[Frontiers in Bioscience, Elite, 8, 358-369, June 1, 2016]

Anticancer activity of drug conjugates in head and neck cancer cells

Debatosh Majumdar[1,2], Mohammad Aminur Rahman[2], Zhuo Georgia Chen[2], Dong M. Shin[2]

[1]Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, Emory University, 1365 C Clifton Road, Atlanta, GA 30322. [2]Glycosyn LLC, Medford, MA 02155

TABLE OF CONTENTS

1. ABSTRACT

Sexually transmitted oral cancer/head and neck cancer is increasing rapidly. Human papilloma virus (HPV) is playing a role in the pathogenesis of a subset of squamous cell carcinoma of head and neck (SCCHN). Paclitaxel is a widely used anticancer drug for breast, ovarian, testicular, cervical, non-small cell lung, head and neck cancer. However, it is water insoluble and orally inactive. We report the synthesis of water soluble nanosize conjugates of paclitaxel, branched PEG, and EGFR-targeting peptide by employing native chemical ligation. We performed a native chemical ligation between the N-hydroxy succinimide (NHS) ester of paclitaxel succinate and cysteine at pH 6.5 to give the cysteine-conjugated paclitaxel derivative. The thiol functionality of cysteine was activated and subsequently conjugated to multiarm thiol-PEG to obtain the paclitaxel branched PEG conjugate. Finally, we conjugated an EGFR-targeting peptide to obtain conjugates of paclitaxel, branched PEG, and EGFR-targeting peptide. These conjugates show anticancer activity against squamous cell carcinoma of head and neck cells (SCCHN, Tu212).

2. INTRODUCTION

Cancer is one of the deadliest diseases causing a large number of deaths worldwide. In 2012, about 8.2 million people died of cancer-related causes globally, 14.1 million new cases reported, and 32.6 million people living with cancer. It is estimated that by 2030, there will be 13.1 million deaths in cancer (1). According to the report of the International Head and Neck Cancer Epidemiology (INHANCE) Consortium, the worldwide incidence of squamous cell carcinoma of head and neck (SCCHN) exceeded 500,000, and mortality from this cancer exceeded 320,000 in the year 2008 (2). Cancers of oral cavity, pharynx, and larynx comprise 5% of all malignancies globally. It is estimated that in 2014 in the United States, there will be 12000 deaths in SCCHN and 55070 new cases of SCCHN (3). Human papillomavirus (HPV) is now recognized to play a role in the pathogenesis of a subset of squamous cell carcinoma of head and neck (SCCHN), particularly those that arise from the middle part of the throat including the soft palate, the base of the tongue, and the lingual and palantine tonsils within the oropharynx (4). HPV is the most common sexually transmitted infection (STI) (5,6). A person can get HPV by having oral, vaginal, or anal sex with someone who has the virus (5,6). In the United States, more than half of the oropharyngeal/head and neck cancers are associated with HPV-16 (7,8). The incidence of HPV-associated oropharyngeal/head and neck cancer has increased during the past two decades, especially among men. If the recent trends continue, then in the United States HPV will cause more oropharyngeal/head and neck cancers than cervical cancers by the year 2020 (7,8).

Paclitaxel, a diterpenoid derived from the bark of the Pacific yew tree *Taxus brevifolia*, is one of the most widely used antitumor agents (9-11). It exhibits effective antitumor activity against various cancers such as breast, ovarian, cervical, testicular, non-small cell lung, and head and neck cancers (9-12). Horwitz and co-workers have shown that paclitaxel binds to and affects the tubulin-microtubule equilibrium, decreasing both the critical concentration of tubulin as well as the induction time for polymerization (13,14). The absence of free tubulin prevents the assembly of a normal mitotic spindle, which ultimately leads to cell death.

Although paclitaxel is an effective chemotherapeutic agent in clinical settings, disadvantages associated with its use include poor water solubility; significant toxicities due to its lack of selectivity towards cancer cells, the use of excipient and the high

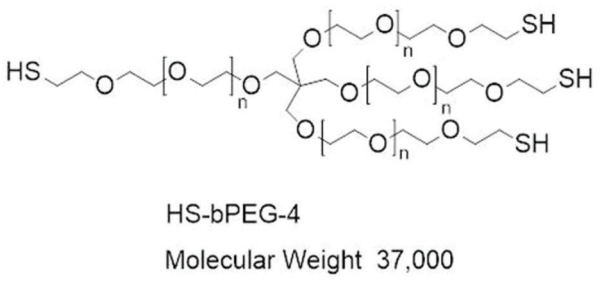

Figure 1. Branched four-arm thiol polyethylene glycol ether.

doses required; drug resistance and inability to cross the blood-brain barrier. Like many chemotherapeutics, poorly water-soluble and orally inactive paclitaxel requires modification prior to intravenous administration. Unfortunately excipients can cause undesired side effects while increasing the treatment cost. Paclitaxel is currently supplied with the excipient containing Cremophor EL and dehydrated ethyl alcohol at a 1:1 (*v*/*v*) ratio (15). Cremophor presents a number of serious concerns when administered intravenously, including various intrinsic toxic side effects, limiting the amount of paclitaxel that can be safely administered (15). The most common morbidity is acute hypersensitivity reaction characterized by dyspnea, flushing, rash, and generalized urticarial (15). Studies have shown that intravenous administration of Cremophor alters the pharmacokinetic profile of paclitaxel. Moreover, systemic use of paclitaxel in large doses can cause hematologic and neurologic toxicities. Life-threating hypersensitivity reactions have been observed in some patients despite appropriate premedication such as histamine H_2 antagonists, dexamethasone, and diphenhydramine. In order to reduce the undesired side effects of paclitaxel therapy, increase its efficacy, and reduce the cost of treatment, it is necessary to develop a paclitaxel agent that has increased bioavailability and is suitable for selective local delivery with limited, or no, toxic side effects. Therefore, as a new preliminary investigation, we synthesized the nanosize conjugates of paclitaxel, branched polyethylene glycol (bPEG), and epidermal growth factor receptor (EGFR)-targeting peptide and investigated their anticancer activity using squamous cell carcinoma of head and neck (SCCHN) (Figures 1-12). Moreover, we included the detail synthesis steps and NMR data.

3. MATERIALS AND METHODS

All commercial materials (Sigma Aldrich, Polymed Therapeutics Inc, CPC Scientific Inc, NOF Corporation) were used without further purification. Branched PEG was purchased from NOF Corporation. All solvents used for extraction and chromatography procedures were used as received from commercial suppliers without further purifications. Unless otherwise mentioned, all reactions were carried out under argon atmosphere in oven-dried glassware using dry solvents under anhydrous conditions. ^{1}H NMR and ^{13}C NMR spectra were measured in deuterated chloroform ($CDCl_3$) and deuterated methanol (CD_3OD) on Varian Inova 600 or Unity 600 NMR spectrometers (Supplementary Information). All proton NMR spectra were recorded at 600 MHz and all carbon NMR spectra were measured at 150 MHz, and reported in parts per million (ppm). Concentration of paclitaxel was determined by UV-Vis spectroscopy with an absorbance peak at wavelength of 227nm.

