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Research Paper

Targeted Sialic Acid–Doxorubicin Prodrugs for Intracellular Delivery and Cancer Treatment

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Purpose. To evaluate a novel targeted anticancer prodrug consisting of several copies of sialic acid (SA, targeting moiety), doxorubicin (DOX), citric acid (multifunctional spacer) and poly(ethylene glycol) (PEG, carrier).
Methods. α, ω bis carboxyl PEG was covalently conjugated with multiple copies of SA and DOX through a citric acid spacer and characterized by proton nuclear magnetic resonance (^{1}HNMR), matrix-assisted laser desorption/ionization-time of flight (MALDI/TOF), and high-performance liquid chromatography (HPLC). The molecular models of conjugates were established using ChemDraw software. Stability, spontaneous and esterase-stimulated drug release was analyzed by HPLC. Cellular internalization (fluorescence microscopy) and cytotoxicity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay] of free DOX and prodrugs were evaluated.
Results. ^{1}HNMR, MALDI/TOF, and HPLC showed the formation of the PEG prodrug conjugates. More than 40% of the drug was released from its conjugate in the presence of esterase enzyme, whereas the conjugate was stable at pH 7.4 in the absence of enzyme. Molecular modeling studies showed stable conformations of conjugates. The targeted prodrug conjugates with two copies of SA and DOX showed enhanced cytotoxicity when compared with non-targeted prodrugs and free DOX.
Conclusions. Targeting of the conjugate to cancer cells by SA with increased copies of targeting moiety and anticancer drug enhanced prodrug uptake by cancer cells and cytotoxicity of the prodrug.

KEY WORDS: doxorubicin; intracellular delivery; prodrugs; sialic acid; targeting.

INTRODUCTION

The major limitations of conventional cancer chemotherapy are the lack of tumor selectivity and limited antitumor activity. Low tumor specificity of most anticancer drugs often results in severe adverse side effects on healthy organs, leading to high systemic toxicity and preventing the use of high drug doses required for the effective killing of cancer cells. The selectivity of chemotherapy can be substantially increased by conjugating (directly or by using a complex drug delivery system) an anticancer drug with a targeting moiety, which provides two major functional roles. First, it directs the anticancer drug specifically to tumor cells, enhancing drug accumulation in tumor and therefore prevents adverse side effects. Second, it works as a penetration enhancer increasing the drug influx by tumor cells.

Nevertheless, penetration of an anticancer drug into the cytoplasm of tumor cells does not automatically guarantee its high cell death-inducing activity even if the drug is potentially highly cytotoxic. Cancer cells develop several mechanisms of cellular defense against cytotoxic agents. Two major mechanisms limit cytotoxicity of the internalized drug. First, the most potent low molecular weight water-soluble anticancer drugs can be removed from the cytoplasm by special active drug efflux pumps (1–4). Second, powerful detoxification mechanisms decrease drug toxicity during its journey through the cytoplasm to the intracellular target, e.g. nucleus (3,5,6). One approach to protect an anticancer drug from membrane active efflux pumps and cytoplasmic detoxification consists of changing a mechanism of cellular drug internalization by conjugating low molecular weight drug with a high molecular weight polymer or incorporating the drug into a nanocarrier (i.e. liposomes, nanoparticles, micelles, etc.) (7). While small water-soluble drugs enter cells by diffusion, high molecular weight substances or nanocarriers are internalized by endocytosis in membrane-limited organelles, which protect the drug from degradation

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ABBREVIATIONS: CA, citric acid; DDS, drug delivery system; DOX, doxorubicin; FITC, fluorescein 5(6)-isothiocyanate-EDC N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide; ^{1}HNMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HPMA, hydroxypropylmethacrylamide.; LHRH, luteinizing hormone-releasing hormone; MALDI/TOF, matrix-assisted laser desorption/ionization-time of flight; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); PEG, polyethylene glycol; SA, sialic acid.

in the cytoplasm of cancer cells and therefore increase its cell inducing activity (7–9). However, endocytosis is a relatively slow process which requires a high concentration of the drug in intracellular space to provide for an intracellular drug concentration that is capable of killing a cancer cell. Incidentally, conjugation of the high molecular weight drug to penetration enhancer/targeting moiety enhances endocytosis and therefore increases cytotoxicity of the complex (10).

In addition to changing the internalization mechanism and enhancing intracellular drug toxicity, the conjugation of an anticancer drug to a high molecular weight carrier or its incorporation into other nanocarrier delivery system results in several other improvements. First, it converts an active drug into a so-called "prodrug"—a form of a drug that remains inactive during its delivery to the site of action and is activated by specific conditions at the targeted site (tumor or cancer cell). A prodrug can be cleaved and activated inside cells (prodrugs of the first class) or two or more parts of the prodrug can be combined together (prodrugs of the second class) to form active substance or substances with high anticancer activity (11). A special type of prodrug, targeted drug delivery system (DDS), has been developed during the past few decades. Targeted DDS usually includes three components: (1) a drug, (2) a targeting moiety and (3) a carrier (10). The carrier binds the DDS components together and facilitates the solubility of the whole complex. The drug (therapeutic component) provides the treatment. The targeting moiety/penetration enhancer substantially increases the internalization of active components specifically into targeted cells enhancing the specific activity of the whole DDS and decreasing adverse side effects on healthy tissues. The present manuscript describes a novel type of targeted anticancer drug delivery system which allows for incorporating several copies of an anticancer drug and a targeting moiety. We selected polyethylene glycol (PEG) as a carrier, doxorubicin (DOX) as an anticancer drug, and sialic acid (SA) as a targeting moiety/penetration enhancer for the proposed targeted anticancer prodrug.

Based on our previous experience (8,12–14), we selected PEG as a carrier for the DDS. PEG is a highly aqueous soluble nontoxic polymer commercially available with different molecular weights and modifications. Therefore, it is widely used for different medical applications including the delivery of anticancer agents. However, native mPEG has only one site available for the conjugation, and therefore only either one drug molecule or one other than the drug active ingredient (i.e. targeting moiety) might be conjugated. This limits the loading capacity of such linear polymeric carriers. The most frequently used method to overcome this shortcoming is the use of so-called branched polymers (7,15). However, such types of DDS include several copies of polymer and therefore might have an excessive molecular weight, which in turn can decrease the efficacy of the drug. Recently, we proposed a novel method of increasing the payload of linear PEG polymeric structures, synthesized and characterized several different PEG prodrug conjugates with different copies of active ingredients, including drugs and targeting peptides (8). The method is based on the use of citric acid (CA) as a branched spacer and allows conjugating the high number of copies of active components to one molecule of PEG. The system substantially limits steric hindrance of components and the number of such copies is limited mainly by the solubility of the whole complex. In the present research we used a similar approach to conjugate one and two copies of DOX and SA to one molecule of PEG.

A traditional well-established anticancer drug DOX was used to induce cell death in cancer cells. Among many other chemotherapeutic agents, DOX is one of the most widely used anticancer drugs with high activity in different types of tumors. Even though adriamycin (DOX) and related anthracycline antitumor drugs are the most important antitumor drugs in clinical use, their mode of biological activity is not clearly defined. DOX has antimitotic and cytotoxic activity through a number of proposed mechanisms of actions. It forms complexes with DNA by intercalation between base pairs, and inhibits topoisomerase II activity by stabilizing the DNA-topoisomerase II complex, preventing the religation portion of the ligation–religation reaction catalyzed by topoisomerase II (16). DOX induces severe adverse effects including nausea, vomiting, diarrhea, myelosuppression and a dose-limiting cardiotoxicity. Therefore, its targeting to tumor cells is required. In addition, DOX is a well-known substrate for cell membrane drug efflux pumps and intracellular detoxification (17), which in turn may be limited by changing the mechanism of drug internalization to endocytosis in membrane limited endosomes.

