 4 h, then rinsed with 1× PBS, and fixed with paraformaldehyde. The cells were then stained with DAPI and imaged using a confocal microscope. The concentration of doxorubicin present in the inulin-conjugate and the concentration of doxorubicin were equal. Cells with the inulin-conjugate were imaged first and the settings used to capture these images were maintained for imaging the cells with doxorubicin and control cells for comparison purposes.

All cell nuclei were stained blue using the DAPI reagent (Figure 5A). Since doxorubicin excites at 485 nm and emits at 590 nm, a series of images can be captured sequentially and overlaid to determine the localization of doxorubicin or inulin-doxorubicin conjugate in the nucleus. The inulin-doxorubicin conjugate was present in greater quantities in the cellular nucleus as compared to doxorubicin in SW620 cells (Figure 5B). In fact, there is little detection of doxorubicin at equal setting when comparing the conjugate to doxorubicin (Figure 5B); however, when the signal was amplified in the cells exposed to doxorubicin, doxorubicin was present in the cellular nuclei (data not shown). Control cells indicated no contribution of natural fluorescence when excited at 485 nm. While doxorubicin penetrates the cell membrane non-specifically via electrostatic or hydrophobic-interactions [32], inulin may be acting as a targeting ligand that interacts non-specifically with carbohydrate receptors on cell membrane. Therefore, the observed increase in the amount of doxorubicin present in the cellular nucleus when the drug is conjugated to inulin may be due by additional internalization mechanisms of receptor-mediated endocytosis, allowing the conjugate to pass through the cell membrane and accumulate intracellularly. Once inside the

cells, the conjugate may form a complex with proteasomes similarly as free doxorubicin, which is then transported to the cell nucleus [32].

5. Transport studies

Caco-2 and HT29-MTX cells were combined to form confluent monolayers with tight junctions to serve as a model for permeation and transport studies. Caco-2 and HT29-MTX cells were combined in a 1:1 ratio and grown on Transwell plates for 21–24 days or until TEER measurements leveled. TEER measurements, after 21 days, measured between 400 and 500 $\Omega \cdot \text{cm}^2$ and were much higher than resistance values measured in human small intestinal epithelia (50–100 $\Omega \cdot \text{cm}^2$) [33–35] and those previously published from our laboratory cellular [36–46]. Variation in the TEER measurements can be contributed to culture conditions such as seeding density, passage number, and which medium was used. A small variation (10–30 Ω) in TEER measurements can also be contributed to the chopstick and EVOM volt-ohm meter. Since these *in vitro* TEER values are much higher than *in vivo* TEER values, results and conclusions developed from these models and experiments may underestimate permeation and transport values observed in *in vivo* conditions.

A co-culture of Caco-2 and HT29-MTX was exposed to 25 $\mu\text{g/mL}$ of doxorubicin or inulin-doxorubicin conjugate in HBSS for 3 h. The UV/VIS plate reader determined the concentration of doxorubicin or inulin-doxorubicin conjugate transported across the cell monolayer. Using Equation 4, doxorubicin permeability was calculated at $4.5 \times 10^{-6} \text{ cm/s}$ and the inulin-doxorubicin conjugate at $2.6 \times 10^{-6} \text{ cm/s}$ (Figure 6). TEER values monitored during the experiment demonstrated no decrease in value as compared to the control cells indicating no compromise in tight junctions. Therefore, the transport of doxorubicin or conjugate was completed transcellularly rather than paracellularly. The slightly lower permeability coefficient for the conjugate is expected since it is a slightly larger molecule to transport transcellularly as well as less able to penetrate the mucosal layer secreted by the HT29-MTX cells.

The apparent permeability values of doxorubicin have been determined in other model systems, but not for co-cultures of Caco-2 and HT29-MTX cell lines. The closest comparison was determining P_{app} for doxorubicin across a Caco-2 monolayer using either HBSS or with fasted state simulated intestinal fluids (FaSSIF). The HBSS P_{app} was $2.8 \times 10^{-5} \text{ cm/s}$ and FaSSIF was $2.9 \times 10^{-5} \text{ cm/s}$ [47]. These slightly higher values can be contributed to the single Caco-2 layer which does not possess the mucus layer secreted by the HT29-MTX cells. Additional P_{app} values have been computed for doxorubicin across human red blood cells ($2.4 \times 10^{-7} \text{ cm/s}$), Ehrlich ascites tumor cells ($2.4 \times 10^{-5} \text{ cm/s}$), leukemia cells ($7.4 \times 10^{-5} \text{ cm/s}$), and has been estimated on the order of 10^{-6} cm/s for hepatocyte membranes from male Wistar rats [48–51]. These values indicate that the magnitude and reported values of transport studies with doxorubicin and inulin-doxorubicin conjugates completed in this project are within the realm of other published results.