Compound 6: Cysteine (200mg, 1.65mmol) was dissolved in phosphate buffer (20mL, pH 6.5) under continuous stirring. Solution of NHS-activated Taxol-succinate ester (50mg, 47μmol, in 20mL CH_3CN/H_2O) was added dropwise into the cysteine solution. The stirring continued for 24h. Acetonitrile was removed under reduced pressure. Then the reaction mixture was extracted with CH_2Cl_2/MeOH (20mL, 3 times, CH_2Cl_2/MeOH =19/1). The organic layer was washed with brine solution (20mL, 3 times), dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography (Eluent: EtOAc: Hexane=1:1, 1%CH_3COOH→EtOAc: Hexane=7:3, 1% CH_3COOH → EtOAc, 1% CH_3COOH→ CH_2Cl_2/MeOH =19/1, 1% CH_3COOH) to give the pure product (34mg, 32μmol) in 68% yield. (Figures 5-6). ^{1}H NMR (600MHz; $CDCl_3$) δ_H 8.09 (d, *J* = 7.2Hz, 2H), 7.75 (d, *J* = 7.2Hz, 2H), 7.64-7.56 (m, 2H), 7.53-7.46 (m, 3H), 7.44-7.36 (m, 5H), 7.33-7.28 (m, 1H), 6.78 (d, *J* = 7.8Hz, 1H), 6.30 (s, 1H), 6.18 (t, *J* = 9.0Hz, 1H), 5.93 (dd, *J* = 9Hz, 3.6Hz, 1H), 5.67 (d, *J* = 7.2Hz, 1H), 5.48 (d, *J* = 4.2Hz, 1H), 4.96 (d, *J* = 10.2 Hz, 1H), 4.62-4.58 (m, 1H), 4.42-4.39 (m, 1H), 4.27 (d, *J* = 6.0Hz, 1H), 4.18 (d, *J* =9.6Hz, 1H), 3.73 (d, *J* = 6Hz, 1H), m (2.90-2.65, 4H), 2.60-2.45 (m, 3H), 2.42 (S, 3H), 2.30-2.10 (m, 5H), 1.90-1.80 (m, 4H), 1.66 (s, 3H), 1.40 (t, *J* = 6.0Hz, 1H), 1.21 (s, 3H), 1.13 (s, 3H). ^{13}C (150MHz, $CDCl_3$) δ_C 172.4, 172.3, 171.9, 171.7, 171.2, 170.2, 168.2, 166.6, 142.3, 136.6, 133.6, 133.4, 132.8, 132.1, 130.1, 129.2, 129.0, 128.6, 127.3, 126.8, 84.3,81.0, 78.6, 75.6, 74.9, 74.4, 71.9, 71.8, 60.4, 58.2, 53.7, 53.2, 45.7, 43.0, 35.5, 35.3, 30.4, 29.1, 26.6, 26.2, 25.2, 22.6, 21.9, 21.0, 20.8, 20.5, 14.7, 14.1, 9.6. HRMS-ESI (*m*/*z*) [M-H$^+$] calculated for $C_{54}H_{59}N_2O_{18}S$, 1055.3489; found, 1055.3472.

Compound 7: Paclitaxel-succinate-cysteine derivative 6 (22mg, 20μmol) was dissolved in DMF (4mL) under continuous stirring. Solution of 2,2′-dithiodipyridine (10mg, 45μmol, 2mL DMF) was added dropwise into the reaction mixture. Then pyridine (100μL) was added into the reaction mixture. Stirring was continued for 24h. Solvent was removed under reduced pressure and the reaction mixture was purified using column chromatography (Eluent: EtOAc: Hexane=1:1, 1%CH_3COOH→EtOAc: Hexane=7:3, 1% CH_3COOH → EtOAc, 1% CH_3COOH→ CH_2Cl_2/

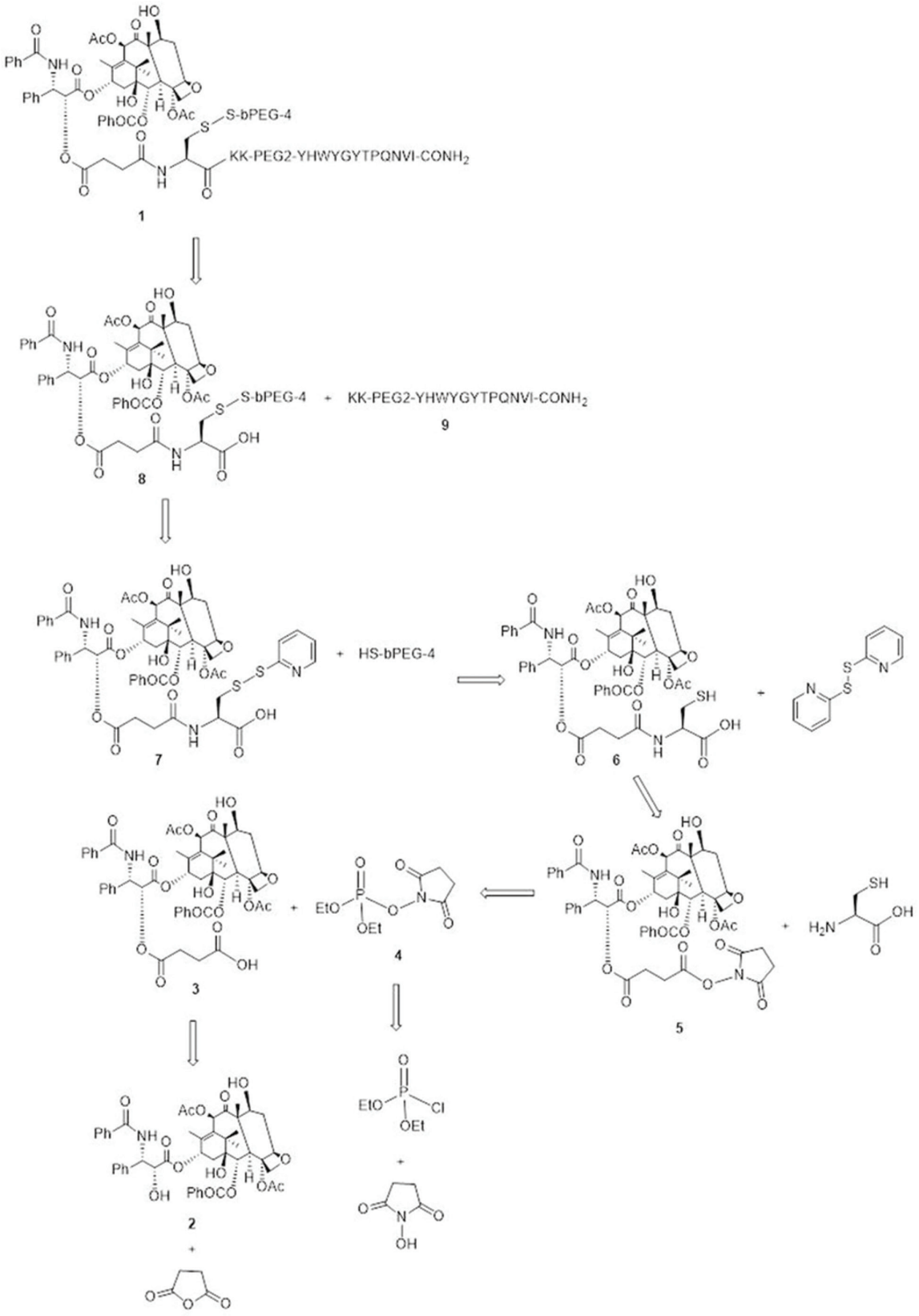

Figure 2. Retrosynthetic analysis for synthesis of conjugates **1** and **8**.