SA was chosen as a targeting moiety/penetration enhancer to limit adverse side effects of DOX on healthy organs and enhance the internalization of relatively high molecular weight prodrug by cancer cells. In general, tumor-targeted drug delivery depends upon the physiological differences between the cancer cells over to normal cells. Tumors differ from normal tissues in several aspects including distinctions in the extra and intracellular environment, pH, microflora, overexpression of certain receptors, antigens and some substances on the surface and inside the tumor cells, etc. Potentially all of these mentioned differences can be used for tumor-specific targeting. Tumor-directed antibodies, ligands to extra- and intracellular receptors, and biological pairs have been extensively used for tumor targeting (10). Recently our lab successfully used a modified peptide synthetic analog of luteinizing hormone-releasing hormone (LHRH) decapeptide as a targeting moiety to tumors overexpressing LHRH receptors (8,12,13,18). The use of this peptide prevented an accumulation of DDS in healthy organs, and enhanced both drug accumulation in tumors and its internalization by cancer cells. Therefore, conjugation of LHRH peptide to DDS substantially increases antitumor efficacy of anticancer drugs and limits their adverse side effects. However, a different targeting approach is needed for other types of tumors, especially for cancers that do not overexpress LHRH receptors. Gangliosides, SA-containing glycosphingolipids, have attracted great interest for more than 20 years in the search for target molecules of relevance for tumor growth and formation of metastases and as potential targets for immunotherapy (19). In most cases SA, overexpressed on the cell surface of many cancer cells, has been used as a target and cancer-specific antigen. On the other hand, a special type of lectins, the so-called C-lectins (which include selectins and pentraxins) were identified on the surface of the plasma membrane of cancer cells (20). Selectins are adhesion molecules that mediate calcium-

dependent cell–cell interactions among leukocytes, platelets, and endothelial cells and are believed to be responsible for adhesion of several types of cancer cells to endothelial cells and therefore for spreading of tumor metastasis. Consequently, SA, a well-known ligand to selectins, potentially could be used for targeting of anticancer agents to tumors that overexpress selectins.

We hypothesize that the use of the proposed SA-DOX targeted prodrug conjugates with different copy numbers of DOX and SA will significantly increase the efficacy of cancer treatment by: (1) targeting anticancer drug specifically to cancer cells and (2) enhancing cytotoxicity of the high molecular weight drug-conjugate. The main objective of the present work is to verify this hypothesis and to study the targeting ability of SA as a penetration enhancer in intracellular delivering of DOX-PEG prodrug conjugates. For this purpose, the number of copies of DOX and SA were varied to evaluate cytotoxicity and targeting ability of the studied prodrugs. CA was used as a multifunctional spacer to incorporate multiple copies of DOX and SA simultaneously with α,ω bis (2-carboxyethyl) PEG polymer. Prodrug conjugates SA and DOX were characterized by different methods including molecular modeling. In addition, cellular entry dynamics of free DOX was compared using fluorescent microscopy, with PEG conjugates either with or without SA. Rate of drug release from the conjugates by hydrolysis was analyzed by high-performance liquid chromatography (HPLC) using esterase enzyme. Finally, *in vitro* cytotoxicity (IC_{50}) of the conjugates was evaluated for prodrugs and compared with free DOX on A2780 human ovarian carcinoma cells.

MATERIALS AND METHODS

Chemicals

DOX HCl was obtained from Sigma Chemical Co. (Atlanta, GA). α,ω bis (2-carboxyethyl) PEG [M_w∼3,000 Da], SA, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide HCl (EDC.HCl) and fluorescein 5(6)-isothiocynate (FITC) were obtained from Fluka (Allentown, PA). *N,N*-diisopropyl-ethylamine, 4-(methylamino) pyridine (DMAP) were purchased from Sigma-Aldrich (St. Louis, MO). Dialysis membrane of molecular weight cut off of 3,500 Da (Spectra Pore), CA, dimethyl sulphoxide (DMSO), dimethyl formamide (DMF), dichloromethane (DCM), acetone, methanol, acetonitrile, ethanol, and diethyl ether were purchased from Fischer Scientific (Rochester, NY). Jupiter and Atlantis column were purchased from Phenomenex (Torrance, CA, USA) and Waters (Waters USA), respectively.

Cell Line

The human ovarian carcinoma A2780 cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ). Cells were grown at 37°C in a humidified atmosphere of 5% CO2 (v/v) in air. All of the experiments were performed on the cells in exponential growth phase.

Synthesis of Prodrug Conjugates

Synthesis of α,ω,-bis(2-Carboxyethyl) PEG-CA Conjugates. Synthesis of α,ω,-bis(2-carboxyethyl) PEG-CA conjugates is schematically presented on Fig. 1. CA was used as a multifunctional spacer and is chemically 2-hydroxy-1,2,3-propanetricarboxylic acid containing one hydroxyl and three carboxyl functional groups for chemical conjugation. To obtain the conjugates (compound 3 in Fig. 1), α,ω, bis(2-carboxyethyl) PEG (1 in Fig. 1) was conjugated with CA (2 in Fig. 1) by a one-step condensation method as reported earlier (8). Briefly, bis(2-carboxyethyl) PEG (100 mg, 0.033 mM) and 2 moles of CA (12.8 mg, 0.066 mM) were dissolved in 6.0 ml of anhydrous dimethylformamide (DMF) and 25 ml of anhydrous DCM. EDC.HCl (13.0 mg, 0.0678 mM) was added to the reacting solution as a coupling agent, and DMAP (4.00 mg, 0.032 mM) was used as a catalyst. The reaction solution was stirred continuously for 24 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted CA and EDC.HCl were removed by dialysis using Spectra/Por membrane molecular mass cutoff, ∼2,000 Da) in DMF as a solvent. Additionally, the conjugate was purified by a size-exclusion Sephadex G10 column. The conjugate was dried under vacuum at room temperature.

Synthesis of α,ω,-bis(2-Carboxyethyl) PEG-CA-SA Conjugates. Synthesis of α,ω,-bis(2-carboxyethyl) PEG-CA-SA conjugates is schematically presented in Fig. 1 (compound 5). Either one or two copies of SA (4 in Fig. 1) were conjugated with α,ω,-bis (2-carboxyethyl) PEG–citrate acid (PEG-CA) conjugate (M_w 3,382.24 Da) (compound 3 in Fig. 1). Briefly, PEG-CA (40 mg, 0.0118 mM) and SA (3.6 mg, 0.0118 mM for one copy of SA) were dissolved in 5 ml of anhydrous DMSO and 10 ml of anhydrous DCM. The reaction mixture was allowed to stir for 15 min. EDC.HCl (3 mg, 0.0156 mM) was added to the above solution as a condensing agent, and DMAP (2.0 mg, 0.016 mM) was used as a catalyst. The reaction was stirred continuously for 24 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted SA and EDC.HCl were removed by dialysis using Spectra/Por dialysis membrane (molecular mass cutoff ∼2,000 Da) in DMSO as a solvent. The conjugate was dried under a vacuum at room temperature. On the other hand, bis PEG polymer conjugates with two copies of SA was prepared by using two molar ratios of SA and EDC.HCl, respectively.