A new inulin-doxorubicin conjugate has been synthesized and characterized. Previously, doxorubicin has been conjugated to the hydroxyl groups which are located in or in close proximity to the quinone ring, formed with acid labile bonds or non-cleavable carbamate linkages, and delivered by injection. However, the conjugate formed here uses the amine group for linkage, is enzymatically degradable, does not reduce the activity of the drug, and is designed for oral drug delivery.

Cytotoxicity, ctDNA binding experiments, confocal imaging, and transport studies have been completed on doxorubicin and the inulin-doxorubicin conjugate. The synthesis of the inulin-doxorubicin conjugate was intended to combine the positive attributes of each individual molecule to elicit new properties which are advantageous for cancer treatment.

Overall, the inulin-doxorubicin conjugate demonstrated it is more potent and toxic than doxorubicin. A more potent conjugate could be beneficial to the medical community because less doxorubicin could be given while still achieving the same therapeutic effect. Doxorubicin is an effective drug; however, once a certain amount of this chemotherapeutic is administered it can no longer be used by the patient due to deadly cardiotoxicity. Perhaps utilizing the inulin-doxorubicin conjugate would allow patients to continue the use of the doxorubicin for longer periods of time with reduced cardiotoxicity.

Literature research has publicized the importance of inulin in human diets and its potential for role in colon cancer prevention [52]. Inulin type fructans consumed in human and rat diets reduced colon cancer risks by reducing exposure to genotoxic carcinogens in the gut or by reducing their genotoxic impacts [53]. In this same paper, inulin based products have shown reduction in metastasis activities of colon tumor cells and inhibition of growth. Inhibition of growth could be a driving mechanism for the observed increased toxicity of the inulin-doxorubicin conjugate. Lastly, nontoxic potentiation of cancer chemotherapy by dietary inulin has also been cited [54]. Seven days before administration of six different cytotoxic drugs, inulin was incorporated into male rat diets. For all experiments, inulin considerably potentiated the therapeutic effects induced by six different cytotoxic drugs commonly utilized in human cancer treatment. In this paper and others, these non-digestible carbohydrates appeared to decrease serum glucose level in rats and humans as well as insulin levels [55], each of which are important regulators of cell and/or tumor proliferation [56].

In each of these cited cases inulin was used as a dietary supplement and not physically bonded to the chemotherapeutic drugs. It is hypothesized that due to the intimate contact between cancer cells and doxorubicin that these effects above could be enhanced. The following information about inulin-doxorubicin can be contributive to its increased cytotoxicity: i) the inulin-doxorubicin conjugate is uptaken into these cells (see confocal results) in quantities higher than doxorubicin, ii) an increase in molecular size could prevent “efflux pumps” from removing the inulin-conjugate as effectively as doxorubicin, iii) the presence of inulin inside of the cell in greater concentrations than outside of the cell could play larger effects with the insulin and glucose levels needed for tumor proliferation and iv) binding studies indicate that the inulin-doxorubicin conjugate more readily binds to ctDNA as compared to doxorubicin.

Lastly, the determination of permeability coefficients of doxorubicin and the inulin-doxorubicin conjugate are a first of its kind when using the co-culture of Caco-2 and HT29-MTX cell lines. The combination of these cell lines more closely exhibits properties found with native GI tract and these results are potentially more true to *in vivo* conditions than those previously completed with just Caco-2 monolayers.

Acknowledgments

This research was supported in part by a grant from the NIH/NCI Center for Oncophysics (CTOPSO54-CA-143837). C.A.S. acknowledges the National Science Foundation for a Graduate Research Fellowship.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000; 100:57–70. [PubMed: 10647931]
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144:646–674. [PubMed: 21376230]
3. Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med. 2002; 53:615–627. [PubMed: 11818492]

4. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature*. 2006; 445:106–110. [PubMed: 17122772]
5. Reya T, Morrison SJ, Clarke MF, Weisman IL. Stem cells, cancer, and cancer stem cells. *Nature*. 2001; 414:105–111. [PubMed: 11689955]
6. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med*. 2006; 355:1253–1261. [PubMed: 16990388]
7. Airley, R., editor. *Cancer Chemotherapy*. Hoboken, John Wiley & Sons Ltd; 2009.
8. Weiss RB. The anthracyclines: will we ever find a better doxorubicin? *Semin. Oncol*. 1992; 19:670–686.
9. Blasiak J, Gloc E, Warszawski M. A comparison of the *in vitro* genotoxicity of anticancer drugs idarubicin and mitoxantrone. *Acta Biochim Pol*. 2002; 49:145–155. [PubMed: 12136935]
10. Lum BL, Svec JM, Torti FM. Doxorubicin: alteration of dose scheduling as a means of reducing cardiotoxicity. *Drug Intel Clin Pharm*. 1985; 19:259–264.
11. Gabizon AA. Pegylated liposomal doxorubicin: metamorphosis of an old drug into a new form of chemotherapy. *Cancer Investig*. 2001; 19:424–436. [PubMed: 11405181]
12. Barraud L, Merle P, Soma E, Lefrancois L, Guerret S, Chavallier M, Dubernet C, Couvreur P, Treppe C, Vitvitski L. Increase of doxorubicin sensitivity by doxorubicin-loading into nanoparticles for hepatocellular carcinoma cells. *J Hepatol*. 2005; 42:736–743. [PubMed: 15826724]
13. Binns J, Stevens HNE, McEwen J, Pritchard G, Brewer FM, Clarke A, Johnson ES, McMillan I. The tolerability of multiple oral doses of Pulsincap capsules in healthy volunteers. *J Control Release*. 1996; 38:151–158.
14. Wilson CG, Bakhshae M, Stevens HNE, Perkins AC, Frier M, Blackshaw EP, Binns JS. Evaluation of a gastro-resistant pulsed release delivery system (Pulsincap) in humans. *Drug Deliv*. 1997; 4:201–206.
15. Niness KR. Inulin and oligofructose: what are they? *J Nutr*. 1999; 129:1402S–1406S. [PubMed: 10395607]
16. Molteni L. Methods in enzymology: dextran and inulin conjugates as drug carriers. *Drug Enzym Target*. 1985; 112:285–298.
17. Tripodo G, Pitarresi G, Palumbo FS, Craparo EF, Giam-mona G. UV-Photocrosslinking of inulin derivatives to produce hydrogels for drug delivery application. *Macromol Biosci*. 2005; 5:1074–1084. [PubMed: 16245273]
18. Park SE, Nho YC, Lim YM, Kim HI. Preparation of pH-sensitive poly(vinyl-g-methacrylic acid) and poly(vinyl alcohol-g-acrylic acid) hydrogels by gamma ray irradiation and their insulin release behavior. *J Appl Polym Sci*. 2003; 91:636–643.
19. Vervoort L, Kinget R. *In vitro* degradation by colonic bacteria of inulin HP incorporated in Eudragit films. *Int J Pharm*. 1996; 129:185–190.
20. Vervoort L, Van den Mooter G, Augustijns P, Busson R, Top-pet S, Kinget R. Inulin hydrogel as carrier for colonic drug targeting. I Synthesis and characterization of methacrylated inulin and hydrogel formation. *Pharm Res*. 1997; 14:1730–1736. [PubMed: 9453061]
21. Maris B, Verheyden L, Reeth K, Samyn C, Augustijns P, Kinget R, Van den Mooter G. Synthesis and characterization of inulin-azo hydrogels designed for colon targeting. *Int J Pharm*. 2001; 213:183–190.
22. Verraest DL, Peters JA, Batelaan JG, van Bekkum H. Carboxymethylation of inulin. *Carbohydr Res*. 1995; 271:101–112. [PubMed: 7648576]
23. Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther*. 1995; 2:702–709. [PubMed: 8750009]
24. Lam W, Leung CH, Chan HL, Fong WF. Toxicity and DNA binding of dextran-doxorubicin conjugates in multidrug-resistant KB-V1 cells: optimization of dextran size. *Anti-Cancer Drugs*. 2000; 11:377–384. [PubMed: 10912954]
25. Hilgendorf C, Spahn-Langguth H, Regardh CG, Lipka E, Amidon GL, Langguth P. Caco-2 versus Caco-2/HT29-MTX co-cultured cell lines: permeability via diffusion, inside-and outside-directed carrier-mediated transport. *J Pharm Sci*. 2000; 89:63–75. [PubMed: 10664539]