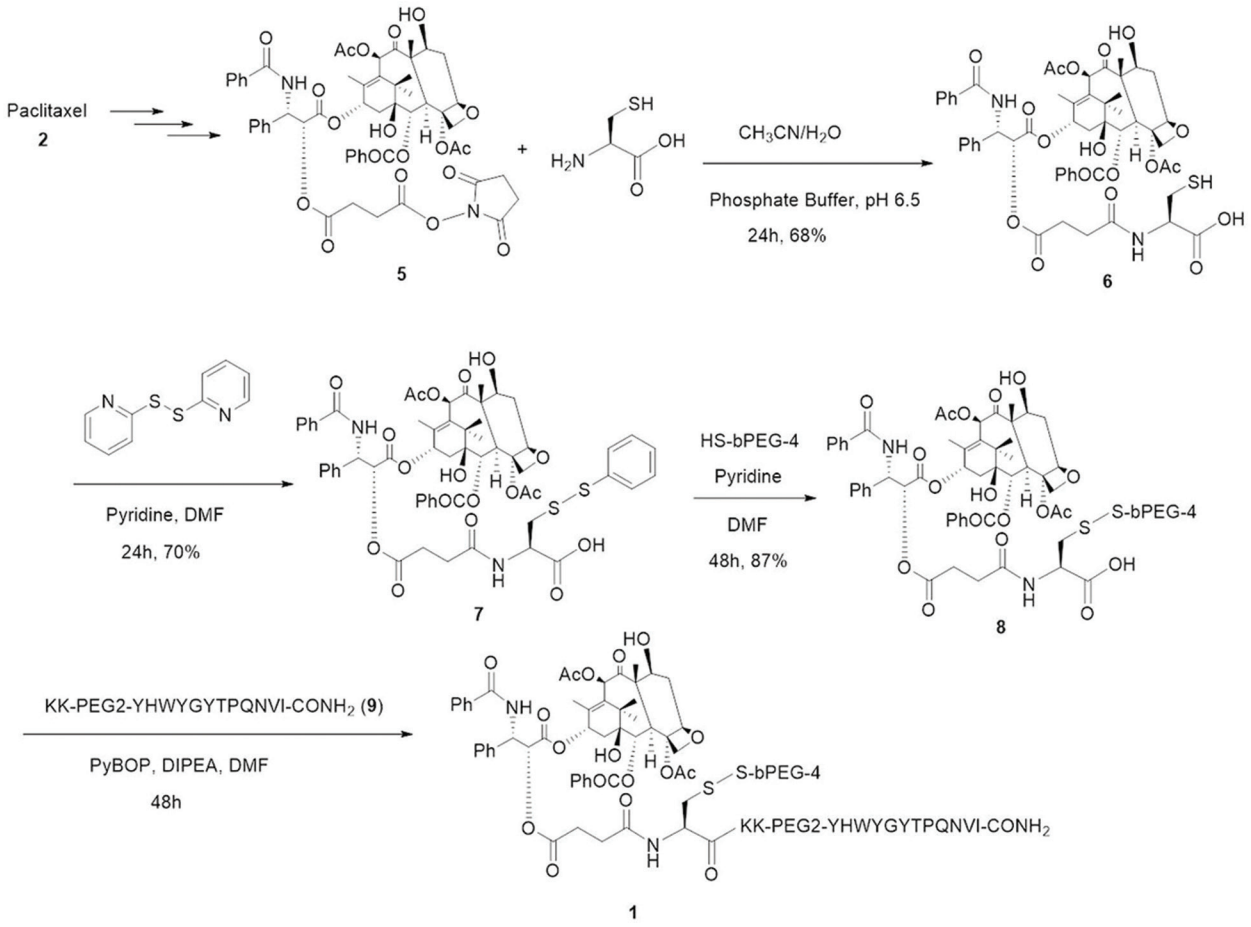

Figure 3. Synthesis of paclitaxel-branched PEG and paclitaxel-branched PEG-EGFR-targeting peptide conjugates.

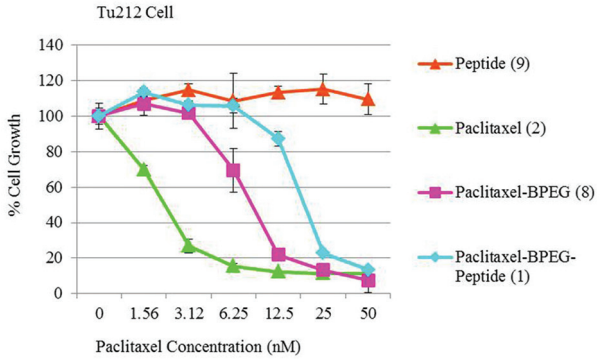

Figure 4. Sulforhodamine B (SRB) assay for cell growth determination of head and neck cancer cell line Tu212. Cells were seeded in a 96-well plate (3000 cells/well). After 16h cells were treated with paclitaxel and paclitaxel polymer conjugates at various concentrations, and incubated for 72h. Cells were then fixed with 10% trichloroacetic acid, stained with 0.4% SRB for 10 min and bound SRB was dissolved in 10mmol/L Tris base (pH 10.5). Cell growth was assessed by OD determination at 492 nm using a microplate reader. The percentage of survival was calculated based on the absorbance values relative to the control samples. The no-treatment group was considered as 100% cell growth and used as a control, and the treatment groups were compared to this control.

MeOH =19/1, 1% CH_3COOH, CH_2Cl_2/MeOH =9/1, 1% CH_3COOH) to give the pure product 7 (16mg, 14μmol) in 70% yield. (Figures 7-8). ^1H NMR (600 MHz; $CDCl_3$) δ_H 8.38 (bs, 1H), 8.08 (d, *J* = 6.0Hz, 2H), 7.72 (d, *J* = 7.8Hz, 2H), 7.63 (t, *J* = 7.8Hz, 1H), 7.58 (t, *J* = 7.8Hz, 1H), 7.55-7.22 (m, 12H), 7.16 (bs, 2H), 6.26 (s, 1H), 6.15 (t, *J* = 7.8Hz, 1H), 5.88 (dd, *J* = 8.4Hz, 3.6Hz, 1H), 5.63 (d, *J* = 7.2Hz, 1H), 5.44 (d, *J* = 3.6Hz, 1H), 4.92 (d, *J* = 9.6Hz, 1H), 4.55 (bs, 1H), 4.40-4.37 (m, 1H), 4.25 (d, *J* = 6.0Hz, 1H), 4.20 (d, *J* = 8.4 Hz, 1H), 3.74 (d, *J* = 6.0Hz, 1H), 3.26 (d, *J* = 11.4Hz, 1H), 2.95 (bs, 1H), 2.72-2.60 (m, 2H), 2.58-2.30 (m, 6H), 2.30-2.20 (m, 1H), 2.17 (s, 3H), 2.07-2.03 (m, 1H), 1.86-1.81 (m, 4H), 1.63 (s, 3H), 1.18 (s, 3H), 1.09 (s, 3H). ^{13}C (150 MHz, $CDCl_3$) δ_C 175.4, 171.8, 171.2, 170.6, 169.9, 168.1, 167.3, 166.9, 158.2, 148.8, 142.6, 138.0, 136.9, 133.6, 133.5, 132.7, 131.9, 130.1, 129.2, 129.0, 128.6, 128.6, 128.5, 127.2, 126.7, 122.3, 122.0, 84.4, 81.0, 78.9, 75.5, 75.0, 74.3, 72.0, 71.8, 58.4, 53.0, 52.6, 45.5, 43.1, 40.5, 35.5, 35.3, 30.4, 29.0, 26.7, 22.6, 22.0, 20.8, 20.4, 14.8, 9.5. HRMS-ESI (*m*/*z*) [M-H^+] calculated for $C_{59}H_{62}N_3O_{18}S_2$, 1164.3475; found, 1164.3472.