Synthesis of α,ω,-bis(2-Carboxyethyl) PEG-Citrate-SA-DOX Conjugates. Synthesis of α,ω,-bis(2-carboxyethyl) PEG-Citrate-SA-DOX conjugates is schematically presented in Fig. 1 (compound 7). DOX (6 in Fig. 1) was conjugated with α,ω,-bis (2-carboxyethyl) PEG-citrate acid-SA (PEG-CA-SA) conjugate (5 in Fig. 1) (M_w 3,690.52 Da). Briefly, PEG-CA-SA (25.0 mg, 0.0067 mM) and DOX (4.0 mg, 0.0068 mM for conjugating one copy of DOX) were dissolved in 5 ml of anhydrous DMSO and 10 ml of anhydrous DCM. The reaction mixture was allowed to stir for 30 min. EDC.HCl (2.0 mg, 0.010 mM) was added to the above solution as a condensing agent, and DMAP (1.0 mg, 0.008 mM) was used as a catalyst. The reaction was stirred continuously for 24 h at room temperature. The carbodiimide urea formed during the reaction was removed by filtration. The unreacted DOX and EDC.HCl were removed by dialysis

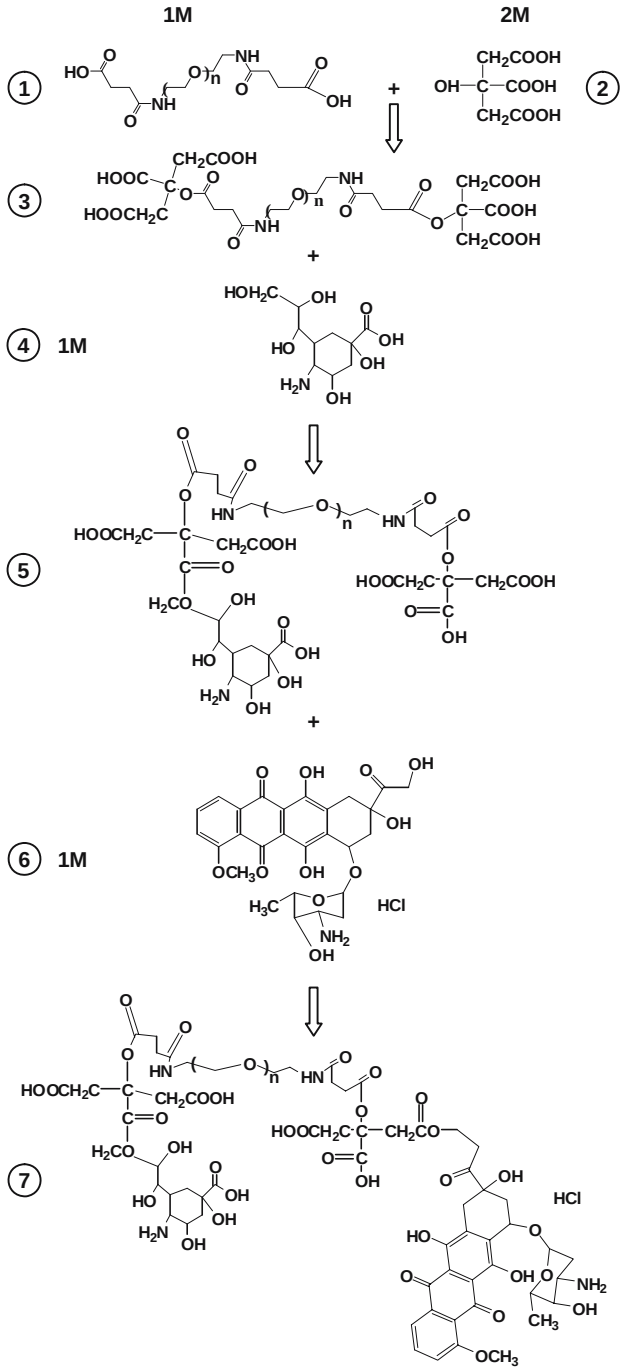


Fig. 1. Synthesis of anticancer prodrug conjugate. bis PEG (*1*) polymer was conjugated with 2 moles of CA (*2*) using EDC.HCl as a coupling agent to obtain α,ω bis PEG_{3000}-CA conjugate (*3*). α,ω-bis (2-carboxyethyl) PEG-CA (*3*) conjugates was coupled to SA (*4*) with one and two moles to obtain SA-PEG-CA conjugate (*5*). Further, SA-DOX-PEG-CA conjugate (*7*) was prepared by coupling one and two copies of DOX (*6*) with SA-PEG-CA conjugate (*5*).

using Spectra/Por dialysis membrane (molecular mass cutoff ~2,000 Da) in DMSO as a solvent. The conjugate was dried under a vacuum at room temperature. Additionally, PEG polymer conjugates with two copies of DOX were prepared by using two molar ratios of DOX and EDC.HCl, respectively.

Synthesis of SA-FITC Conjugates. Hydroxyl group in FITC was used for covalent conjugation with carboxyl group of SA using EDC.HCl as coupling agent. In brief, SA (15 mg, 0.048 mM) and FITC (18 mg, 0.046 mM) were dissolved in 2 ml of anhydrous DMSO and 10 ml of anhydrous DCM. EDC.HCl (9 mg, 0.046 mM) was added as a condensing agent, and DMAP (1.0 mg, 0.008 mM) was used as a catalyst. The reaction was stirred for 24 h under subdued light. The reaction solution was filtered to remove the carbodiimide salt. The conjugate was washed with excess acetone three times to remove free FITC, and the conjugate was precipitated in diethyl ether.

Characterization of Synthesized Prodrugs

Proton nuclear magnetic resonance (^{1}HNMR) spectra for synthesized conjugates were recorded on a Varian 400-MHz spectrophotometer using $DMSOd_6$ as a solvent. The molecular mass of the conjugates was recorded using matrix-assisted laser desorption/ionization-time of flight (MALDI/TOF). In addition, the conjugates were analyzed using HPLC.

The analytical HPLC system employed consisted of a Waters HPLC device equipped with a 996 photo diode array detector. The HPLC pumps, auto sampler and diode array system were monitored and controlled and the analytical data was evaluated using the Empower computer program. Wavelengths used for identification of SA, and identification of other SA conjugates with the diode array detector were 210, 223, 280 nm for DOX-PEG, SA-DOX-PEG, 455 nm for free DOX. The separation of free forms of SA and DOX was carried using Atlantis column (Waters USA) C18 4.6 × 250 mm, 5–120 Å length with a C-18 guard column (Phenomenex) and separation of conjugates were with a Jupiter column C-5 4.6 × 250 mm, 5–300 A. Gradient elution was employed for all the conjugates mentioned above other than SA with a mobile phase consisting of 1% methanol in water, pH 3.5 (solution A) and water (pH 3.5), acetonitrile and methanol mixture of ratio 20:70:10 (solution B) as follows: isocratic elution 100% A, 0–10 min; linear gradient from 100% A /0% B to 50% A/50% B, 10–20 min; isocratic elution 20% A/80% B, 20–35 min; linear gradient from 0% A/100% B,35–40 min and 100% A/0% B, 40–50 min; post-time 10 min before next injection. SA was quantified using isocratic elution: 99% solution A and 1% solution B. The flow rate of the mobile phase was 1 ml/min, and the injection volumes were 20 µl of the standards and reaction mixture.