26. Peppas NA, Kavimandan NJ. Nanoscale analysis of protein and peptide absorption: insulin absorption using complexation and pH-sensitive hydrogels as delivery vehicles. *Eur J Pharm Sci.* 2006; 29:183–197. [PubMed: 16777391]
27. Beaumont PO, Moore MJ, Ahmad K. Role of glutathione s-transferases in resistance of human colon cancer cell lines to doxorubicin. *Cancer Res.* 1998; 58:947–955. [PubMed: 9500455]
28. Oudard S, Thierry A, Jorgensen TJ, Rahman A. Sensitization of multidrug resistant colon cancer cells to doxorubicin encapsulated in liposomes. *Cancer Chemother Pharmacol.* 1991; 28:259–265. [PubMed: 1678995]
29. Emdad L, Lebedeva IV, Su ZZ, Sarkar D, Dent P, Curiel DT, Fisher PB. Melanoma differentiation associated gene-7/interleukin-24 reverses multidrug resistance in human colorectal cancer cells. *Mol Cancer Ther.* 2007; 6:2985–2994. [PubMed: 18025283]
30. Zaman, NT.; Tan, FE.; Joshi, SM.; JYY. Mol Engr Biol Chem Syst. DSpace@MIT; Targeted stimuli-responsive dextran conjugates for doxorubicin delivery to hepatocytes. 2005–01
31. Frederick CA, Williams LD, Ughetto G, Vandermael GA, Vanboom JH, Rich A, Wang AH. Structural comparison of anticancer drug-DNA complexes: adriamycin and daunomycin. *Biochemistry.* 1990; 29:2538–2549. [PubMed: 2334681]
32. Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev.* 2004; 56:185–229. [PubMed: 15169927]
33. Powell DW. Barrier function of epithelia. *Am J Physiol.* 1981; 241:G275–G288. [PubMed: 7032321]
34. Balimane PV, Chong S. Cell culture-based models for intestinal permeability: a critique. *Drug Discov Today.* 2005; 10:335–343. [PubMed: 15749282]
35. Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology.* 1989; 96:736–749. [PubMed: 2914637]
36. Wood KM, Stone GM, Peppas NA. The effect of complexation hydrogels on insulin transport in intestinal epithelial cell models. *Acta Biomaterialia.* 2010; 6:48–56. [PubMed: 19481619]
37. Phillips MR, Peppas NA. Hydrogels modified with carbohydrates for oral protein delivery. *Trans Ann Biomater Meeting.* 2009; 33:267.
38. Carr DA, Peppas NA. Assessment of poly(methacrylic acid-co-N-vinyl pyrrolidone) as a carrier for the oral delivery of therapeutic proteins using Caco-2 and HT29-MTX cell lines. *J Biomed Mater Res A.* 2010; 92A:504–512. [PubMed: 19213059]
39. Shofner J, Phillips MA, Peppas NA. Cellular Evaluation of synthesized insulin/transferrin bioconjugates for oral insulin delivery using intelligent complexation hydrogels. *Macromol Biosci.* 2010; 10:299–306. [PubMed: 20034125]
40. Foss AC, Goto T, Morishita M, Peppas NA. Development of acrylic-based copolymers for oral insulin delivery. *Eur J Pharm Biopharm.* 2004; 57:163–169. [PubMed: 15018971]
41. Blanchette J, Peppas NA. Cellular evaluation of oral chemotherapy carriers. *J Biomed Mater Res A.* 2005; 72A:381–388. [PubMed: 15666363]
42. Peppas NA, Kavimandan NJ. Confocal microscopic analysis of transport mechanisms of insulin across the cell monolayer. *Int J Pharm.* 2008; 354:143–148. [PubMed: 18262739]
43. Torres-Lugo M, Garcia M, Record R, Peppas NA. pH-Sensitive hydrogels as gastrointestinal tract absorption enhancers: transport mechanisms of salmon calcitonin and other model molecules using the Caco-2 model. *Biotechnol Prog.* 2002; 18:612–616. [PubMed: 12052080]
44. Torres-Lugo M, Garcia M, Record R, Peppas NA. Physicochemical behavior and cytotoxic effects of P(MAA-g-EG) nanospheres for oral delivery of proteins. *J Control Rel.* 2002; 80:197–205.
45. Foss AC, Peppas NA. Investigation of the cytotoxicity and insulin transport of acrylic-based copolymer protein delivery systems in contact with Caco-2 cultures. *Eur J Pharm Biopharm.* 2004; 57:447–455. [PubMed: 15093592]
46. Lopez JE, Peppas NA. Cellular evaluation of insulin transmucosal delivery. *J Biomater Sci Polym Ed.* 2004; 15:385–396. [PubMed: 15212324]
47. Ingels F, Beck B, Oth M, Augustijns P. Effect of simulated intestinal fluid on drug permeability estimation across Caco-2 monolayers. *Int J Pharm.* 2004; 274:221–232. [PubMed: 15072798]