Compound 8: Branched four-arm thiol polyethylene glycol ether, HS-bPEG-4 (156mg, 4.2μmol)

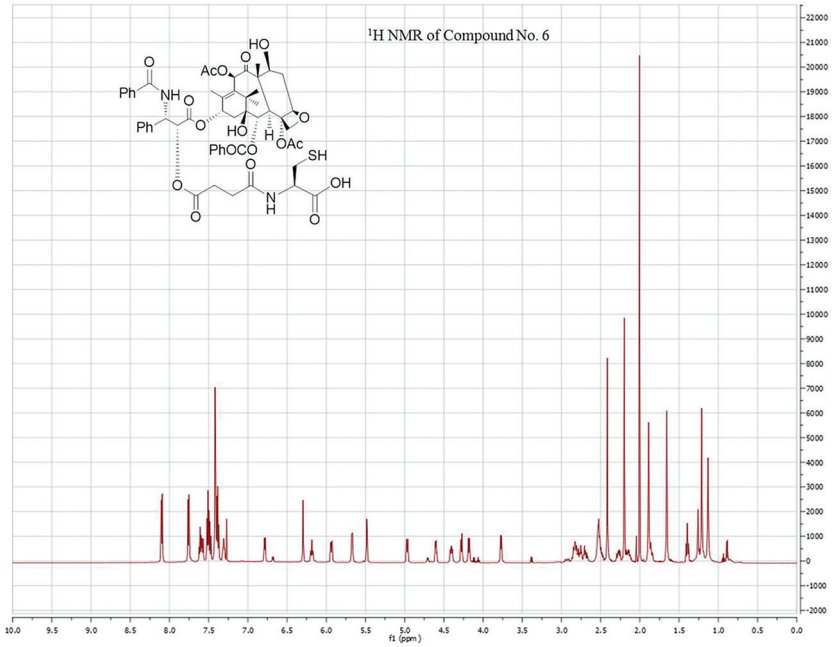

Figure 5. ^{1}H NMR of compound 6.

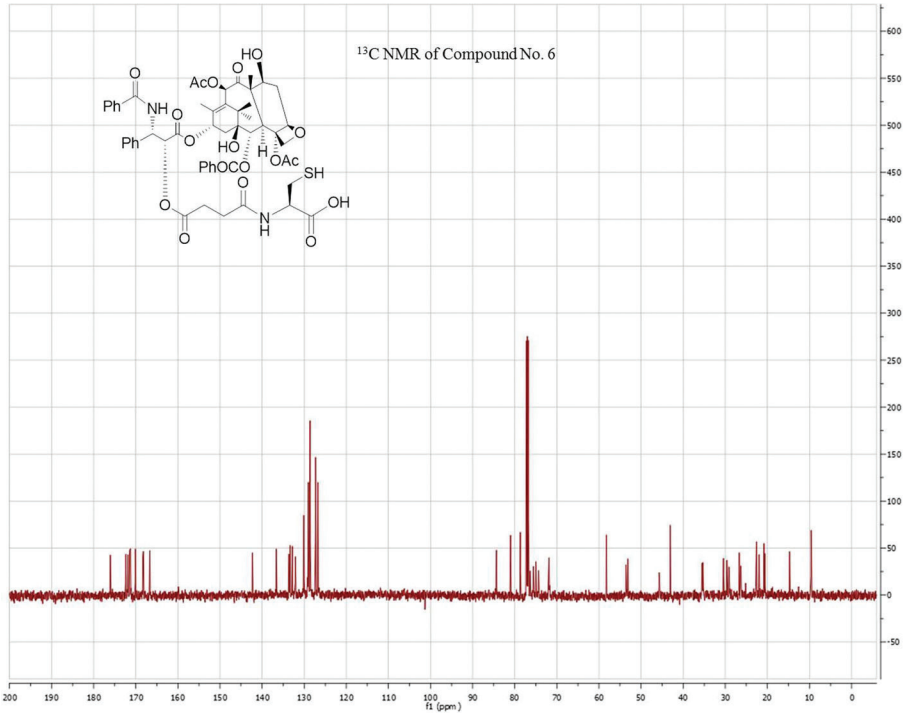

Figure 6. ^{13}C NMR of compound 6.

and compound 7 (30mg, 25.2μmol) were dissolved in DMF (6mL) under stirring conditions. Pyridine (500μL) was added dropwise into the reaction mixture. Stirring was continued for 48h. Then the solvent was removed and the product was purified using size exclusion column chromatography to give compound paclitaxel-branched PEG 8 in 87% yield with about 8% wt paclitaxel loading. (Figures 9-10) ^{1}H NMR (600MHz; $CDCl_3$) δ_C 8.13 (d, *J* = 7.7Hz, 2H), 7.82 (d, *J* = 7.2Hz, 2H), 7.63 (t, *J* =7.4Hz, 1H), 7.53 (t, *J* =7.7Hz, 2H), 7.50 (t, *J* =7.3Hz, 1H), 7.45-7.36 (m, 6H), 7.31-7.24

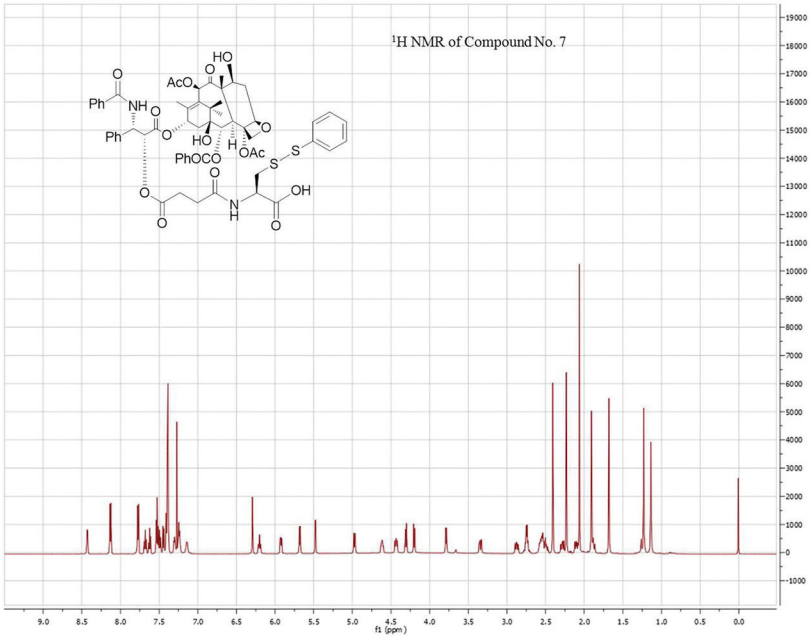

Figure 7. ^{1}H NMR of compound 7.

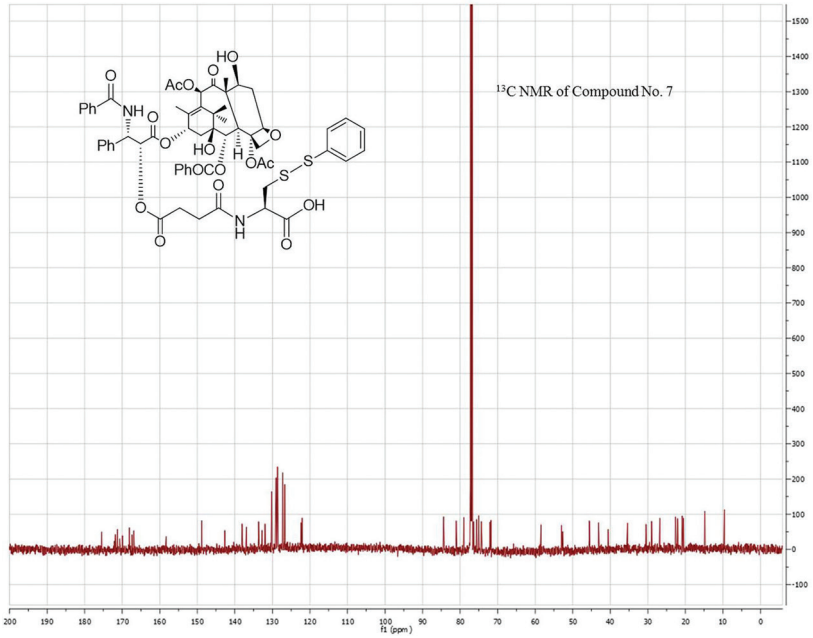

Figure 8. ^{13}C NMR of compound 7.