Free drug was quantified using the external standard method and based on the peak area under the curve. Calibration curves of the standards were made by diluting stock standards in water and DMSO. The samples were prepared and analyzed in triplicate. The linearity of the detector responses, detection, and determination limits were tested for all free drug and its conjugates. The limit of detection and quantitation was set at 0.0009 and 0.0012 µg respectively. Day-to-day reproducibility of the methods were tested by analyzing the reference sample 5 (free drugs) or 10 (conjugates) times. Identity and purity of each peak was monitored (in addition to monitoring retention times and the symmetry of the peak) with a photodiode array detector using the spectral data. The purity match of the sample peaks was obtained from available standard peaks. In the DAD system, purity analysis was made by comparing the spectral data of the sample peaks with those obtained for the

standards. Assay validation studies revealed that the method was rapid, simple, high throughput and convenient for the determination of the conjugate and free drugs. The characteristic UV absorbance pattern and retention time variation are evident of the formation conjugates and further supported by the increase in quantity of free drugs during enzymatic hydrolysis.

Molecular Modeling

Chemical structures for free PEG, and its conjugates with SA and DOX to represent seven ethylene repeat ($–CH_2–O–CH_2–$) units were built using ChemDraw 9.0 Pro (Cambrigesoft Corp, Cambridge, MA). The settings for energy minima and molecular dynamics were of order of 1.000 step interval: 2.0 fs, frame interval: 10 fs, terminated after 10,000 steps, heating/cooling rate: 1.000 Kcal/atom/ps, target temperature: 300°K as reported earlier (8). Distance between the first and last carbon atom was measured for the modeled structure of bis PEG and its conjugation with DOX and SA. RasTop Molecular Visualization Software (Philippe Valadon, San Diego, CA) was used for obtaining the length of free drug, polymer, and its conjugates.

In Vitro Cellular Entry of Free DOX and Prodrugs

A2780 human ovarian cancer cells were seeded in a six-well culture plate (1×10^4 cells/well) and made up to 2 ml final volume supplemented with 10% fetal bovine serum and 1% penicillin. The cells were incubated for 24 h at 37°C with free DOX, 2 DOX-PEG, $1 \times$ SA-$2 \times$ DOX-PEG, or $2 \times$ SA-$2 \times$ DOX-PEG conjugates. At the end of the incubation period (0, 5, 30, 45 and 60 min), the medium and 1×10^7 cells were separated by centrifugation and lyzed with a protease containing lysis buffer from a QIAamp kit (Qiagen, Valencia, CA). Cellular uptake of DOX and its PEG conjugates was estimated using a fluorescence microplate reader (GENios, Tecan Group Ltd, Durham, NC) at excitation 530 nm and emission 635 nm using the calibration curve of standard DOX.

Release of DOX from the Conjugates

The release of the drug from the prodrug conjugates was assessed by HPLC using esterase as hydrolyzing enzyme at pH 7.4. For prodrug conjugate stability studies, 2.7 mg of $1 \times$ DOX-$2 \times$ SA-PEG conjugate was dissolved in 1.0 ml of PBS buffer (pH 7.4) without or with 2.0 mg of esterase in separate vials. Both the vials were stirred continuously and 100 μl of the sample were withdrawn and replaced by an equivalent amount of PBS buffer at 0, 30 min, 1, 3, 6, 48 h. The standard curve of DOX was obtained in a special series of HPLC experiments and used for calculating the release of DOX from the PEG conjugate. The data is presented as a percentage of total DOX released from the conjugates over a 120 h period.

Cellular Internalization

Cellular internalization of FITC-labeled SA, PEG-DOX and SA-PEG-DOX conjugates were studied using the fluorescence microscope Axiostar plus (Carl Zeiss Micro-Imaging Inc, Thornwood, NY). To assess intracellular distribution of the compounds SA was labeled with FITC (green fluorescence), while DOX possess intrinsic fluorescence (red). Fluorescent and light images were recorded. To compare intracellular distribution of SA and DOX, fluorescent images of SA-FITC (green fluorescence, "FITC" filter) and DOX (red fluorescence, "TRITC" filter) were overlaid with the light image. Co-localization of SA and DOX gives yellow color on the composite image.

In Vitro Cytotoxicity

The cytotoxicity of free DOX, SA, and their PEG conjugates was assessed in A2780 ovarian carcinoma cells using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (21, 22). To measure cytotoxicity, cells were separately incubated in a microtiter plate with different concentrations of free DOX, free SA, PEG-DOX and PEG-DOX-SA conjugates. Control cells received an equivalent volume of fresh medium. The duration of incubation was 24 h. Based on these measurements, IC_{50} doses of free DOX and its polymeric conjugates (the concentrations of active ingredients necessary to inhibit the cell growth by 50%) were calculated. A decrease in the IC_{50} dose indicates an increase in drug toxicity. In some of the experiments the IC_{50} doses of $1 \times$ SA-PEG-$1 \times$ DOX peptide was measured in human A2780 ovarian cancer cells in the presence of 34 different concentrations of free SA. The concentration of the conjugate in these experiments was 10 times higher than the IC_{50} dose.

Statistics

All *in vitro* experiments were performed in quadruplicate. The results are expressed as mean ± SD from 4–8 independent measurements. Statistical analysis was performed as a one-way analysis of variance (ANOVA) and comparisons among groups were performed by an independent sample *t*-test.

RESULTS

Characterization of Synthesized Prodrug Conjugates

The conjugates formed were characterized by ^{1}HNMR and the mass of the conjugates was measured using MALDI/TOF mass spectrometry. ^{1}HNMR spectra were recorded on a 400 MHz spectrophotometer using DMSO-d6 as a solvent. A typical ^{1}HNMR spectrum of PEG-DOX is shown in Fig. 2a. The peaks at δ1.22 (3H,d,CH3-C-5′), 3.0–3.8 broad peaks resulting from PEG backbone, 3.24 (3H,s,O CH3-H PEG), 3.99 (3H,s,CH3-O-C1), 4.45–5.0 (2H,m,OH-C-8 and OH-C4′), 6.80 (5H,m,aromatic), 7.94 (2H,d,CH-2 and CH-4) indicating formation of the PEG –DOX conjugate. In addition, the mass of the conjugates was measured using MALDI/TOF (Voyager De-Pro) system with α-cyano-4-hydroxycinnamic acid as a matrix (Fig. 2b). The mass of bis DOX-PEG conjugate was found to be 3,411 Da, whereas, PEG-SA-DOX conjugate showed a mass of 4,210 Da (data not shown). The total mass of the compounds showed formation of the PEG conjugates.