48. Dalmark M, Storm HH. A fickian diffusion transport process with features of transport catalysis. Doxorubicin transport in human red blood cells. *J Gen Physiol.* 1981; 78:349–364. [PubMed: 7288392]
49. Dalmark M, Hoffmann EK. Doxorubicin (Adriamycin) transport in Ehrlich ascites tumour cells: compariosn with transport in human red blood cells. *Scand J Clini Lab Investig.* 1983; 43:241–248.
50. Dordal MS, Winter JN, Atkinson AJ. Kinetic analysis of P-glycoprotein-mediated doxorubicin efflux. *J Pharmacol Exp Ther.* 1992; 263:762–766. [PubMed: 1359116]
51. Hilmer SN, Cogger VC, Muller M, Le Couteur DG. The hepatic pharmacokinetics of doxorubicin and liposomal doxorubicin. *Drug Metab Dispos.* 2004; 32:794–799. [PubMed: 15258103]
52. Pool-Zobel BL, Van Loo J, Rowland I, Roberfroid M. Experimental evidences on the potential of prebiotic fructans to reduce the risk of colon cancer. *Br J Nutr.* 2002; 87:S273–S281. [PubMed: 12088529]
53. Pool-Zobel BL. Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. *Br J Nutr.* 2005; 93:S73–S90. [PubMed: 15877900]
54. Taper HS, Roberfroid MB. Nontoxic potentiation of cancer chemotherapy by dietary oligofructose or inulin. *Nutr Cancer.* 2000; 38:1–5. [PubMed: 11341034]
55. Yamashita K, Kawai K, Itakura M. Effects of fructooligosac-charides on blood glucose and serum lipids in diabetic subjects *Nutr. Res.* 1994; 4:961–966.
56. Gioivanucci G. Insulin and colon cancer. *Cancer Causes & Control.* 1995; 6:164–179. [PubMed: 7749056]

Figure 1.
Structure of inulin-doxorubicin conjugate.

Figure 2. IC₅₀ values. Caco-2, HT29-MTX, and SW620 cells were seeded, grown for 48 h, and exposed to increasing amounts of doxorubicin for 4 h. The doxorubicin was removed and growth continued for an additional 96 h before being assayed for cytotoxicity. Error bars represent error propagated over control cells. n = 6–8.

Figure 3.

Effect of 4 h exposure of inulin-doxorubicin conjugate (■) versus doxorubicin (◇) on colon cancer cell proliferation. Caco-2, HT29-MTX, and SW620 cells were seeded, grown for 48 h, and exposed to increasing amounts of inulin-doxorubicin conjugate or doxorubicin for 4 h. The amount of doxorubicin present in the inulin-doxorubicin conjugate and doxorubicin was equal. The inulin-doxorubicin conjugate or doxorubicin was removed and growth continued for an additional 96 h before being assayed for cytotoxicity. Error bars represent error propagated over control cells. $n = 6-8 \pm \text{SD}$.

Figure 4.

Binding studies of the inulin-doxorubicin conjugate (■) versus doxorubicin (◇) with ctDNA. Stock solutions of doxorubicin and inulin-doxorubicin conjugate were mixed with increasing concentrations of ctDNA. Sample fluorescent values were measured on a UV/VIS plate reader and the ratio of F/F_0 was indicative of fluorescent quenching where F is the fluorescent value of the sample and F_0 the fluorescent value with no ctDNA present. $n = 3 \pm \text{SD}$.

Figure 5.

Confocal imaging of SW620 cells incubated with doxorubicin or inulin-doxorubicin conjugate for 4 h. SW620 cells were seeded, grown for 48 h, and exposed to the inulin-doxorubicin conjugate or doxorubicin for 4 h. The amount of doxorubicin ($0.27 \mu\text{g/mL}$) present in the inulin-doxorubicin conjugate and doxorubicin was equal. The inulin-doxorubicin conjugate or doxorubicin was removed, the cells fixed with paraformaldehyde, and stained with DAPI. A) DAPI stain (blue) of cell nuclei, B) fluorescence (red) of inulin-doxorubicin conjugate or doxorubicin, C) overlay of A and B.

Figure 6.

Transport of inulin-doxorubicin conjugate (■) or doxorubicin (◇) across Caco-2 and HT29-MTX co-culture. Caco-2 and HT29-MTX were combined in a 1:1 ratio and seeded on a permeable Transwell® plate and cultured for 21–24 days or until the TEER values leveled. Cells were exposed to 25 µg/mL of the inulin-doxorubicin conjugate or doxorubicin. Doxorubicin concentration was determined using a UV/VIS spectrometer. $n = 4-6 \pm SD$.