(m, 1H), 6.78 (bs, 1H), 6.28 (s, 1H), 6.17 (t, *J* = 9.0Hz, 1H), 5.92-5.86 (m, 1H), 5.66 (d, *J* = 7.0Hz, 1H), 5.47 (d, *J* = 4.2Hz, 1H), 4.97 (d, *J* = 9.5Hz, 1H), 4.63 (bs, 1H), 4.46-4.39 (m, 1H), 4.30 (d, *J* = 8.4Hz, 1H), 4.18 (d, *J* =8.4Hz, 1H), 3.80-3.50 (m, CH_2 of PEG), 3.41 (bs, 1H), 2.97-2.90 (m, 1H), 2.89-2.81 (m, 1H), 2.76 (t, 2H), 2.62-2.48 (m, 3H), 2.42 (s,3H), 2.32-2.20 (m, 5H), 1.93-1.83 (m, 4H), 1.67 (s, 3H), 1.25 (s, 3H), 1.22 (s, 3H), 1.12 (s, 3H). ^{13}C (150 MHz, CD_3OD) δ_C 176.5, 173.7, 171.6, 171.4, 171.3, 170.5, 167.7, 162.0, 150.3, 142.5, 139.4, 138.6, 135.6, 134.9, 134.7, 133.0, 131.5, 131.3, 130.2, 129.9, 129.7, 128.8, 122.4, 121.1, 85.9, 82.3, 79.0, 77.5, 76.8, 76.3, 76.0, 72.9, 59.3, 55.4, 44.7, 40.1, 39.5, 37.6, 36.5, 31.5, 30.8, 30.2, 27.1, 23.4, 22.5, 21.0, 15.2, 10.6.

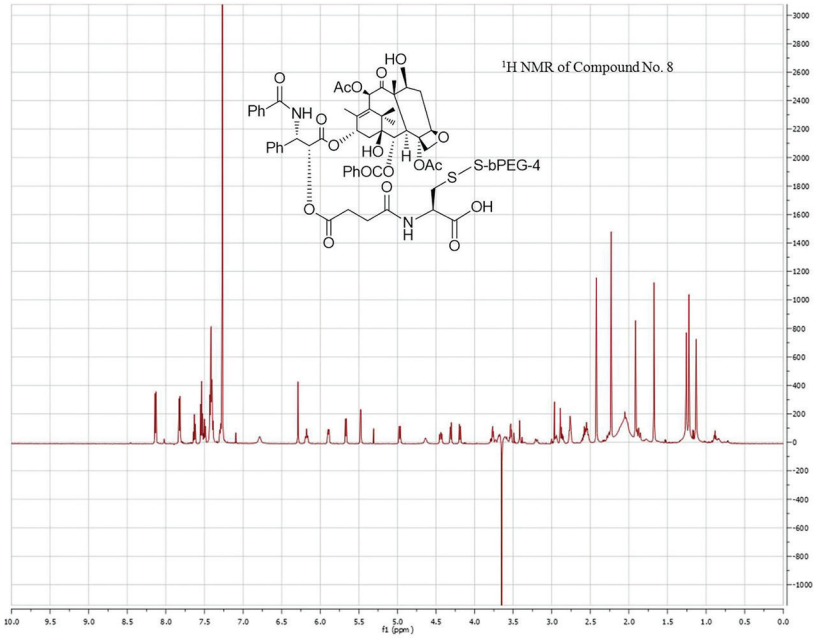

Figure 9. ^{1}H NMR of compound 8

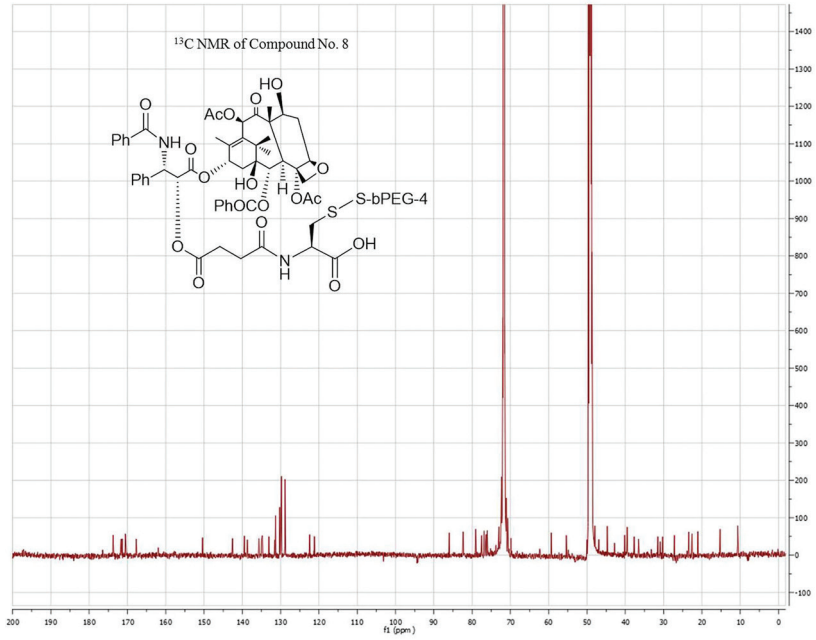

Figure 10. ^{13}C NMR of compound 8

Compound 1: Compound 8 (23mg, 5.57x10^{-1}µmol) and compound 9 (54mg, 27.6µmol) were dissolved in DMF (1mL) under stirring conditions at 0^{o}C, followed by the addition of PyBOP (12mg, 23µmol, 250µL DMF) and DIPEA (100µL). The reaction was stirred for 36h. The solvent was removed under reduced pressure, redissolved in MeOH and the product was purified by size exclusion column chromatography LH-20 to give the conjugate paclitaxel-branched PEG-EGFR-targeting peptide, 1 (Figures 11-12) with about 12% wt peptide loading. Peptide loading was determined using UV-Vis spectroscopy.

Cell line: The SCCHN Tu212 cell line was established from a hypopharyngeal tumor and was

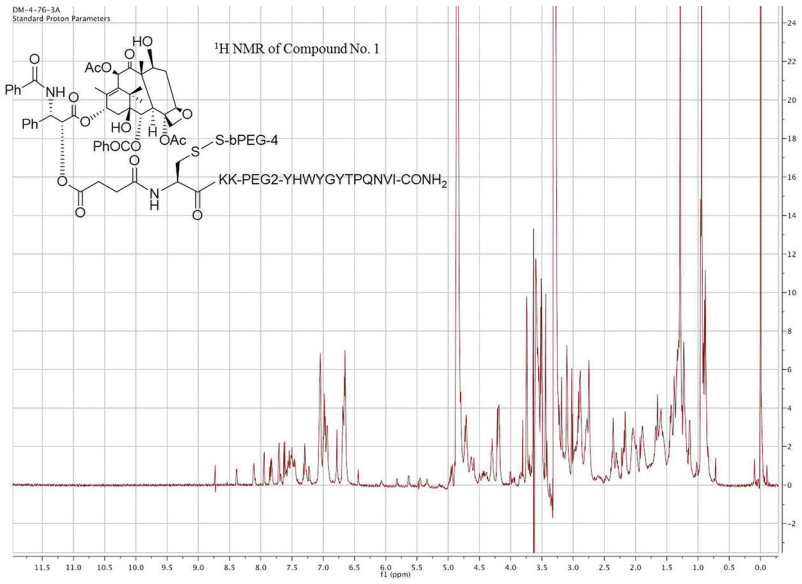

Figure 11. ^{1}H NMR of compound 1.