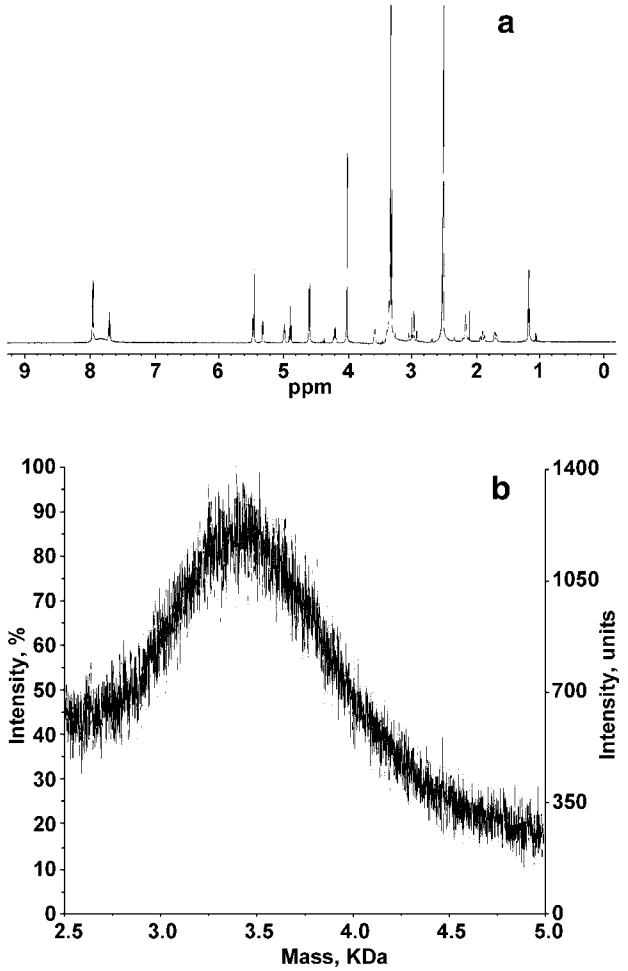


Fig. 2. Characterization of synthesized polymer conjugates. Typical images of ^{1}HNMR spectra of DOX-bis PEG-conjugate (**a**) and MALDI/TOF chromatogram (**b**). ^{1}HNMR spectra were recorded on 400 MHz spectrophotometer using DMSO-d6 as a solvent. Mass of bis 1DOX-PEG conjugate (3.4 KDa), was measured by MALDI/TOF (Voyager De-Pro) system using α-cyano-4-hydroxycinnamic acid as a matrix. The total mass of the conjugate indicate formation of the DOX PEG conjugate.

In addition, DOX, SA and their PEG-based prodrugs conjugates were analyzed by HPLC to verify the identity of compounds. Typical HPLC chromatograms are shown in Fig. 3a–c. Typically SA showed the retention time at 3.76 min (210 nm absorbance) as indicated in Fig. 3a. Under the same conditions, DOX.HCl showed the retention at 22.97 min on UV absorbance 479 nm as indicated in Fig. 3b. On the other hand, DOX-PEG conjugate showed one sharp peak at 8.78 min at 210 nm, with several small peaks having shoulders (Fig. 3c).

Molecular Modeling Studies

The structural stability of the linear bis PEG polymer along with DOX and SA with different number of copies was analyzed. Structures were built in Chemdraw software, and evaluated for the energy minima and molecular dynamics for DOX, bis PEG, SA, and polymeric conjugates containing DOX and SA. The molecular dynamics conformational distance for free DOX (C1 to C27) was observed to be 9.2 Å (figure not shown). The molecular dynamics distance for free bis PEG (C1 to C 24) was 6.40 Å (figure is not shown). On the other hand, the molecular dynamics conformational distances for (1) DOX-PEG-CA, (2) 1 × DOX-PEG-CA, and (3) 2 × DOX-2 × SA-PEG-CA-PEG conjugate were 8.58 Å, 5.38 Å, and 26.77 (C1-109 in Fig. 4) respectively.

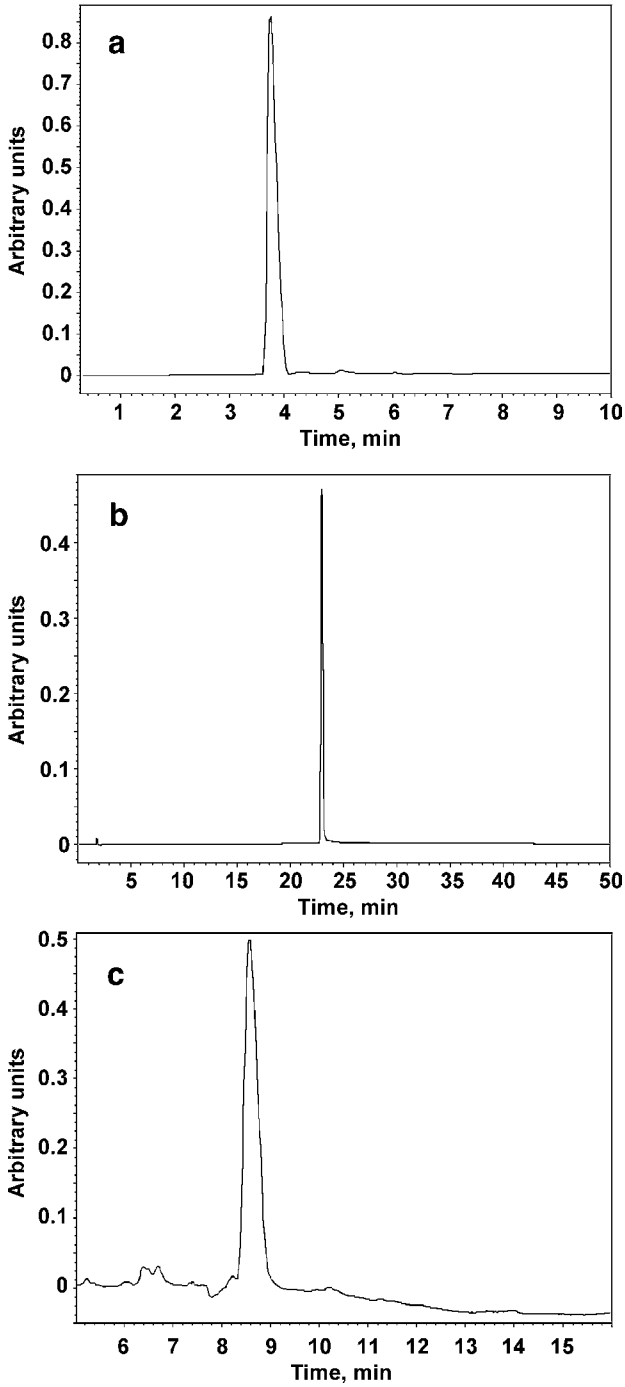


Fig. 3. Typical HPLC chromatograms of SA (A, 3.7 min retention time), DOX (B, 23.0 min retention time) and DOX-PEG conjugate (C, 8.6 min retention time).

In vitro Cellular Entry of Conjugates

It was found that the intracellular uptake of free (Fig. 5a, curve 1) and conjugated DOX (Fig. 5a, curves 2–4) slowly increased, reaching a plateau after 60 min of the incubation. The maximum cellular uptake reached 11–13% of the applied DOX dose for free DOX and two copies of DOX conjugated with PEG or PEG containing one copy of SA per 1 molecule of the polymer. The increase in the number of SA copies to two substantially enhanced the intracellular uptake of the conjugate (Fig. 5a, curve 4). First, 2 × DOX-2SA-PEG was internalized very fast by ovarian cancer cells. In fact, about 20% of the applied dose were internalized into the cells 5 min after the beginning of the incubation of the cells with 2 × DOX-2SA-PEG conjugate solution. Second, the percent of the internalized drug was substantially higher for 2 × DOX-2SA-PEG conjugate when compared with free DOX, 2 × DOX-PEG and 2 × DOX-1 × SA-PEG and reached approximately 30% of applied dose.

Release of DOX from the PEG Conjugates

The stability of conjugates in PBS buffer and release of the drug from the PEG conjugates containing DOX and SA after hydrolysis by esterase enzyme at pH 7.4 was assessed by HPLC (Fig. 5b). The standard curve of DOX was obtained in a special series of HPLC experiments and used for calculating drug release from the conjugates. Only a traceable amount of DOX was released from the conjugates after incubation in the buffer without hydrolyzing enzymes (data are not shown). In contrast, more than 40% of DOX was released from different conjugates in the presence of hydrolyzing enzymes. The typical time-profile of DOX release from a 1 × DOX-2 × SA-PEG conjugate in the presence of esterase is presented in Fig. 5B.