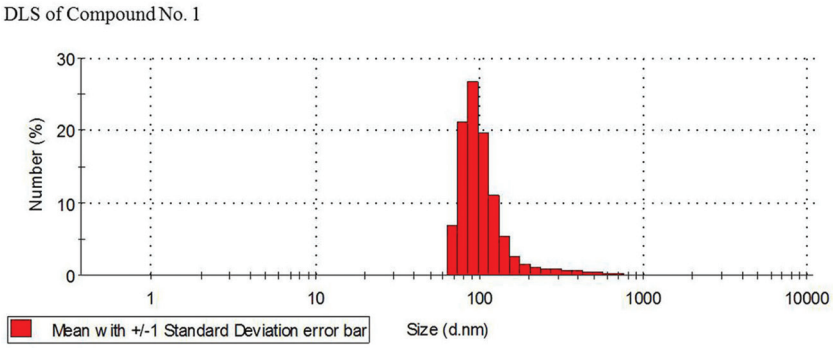

Figure 12. DLS of compound 1.

generously provided by Dr. Gary L. Clayman, University of Texas M.D. Anderson Cancer Center, Houston, TX. This cell line was cultured in DMEM/F12 (1:1) with 10% heat inactivated fetal bovine serum (FBS). Cells were maintained in a humidified incubator at 37^{o}C, 5% CO_2.

4. RESULTS

In an effort to address the issues of cancer therapy using paclitaxel, we have synthesized a water soluble nanosize conjugate of paclitaxel, branched polyethylene glycol (BPEG), and EGFR-targeting peptide, 1 (Figures 2-3). In the case of linear PEG of molecular weight ~40,000, the loading was 4% by weight of paclitaxel. Here, we used branched four-arm thiol PEG (HS-bPEG-4) of average molecular weight 37,000 (Figure 1), and loading was 8% by weight of paclitaxel.

The EGFR-targeting peptide used in this study (YHWYGYTPQNVI) binds specifically and efficiently to EGFR with a dissociation constant of ~22nM, but with much lower mitogenic activity than EGF (16,17). In order to increase the efficacy of paclitaxel chemotherapy and reduce undesired side effects, we used EGFR-targeting peptide as a ligand to target cancer cells that overexpress EGFR.

We used a Y-shaped linker system where three functional moieties – ester, amide and disulfide bonds – are involved. We used cysteine in this linker system. Cysteine has three different functional groups, -NH_2, -CO_2H, and -SH, which we utilized to conjugate three different moieties. The NH_2 group of cysteine was used to conjugate the paclitaxel-hemisuccinate derivative via succinamide linkage, the SH group of cysteine was used to conjugate the multi-arm thiol-PEG via disulfide bond, and the CO_2H group of cysteine was used to conjugate the EGFR-targeting peptide ligand via an amide bond.

Paclitaxel 2 was reacted with succinic anhydride to give paclitaxel hemisuccinate 3 in 74% yield (Figure 2,

retrosynthetic analysis) (18). Diphenyl chlorophosphate was treated with N-hydroxysuccinimide in the presence of triethyl amine at 0ºC to obtain *N*-hydroxysuccinimido diphenyl phosphate (SDPP) 4 (Figure 2) (19). Paclitaxel hemisuccinate 3 was reacted with SDPP 4 in the presence of Et_3N to give paclitaxel hemisuccinate-NHS ester 5 in 67% yield (Figure 2) (18). Paclitaxel hemisuccinate-NHS ester 5 was treated with cysteine in a phosphate buffer solution (pH 6.5) which underwent oxo-ester mediated native chemical ligation to give paclitaxel hemisuccinate-cysteine amide 6 in 68% yield (Figure 3) (20,21). In the next step, the paclitaxel hemisuccinate-cysteine amide 6 was treated with 2, 2′ dithiodipyridine/pyridine to give the disulfide derivative 7 in with 70% yield (Figure 3). The newly formed disulfide bond in compound 7 is highly reactive towards an incoming thiol group. In order to load the cysteine functionalized paclitaxel derivative 7 on the four-arm thiol PEG (HS-bPEG-4), compound 7 was reacted with HS-bPEG-4/pyridine and the product was purified by size-exclusion column chromatography to give the polymer-bound paclitaxel product 8 in 87% yield with about 8% wt paclitaxel loading (Figure 3). The progress of reaction and purity of the product was assessed by NMR. In the next step, compound 8 was conjugated with EGFR-targeting peptide 9 using PyBOP/DIPEA to give the nanosize conjugate of paclitaxel, branched PEG, and EGFR-targeting peptide 1 with about 12% wt peptide loading (Figure 3). Dynamic light scattering (DLS) study demonstrated that the hydrodynamic diameter of this nanosize conjugate is about 90nm (Figure 12).

To evaluate the ability of the newly synthesized paclitaxel-branched polymer conjugates to inhibit cell proliferation, we performed cell growth inhibition assays using the SCCHN cell line, Tu212, which overexpresses EGFR. Tu212 were seeded in 96 well plates (3000 cells/well). After 16h cells were treated with the following compounds at various concentrations: paclitaxel 2, paclitaxel-branched PEG 8, paclitaxel-branched PEG-EGFR-targeting peptide 1, and incubated for 72h. Cell growth inhibition assay was performed using sulforhodamine B (SRB, Figure 4) (22). OD was measured at 492nm. An untreated group was considered as 100% cell growth and used as a control, and the treatment groups were compared to this control. Parallel control (without treatment) group for compounds 1, 2, 8, and 9 showed no growth inhibition (Figure 4). At a concentration of 1.56nM, 30% growth inhibition was observed for compound 2, whereas no growth inhibition was observed for compounds 1, 8, and 9 (Figure 4). At a concentration of 3.12nM, 73% growth inhibition was observed for compound 2, whereas no growth inhibition was observed for compounds for 1, 8, and 9 (Figure 4). At a concentration of 6.25nM, 85% growth inhibition was observed for compound 2, 30% growth inhibition was observed for compound 8, and no growth inhibition was observed for compounds 1 and 9 (Figure 4). At a concentration of 12.5nM, about 88% cell growth inhibition was observed for compound 2 and 78% cell growth inhibition for compound 8, 13% cell growth inhibition for compound 1, and no growth inhibition was observed for compound 9 (Figure 4). At a concentration of 25nM, about 88% cell growth inhibition was observed for compound 2 and 87% cell growth inhibition was observed for compound 8, and 77% cell growth inhibition was observed for compound 1, and no growth inhibition was observed for compound 9 (Figure 4). At a concentration of 50nM, about 89% cell growth inhibition was observed for compound 2 and 92% cell growth inhibition was observed for compound 8, and 86% cell growth inhibition was observed for compound 1, and no growth inhibition was observed for compound 9 (Figure 4). Based on these data, the IC_{50} value of paclitaxel-branched PEG 8 (8.05nM) was 5-fold higher than that of paclitaxel 2 (1.47nM), whereas paclitaxel-branched PEG-EGFR-targeting peptide 1 had a much higher IC_{50} value (21.74nM). This study shows that the paclitaxel-branched PEG 8 and paclitaxel-branched PEG-EGFR-targeting peptide 1 conjugates retained their cytotoxicity against squamous cell carcinoma of head and neck (SCCHN) which can be caused by sexually transmitted infections.

5. DISCUSSIONS

PEG is a biocompatible polymer frequently used in drug delivery and approved by the FDA (23-26). PEGylation of drugs is well known for its ability to solubilize highly insoluble small molecule drugs, increase the circulating half-life, alter the biodistribution of parent drugs, and protect the drug from RES uptake (27-32). In fact, studies from various research groups have demonstrated the dramatic enhancement of circulating half-life, solubility, and *in vivo* efficacy of native anticancer drugs achieved by employing a prodrug strategy in conjunction with nonimmunogenic high molecular weight PEG ether (33). Previously, paclitaxel linear PEG esters have been used and shown to function *in vitro* as prodrugs.

The epidermal growth factor receptor (EGFR) is overexpressed in SCCHN and in a wide variety of solid tumors including lung and colorectal cancer (34-38). The prognostic significance of EGFR overexpression has stimulated immense interest in this receptor as an anticancer drug target.

Previously, ester bonds have been used to conjugate anticancer drugs to the polymer or peptide and they are known to be cleaved by esterase enzyme, thus releasing the drug (39-42). In our case, paclitaxel is conjugated to the succinate linker via the ester bond. The amide bonds are known to undergo hydrolysis by peptidases and proteases.

There are several examples where disulfide bonds have been used to conjugate a chemotherapeutic

drug to an antibody (43). Disulfide bonds are stable at physiological pH. In cancer cells, the glutathione concentration is reported to be in the millimolar range, whereas the level of glutathione in circulation in the blood is very low (micromolar range) (43,44). The high concentration of glutathione can cause the scission of disulfide bonds and release of the drug inside the cancer cell. Hence, we have used four-arm thiol PEG to conjugate to the thiol of the cysteine linker via disulfide bonds.

In conclusion, we have described the synthesis of nanosize conjugates consisting of paclitaxel, branched PEG, and EGFR-targeting peptide, and evaluated their anticancer activity. Cell proliferation assay demonstrated that the conjugates have retained cytotoxicity against SCCHN cells. The IC_{50} values of paclitaxel-branched PEG 8 and paclitaxel-branched PEG-EGFR-targeting peptide 1 were increased compared to that of paclitaxel due to conjugation to the polymer. This phenomenon has also been observed by other researchers (18,45). However, under *in vivo* conditions, the circulation time and pharmacokinetics of drug-polymer conjugate and free drug can be different. The conjugates, paclitaxel-branched PEG 8, and paclitaxel-branched PEG-EGFR-targeting peptide 1, are water soluble and hence suitable for intravenous administration. Loading of paclitaxel can be increased by using the branched PEG containing more arms.

Moreover, this synthetic methodology can be used to conjugate other drugs such as docetaxel, other peptide ligands, non-peptidic ligands such as folic acid, and other antibody ligands (46). Compound 7 contains a carboxyl functionality as well as an activated thiol functionality. An antibody and a nanoparticle can be conjugated to compound 7 either through the thiol functionality or through the carboxyl functionality (46). We are currently synthesizing various paclitaxel-PEG-ligand conjugates using other peptide and small molecule ligands, and also an antibody ligand to synthesize an antibody drug conjugate (ADC). We will evaluate their cytotoxicity against various cancer cell lines and conduct biological studies. Taken together, current study shows that paclitaxel-polymer conjugates can be useful for the therapy of various cancers such as breast, ovarian, cervical, testicular, non-small cell lung, and head and neck cancers.

6. ACKNOWLEDGEMENTS

This work was supported by grants from the Specialized Program of Excellence (SPORE) in Head and Neck Cancer (P50CA128613) and National Cancer Institute (NCI) Centers of Cancer Nanotechnology Excellence (CCNE) Program (U54CA119338).

7. REFERENCES

1. http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx
2. http://inhance.iarc.fr/about.php
3. R. Siegel, J. Ma, Z. Zou, A. Jemal. Cancer statistics, 2014. *CA Cancer J Clin* 64, 1, 9-29 (2014) DOI: 10.3322/caac.21208
4. C. Fakhry, M. L. Gillison. Clinical implications of human papillomavirus in head and neck cancers. *Journal of Clinical Oncology*. 24, 17, 2606-11 (2006) DOI: 10.1200/JCO.2006.06.1291
5. http://www.cdc.gov/std/HPV/STDFact-HPV.htm
6. http://www.oralcancerfoundation.org
7. V. Jayaprakash, M. Reid, E. Hatton,M. Merzianu, N. Rigual, J. Marshall, S. Gill, J. Frustino, G. Wilding, T. Loree, S. Popat, M. Sullivan. Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis. *Oral Oncology*. 47, 11, 1048-54 (2011) DOI: 10.1016/j.oraloncology.2011.07.009
8. A. K. Chaturvedi, E. A. Engels, R.M. Pfeiffer, B. Y. Hernandez, W. Xiao, E. Kim, B. Jiang, M.T. Goodman, M. Sibug-Saber, W. Cozen, L. Liu, C. F. Lynch, N. Wentzensen, R. C. Jordan, S. Altekruse, W. F. Anderson, P. S. Rosenberg, M. L. Gillison. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of Clinical Oncology* 29, 32, 4294-301 (2011) DOI: 10.1200/JCO.2011.36.4596
9. M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon, A. T. McPhail. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc* 93, 9, 2325-7 (1971) DOI: 10.1021/ja00738a045
10. K. C. Nicolaou, W. M. Dai, R. K. Guy. Chemistry and biology of taxol. *Angewandte Chemie-International Edition* 33, 1, 15-44 (1994) DOI: 10.1002/anie.199400151
11. D. Majumdar, S. Singhal. Medicinal chemistry and nanomedicine for reproductive cancer therapeutics. *Frontiers in Bioscience (Elite Ed)* 4, 2029-44 (2012) DOI: 10.2741/E523
12. D. A. Fuchs, R. K. Johnson. Cytologic evidence that taxol, an antineoplastic agent from Taxus brevifolia, acts as a mitotic spindle poison. *Cancer Treat Rep* 62, 8,1219-22 (1978)

13. P. B. Schiff, J. Fant, S. B. Horwitz. *Nature* 277, 5698, 665-7 (1979)
DOI: 10.1038/277665a0

14. P. B. Schiff, S. B. Horwitz. *Proc Natl Acad Sci U S A* 77, 3, 1561-5 (1980)
DOI: 10.1073/pnas.77.3.1561

15. H. Gelderblom, J. Verweij, K. Nooter, A. Sparreboom. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. *Eur J Cancer* 37, 13, 1590-8 (2001)
DOI: 10.1016/S0959-8049(01)00171-X

16. Z. Li, R. Zhao, X. Wu, Y. Sun, M. Yao, J. Li, Y. Xu, J. Gu. Identification and characterization of a novel peptide ligand of epidermal growth factor receptor for targeted delivery of therapeutics. *FASEB J* 19, 14, 1978-85 (2005)
DOI: 10.1096/fj.05-4058com

17. L. Milane, Z. Duan, M. Amiji. Development of EGFR-targeted polymer blend nanocarriers for combination paclitaxel/lonidamine delivery to treat multi-drug resistance in human breast and ovarian tumor cells. *Mol Pharm*, 8, 1, 185-203 (2011)
DOI: 10.1021/mp1002653

18. Y. Luo, G. D. Prestwich. Synthesis and selective cytotoxicity of a hyaluronic acid-antitumor bioconjugate. *Bioconjug Chem* 10, 5, 755-63 (1999)
DOI: 10.1021/bc9900338

19. H. Ogura, S. Nagai, K. Takeda. A novel reagent (N_succinimidyl diphenylphosphate) for synthesis of active ester and peptide. *Tetrahedron Letters* 21, 15, 1467-68 (1980)
DOI: 10.1016/S0040-4039(00)92747-3