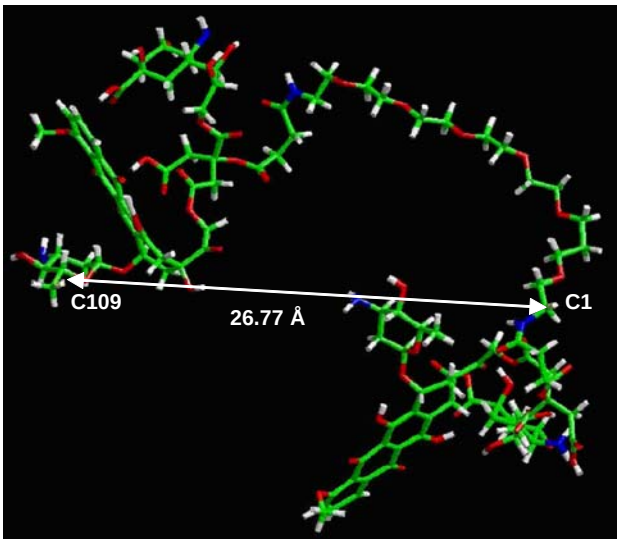


Fig. 4. Molecular modeling studies. Structures of the PEG, free DOX and SA and their conjugates were built in Chemdraw software and allowed to undergo conformational stabilization for energy minima and molecular dynamics. The stabilized structures were than exported to RasTop software to estimate the distances between first and last carbon atoms. Molecular dynamics structure with the distance between C1–C109 was observed to be 26.77 Å for 2SA-2DOX-bis PEG-CA conjugate.

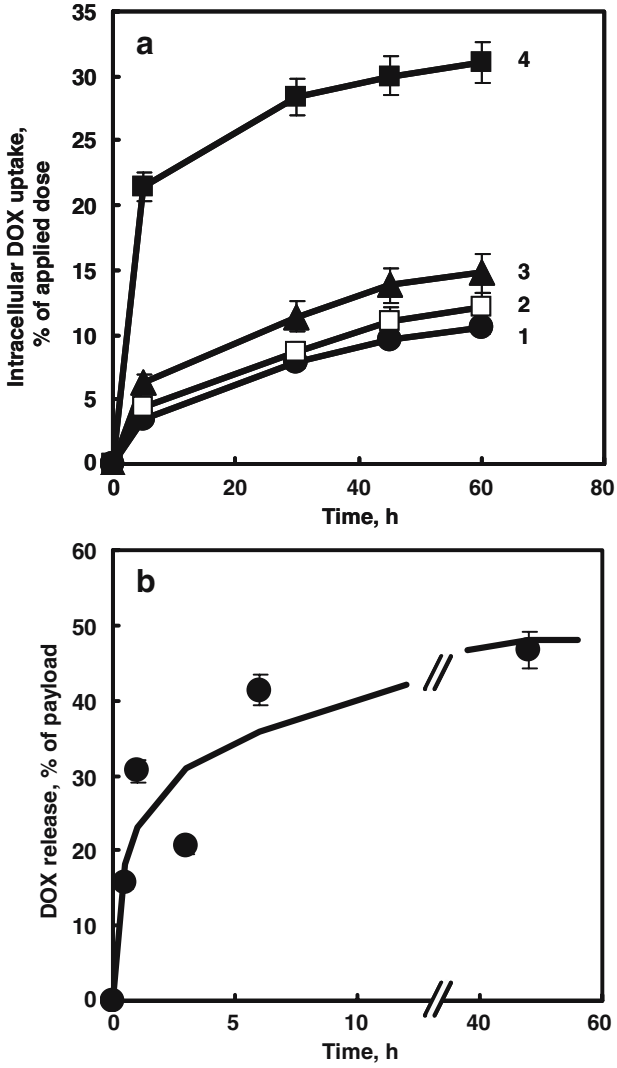


Fig. 5. Cellular entry of free DOX and its conjugates in A2780 ovarian carcinoma cells (**a**) and the release of DOX from the PEG conjugate by HPLC using esterase (pH 7.4) (**b**). The maximum cellular uptake reached 11–13% of applied DOX dose for free DOX and two copies of DOX conjugated with PEG or PEG containing one copy of SA per 1 molecule of the polymer. The increase in the number of SA copies to two substantially enhanced intracellular uptake of the conjugate (**A**, *curve 4*). Means ± SD are shown. *1* DOX; *2* PEG-2 × DOX; *3* 1 × SA–PEG-2 × DOX; *4* 2 × SA–PEG-2 × DOX.

Cellular Internalization of SA-FITC and PEG DOX Conjugates

Cellular internalization and localization of SA and DOX were studied by fluorescence microscopy. To visualize SA inside cancer cells, the compound was labeled with FITC showing green fluorescence. DOX itself possesses intrinsic red fluorescence. Therefore, the superposition of pictures with red (DOX) and green (SA-FITC) fluorescence results in a yellow color of the composite picture. The results shown that free SA is internalized very fast: there was a measurable

amount of the compound starting 5 min after exposure (Fig. 6). In contrast, internalization of free DOX (data not shown) and PEG-DOX was considerably slower: a measurable florescence level of DOX was found inside cancer cells only after 30 min–1 h exposure. The addition of SA to the PEG-DOX prodrug substantially accelerated its uptake by cancer cells (Fig. 6). After internalization by cancer cells, SA and DOX showed similar intracellular distribution and were localized both in the cytoplasm and nucleus with prevailing accumulation in the cytoplasm.

In Vitro Cytotoxicity

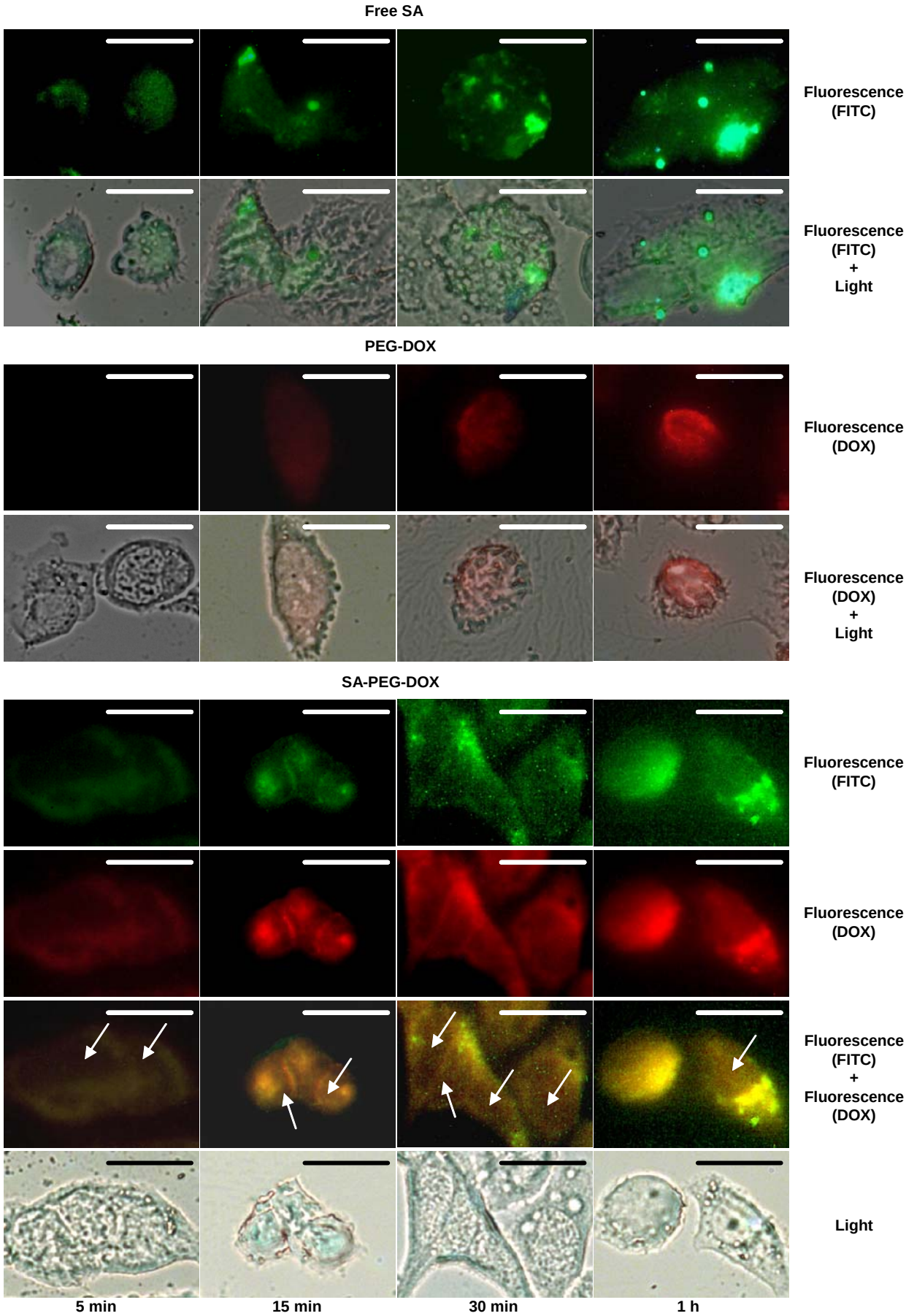
The average cytotoxicity data of non-targeted and targeted PEG-DOX prodrugs with different copies of active components in A2780 human ovarian carcinoma cells are presented in Fig. 7a. The results show that an increase in molecular weight of DOX after conjugation with PEG polymer significantly decreased its cytotoxicity and increased the IC_{50} dose (Fig. 7a, bar 2). Doubling the number of drug copies substantially decreased the IC_{50} dose of the prodrug (Fig. 7a, bar 3). However, cytotoxicity of PEG-2 × DOX prodrug still was approximately 50 times lower when compared with free DOX. The inclusion of a targeting moiety, SA, led to a dramatic increase in the cytotoxicity of the whole prodrug (Fig. 7a, bar 4). An increase in the number of copies of the drug (DOX) and targeting moiety (SA) resulted in a further increase in the cytotoxicity of prodrugs (Fig. 7a, bars 5 and 6). Finally, the conjugate containing 2 copies of SA and 2 copies of DOX was almost 10,000-times more toxic than a non-targeted PEG-1 × DOX prodrug and 84-times more toxic than free DOX (compare bar 6 with bars 2 and 1 in Fig. 7a). To demonstrate that high cytotoxicity of SA-containing DDS is associated with the binding of SA to SA-specific molecules expressed in the plasma membrane of cancer cells (most probably selectins), we incubated A2780 human ovarian carcinoma cells with 34 different concentrations of free SA (ligand for selectins) and then measured the cytotoxicity of the 1 × SA-PEG-1 × DOX conjugate. Data obtained showed that SA as a selectin ligand substantially limited the cytotoxicity of the conjugate in concentration-dependent manner (Fig. 7b).

DISCUSSION

Recently, we developed and evaluated targeted multicomponent anticancer prodrugs with a different number of copies of camptothecin and LHRH peptide as a targeting moiety (8). A similar approach was used in the present research for the conjugation of another anticancer drug–DOX and another targeting moiety–SA simultaneously with α,ω,-bis (2-carboxyethyl) PEG polymer through incorporation of multifunctional CA spacer. Bis PEG polymer with a molecular mass of 3,000 Da, used in both studies, exhibits a moderate decrease in bioavailability and cytotoxicity of anticancer drugs while improving drug pharmacokinetics (10,23–25). Such a relatively low molecular weight polymer does not provide effective passive tumor targeting by the enhanced permeability and retention effect. Therefore, low molecular weight polymeric prodrugs require active targeting, which we achieved by incorporating a special tumor targeting moiety (LHRH peptide or SA) into the prodrug. Our conjugation approach is based on the use of CA as a branched spacer. The advantages of CA as a spacer include a chemical flexibility and versatility due to the presence of one hydroxyl and three carboxyl functional groups for conjugation with drugs and carboxyl terminated PEG (8). In addition, the reactivity of CA is expected to be greater than widely used amino acids or linear polymers. Moreover, such spacers may reduce the steric hindrance for chemical conjugation with bulkier drugs and high molecular weight linear polymers (7). The bis-PEG polymer used here has two carboxyl terminal end groups and therefore is coupled with hydroxyl group in CA on a two molar basis to obtain a total of six carboxyl end groups. The resulting bis PEG-CA moiety is used for conjugating 1 and 2 copies of SA and DOX simultaneously, by varying the molar ratio of the components as well as the coupling agent. The conjugates formed were characterized by ^{1}HNMR and the mass of the conjugates was measured using MALDI/TOF.

In addition, DOX, SA and their PEG conjugates were analyzed by HPLC. The synthesized conjugates normally show one major chromatographic peak accompanied by several small peaks having shoulders. The resulting shoulder peaks might be formed due to complex polymeric architectures, with multiple hydroxyl and carboxyl groups subjected to a variable extent of steric hindrance or shielding by the larger amino groups. In addition, these peaks are virtually identical in a hydrophilic environment and try to resolve into smaller peaks. The different extents of shielding allow each chromophore, which is being formed due to conjugation, to interact differently and show less intense smaller peaks. It should be stressed that, there have been very few reports on polymeric conjugates involving high molecular weight components. More detailed analysis of PEG prodrug conjugates, including PEG–paclitaxel and dendrimer–paclitaxel conjugates, have been reported by our laboratory (26). On the other hand, HPLC resolved the conjugates of SA-PEG into multiple peaks. The retention time and separation of SA-PEG in reversed-phase HPLC is influenced by the linkage between different hydroxyl and carboxyl groups. The different arrangements of the hydroxyl and carboxyl groups around the periphery of PEG isomers lead to different hydrophilicity of these molecules, thus affecting their separation. In summary, characterization of the synthesized conjugates confirms the formation of prodrugs with DOX, SA and PEG.

SA is a 9-carbon monosaccharide and the one found in humans is *N*-acetylneuraminic acid (Neu5Ac). In most animals (with the notable exception of chickens) Neu5Ac co-exists with *N*-glycolylneuraminic acid, a slightly modified form of SA bearing a hydroxyl group at the *N*-acyl position. The primary hydroxyl group at *N*-acyl position in the SA was used for chemical conjugation with carboxyl terminal in the bis PEG-CA conjugate. Such oligomeric and polymeric reactions that form a conjugate with other carbohydrates including *N*-acetyl glucosamine have been reported in the past (27). Either one or two copies of SA were coupled with a bis PEG-CA conjugate using equimolar ratios of coupling agent. DOX hydrochloride, is chemically (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacenedione hydrochloride with M_w ~580. It should be noted that naphthacenedione ring in DOX has 3 hydroxyl groups for


Free SA
Fluorescence (FITC)
Fluorescence (FITC) + Light
PEG-DOX
Fluorescence (DOX)
Fluorescence (DOX) + Light
SA-PEG-DOX
Fluorescence (FITC)
Fluorescence (DOX)
Fluorescence (FITC) + Fluorescence (DOX)
Light
5 min
15 min
30 min
1 h