20. P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent. Synthesis of proteins by native chemical ligation. *Science* 266, 5186, 776-9 (1994)
DOI: 10.1126/science.7973629

21. Q. Wan, J. Chen, Y. Yuan, S. J. Danishefsky. Oxo-ester mediated native chemical ligation: concept and applications. *J Am Chem Soc* 130, 47, 15814-6 (2008)
DOI: 10.1021/ja804993y

22. V. Vichai, K. Kirtikara. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc* 1, 3, 1112-6 (2006)
DOI: 10.1038/nprot.2006.179

23. R. Duncan. The dawning era of polymer therapeutics. *Nature Reviews Drug Discovery* 2, 5, 347-60 (2003)
DOI: 10.1038/nrd1088

24. M. Thanou, R. Duncan. *Curr Opin Investig Drugs* 4, 6, 701-9 (2003)

25. M. J. Vicent, R. Duncan. Polymer-protein and polymer-drug conjugates in cancer therapy. *Trends in Biotechnology* 24, 1, 39-47 (2006)
DOI: 10.1016/j.tibtech.2005.11.006

26. R. Duncan. Polymer conjugates as anticancer nanomedicines. *Nature Reviews Cancer* 6, 9, 688-701 (2006)
DOI: 10.1038/nrc1958

27. C. D. Conover, H. Zhao, C. B. Longley, K. L. Shum, R. B. Greenwald. Utility of poly(ethylene glycol) conjugation to create prodrugs of amphotericin B. *Bioconjugate Chemistry* 14, 3, 661-6 (2003)
DOI: 10.1021/bc0256594

28. R. B. Greenwald, A. Pendri, C. D. Conover, C. Lee, Y. H. Choe, C. Gilbert, A. Martinez, J. Xia, D. C. Wu, M. Hsue. Camptothecin-20-PEG ester transport forms: the effect of spacer groups on antitumor activity. *Bioorganic & Medicinal Chemistry* 6, 5, 551-62 (1998)
DOI: 10.1016/S0968-0896(98)00005-4

29. P. C. A. Rodrigues, U. Beyer, P. Schumacher, T. Roth, H. H. Fiebig, C. Unger, L. Messori, P. Orioli, D. H. Paper, R. Mulhaupt, F. Kratz. Acid-sensitive polyethylene glycol conjugates of doxorubicin: preparation, *in vitro* efficacy and intracellular distribution. *Bioorganic & Medicinal Chemistry* 7, 11, 2517-24 (1999)
DOI: 10.1016/S0968-0896(99)00209-6

30. E. K. Rowinsky, J. Rizzo, L. Ochoa, C. H. Takimoto, B. Forouzesh, G. Schwartz, L. A. Hammond, A. Patnaik, J. Kwiatek, A. Goetz, L. Denis, J. McGuire, A. W. Tolcher. A phase I and pharmacokinetic study of pegylated camptothecin as a 1-hour infusion every 3 weeks in patients with advanced solid malignancies. *Journal of Clinical Oncology* 21, 1, 148-57 (2003)
DOI: 10.1200/JCO.2003.03.143

31. H. Zhao, B. Rubio, P. Sapra, D. C. Wu, P. Reddy, P. Sai, A. Martinez, Y. Gao, Y. Lozanguiez, C. Longley, L. M. Greenberger, I. D. Horak. Novel prodrugs of SN38 using multiarm poly(ethylene glycol) linkers. *Bioconjugate Chemistry* 19, 4, 849-59 (2008)
DOI: 10.1021/bc700333s

32. P. Sapra, H. Zhao, M. Mehlig, J. Malaby, P.

Kraft, C. Longley, L. M. Greenberger, I. D. Horak. Novel delivery of SN38 markedly inhibits tumor growth in xenografts, including a camptothecin-11-refractory model. Clin Cancer Res. 14, 6, 1888-96 (2008) DOI: 10.1158/1078-0432.CCR-07-4456

33. R. B. Greenwald, Y. H. Choe, J. McGuire, C. D. Conover. Effective drug delivery by PEGylated drug conjugates. *Adv Drug Deliv Rev* 55, 2, 217-50 (2003) DOI: 10.1016/S0169-409X(02)00180-1

34. Y. Yarden, M. X. Sliwkowski. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2, 2, 127-37 (2001) DOI: 10.1038/35052073

35. J. Schlessinger. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell* 110, 6, 669-72 (2002) DOI: 10.1016/S0092-8674(02)00966-2

36. R. N. Jorissen, F. Walker, N. Pouliot, T. P. Garrett, C. W. Ward, A. W. Burgess. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res* 284, 1, 31-53 (2003)

37. S. V. Sharma, D. W. Bell, J. Settleman, D. A. Haber. Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 7, 3, 169-81 (2007)

38. J. Bernier, S. M. Bentzen, J. B. Vermorken. Molecular therapy in head and neck oncology. *Nat Rev Clin Oncol* 6, 5, 266-77 (2009) DOI: 10.1038/nrclinonc.2009.40

39. K. Beaumont, R. Webster, I. Gardner, K. Dack. Design of ester prodrugs to enhance oral absorption of poorly permeable compounds: challenges to the discovery scientist. *Current Drug Metabolism* 4, 6, 461-85 (2003) DOI: 10.2174/1389200033489253

40. R. B. Greenwald, A. Pendri, D. Bolikal. Highly Water Soluble Taxol Derivatives: 7-Polyethylene Glycol Carbamates and Carbonates. *Journal of Organic Chemistry* 60, 2, 331-36 (1995) DOI: 10.1021/jo00107a010

41. R. B. Greenwald, C. W. Gilbert, A. Pendri, C. D. Conover, J. Xia, A. Martinez. Drug delivery systems: water soluble taxol 2'-poly(ethylene glycol) ester prodrugs-design and *in vivo* effectiveness. *J Med Chem* 39, 2, 424-31 (1996) DOI: 10.1021/jm950475e

42. C. Che, G. Yang, C. Thiot, M. C. Lacoste, J. C. Currie, M. Demeule, A. Regina, R. Beliveau, J. P. Castaigne. New Angiopep-modified doxorubicin (ANG1007) and etoposide (ANG1009) chemotherapeutics with increased brain penetration. *J Med Chem* 53, 7, 2814-24 (2010) DOI: 10.1021/jm9016637

43. R. V. Chari. Targeted cancer therapy: conferring specificity to cytotoxic drugs. *Acc Chem Res* 41, 1, 98-107 (2008) DOI: 10.1021/ar700108g

44. A. Meister, M. E. Anderson. Glutathione. *Annu Rev Biochem* 52, 711-60 (1983) DOI: 10.1146/annurev.bi.52.070183.003431

45. H. Lee, K. Lee, T. G. Park. Hyaluronic acid-paclitaxel conjugate micelles: synthesis, characterization, and antitumor activity. *Bioconjugate Chemistry* 19, 6, 1319-25 (2008) DOI: 10.1021/bc8000485

46. L. Ducry, B. Stump. Antibody-drug conjugates: linking cytotoxic payloads to monoclonal antibodies. *Bioconjugate Chemistry* 21, 1, 5-13 (2010) DOI: 10.1021/bc9002019

Key Words: Cancer, Head, Neck, Cancer, Squamous Cell Carcinoma, SCCHN, Paclitaxel, Human Papillomavirus, Oropharynx, Epidermal Growth Factor Receptor, EGFR, Polyethylene Glycol Ether, PEG, Native Chemical Ligation, Peptide, Ligand, Antibody, Cancer Therapy

Send correspondence to: Dong M. Shin, Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365 C Clifton Road, Atlanta, GA 30322, Tel: 404-778-5990; Fax: 1-404-778-5520; E-mail: dmshin@emory.edu