◀ **Fig. 6.** Typical fluorescence and composite images of human A2780 ovarian carcinoma cells incubated for 24 h with FITC-labeled SA, DOX-PEG and SA-DOX-PEG conjugates. The use of SA as a targeting moiety in PEG-DOX conjugate enhanced the internalization of the prodrug by cancer cells. Arrows indicate nuclei. The size of the *scale bar* is 20 μm.

chemical conjugation with reactive compounds. Hydroxyacetyl group at C8 being a primary hydroxyl, is more reactive and less sterically hindered when compared to secondary hydroxyl groups at C'6, C'8, and C'11. Therefore, the primary hydroxyl group (i.e. C8) in DOX is expected to be more reactive than the other hydroxyl groups while conjugating with carboxyl groups of bis PEG-CA conjugate using EDC.HCl as a condensing agent. However, most of the prodrug conjugates show modifications at 3′ amino of the hexopyranosyl ring in DOX by aminolysis (28–30). Other than aminolysis reactions, acetyl group at C8 in DOX.HCl was conjugated with *cis*-4,7,10,13,16,19-docosahexenoic acid to form a hydrazone bond (31). The objective previously described in the literature was to selectively cleave the hydrazone bond to release DOX.HCl in the tumor cells, leading to selective toxicity. It should be noted that the amine at C'3 is in the HCl salt form, and cannot be used for conjugation unless neutralized or de-protected. By doing so, the stability, ionization, and water solubility of the drug may be affected. In addition, amination of 3′ amino of the hexopyranosyl ring in DOX may decrease the activity of the drug due to unavailability of amine group which otherwise would form a complex with DNA, which subsequently forms a DNA-topoisomerase II complex. Studies in this direction were aimed at evaluating the structure activity relationship of the drugs and their sites utilized for conjugating with polymers (7). In another report, DOX was linked with poly-2 hydroxypropylmethacrylamide (HPMA) backbone via a tertrapeptidyl spacer which, in addition, was conjugated with FCE 28069 containing D-galactosamine molecules as liver targeting moieties (32). The drug and D-galactosamine were coupled with tertrapeptidyl spacer molecules using amino functional groups in the moieties to form an amide linkages.

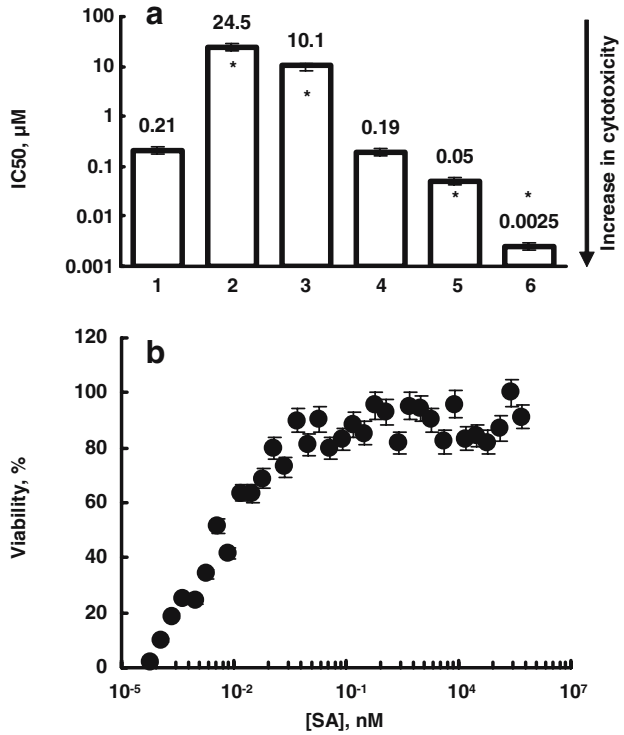


Fig. 7. Cytotoxicity of free DOX and its non-targeted and targeted prodrugs against A2870 human ovarian carcinoma cells (**a**) and competitive inhibition of the cytotoxicity of 1 × SA-PEG-1 × DOX by coincubation with free SA (**b**). The use of two copies of SA and doubling the number of copies of DOX in one molecule of the prodrug substantially enhances toxicity of DOX by decreasing the dose of the drug required to kill 50% of cells (IC_{50} dose). Free SA as a selectin ligand substantially limited cytotoxicity of the conjugate in concentration-dependent manner. Means ± SD are shown. *Asterisk* $P < 0.05$ when compared with free DOX. *1* DOX; *2* PEG-1 × DOX; *3* PEG-2 × DOX; *4* 1 × SA-PEG-1 × DOX; *5* 1 × SA-PEG–2 × DOX; *6* 2 × SA-PEG–2 × DOX.

Many copolymeric architectures including HPMA have a tendency to form unimolecular micelles due to a bound hydrophobic environment at the intramolecular core or multimolecular micellar aggregates. Such phenomenon may prevent enzyme activation of conjugated drug often limiting the activity of such macromolecular prodrugs (33). Therefore, a less complex polymeric DOX prodrug architecture, which is more stable and yet capable of releasing the drug with a desired rate, is needed. Recently, several PEG-peptide-DOX conjugates have been designed with 1:1:1 stoichiometry of PEG chain, amino acid spacer, and the drug. Such conjugates offer an opportunity to prepare an anticancer prodrug with a low polydispersity compared to pendent polymer conjugates (30). Continuing the efforts in this direction, we developed and reported in the present work a novel approach that has the flexibility of conjugating DOX and SA simultaneously to a bis PEG-CA conjugate. Moreover, this methodology does not limit the conjugation of other drug molecules and targeting moieties to the prodrug.

A further characterization of synthesized PEG-based prodrugs was made by molecular modeling. The modeling results indicate the stable structural conformations for bis PEG, DOX and its conjugates. Conformational distances for DOX-PEG-CA were 8.58 Å and 5.38 Å for 1 × DOX-PEG-SA and increased significantly for 2 × DOX-2 × SA-PEG-CA to 26.77 Å.

The biological evaluation supports high anticancer activity of DOX-SA-PEG prodrug conjugates. The results showed that this activity increases by doubling the number of copies of both the anticancer drug (DOX) and the targeting moiety (SA). Doubling the number of SA copies increases the bioavailability of the whole complex and its internalization by cancer cells, while an increase in the DOX content in the prodrug enhances anticancer activity of prodrugs. The targeting moiety accelerates internalization of the prodrugs by cancer cells and substantially enhances their cytotoxicity. Such effect is most probably related to the change in the mechanism of internalization of the prodrugs. In contrast to free low molecular weight drugs, which are internalized by diffusion, relatively high molecular weight non-targeted prodrugs are internalized by endocytosis, which is a much slower process requiring substantially higher drug concentration gradient across the plasma membrane to penetrate into the cells when compared with diffusion (10). The rate of prodrug uptake by endocytosis can be increased by using a targeting moiety as a ligand to certain receptors or molecules overexpressed in cancer cells. The use of such targeting moiety switches the mechanism of internalization from "simple"

endocytosis to receptor-mediated endocytosis enhancing cytotoxicity of the whole prodrug. Further enhancement in cytotoxicity of targeted prodrugs was achieved by doubling the number of copies of both targeting moiety and anticancer drug.

CONCLUSIONS

We proposed and designed a novel architecture of PEG prodrug of DOX based on the use of CA as a spacer and SA as cancer specific targeting moiety. The developed prodrug allows conjugating of several copies of targeting moiety and anticancer drug. Biological evaluation of synthesized conjugates showed that targeted prodrugs containing two copies of targeting moiety and two copies of anticancer drug possesses substantially higher cytotoxicity when compared with free drug and non-targeted conjugates.

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