Low-molecular-weight polymer–drug conjugates for synergistic anticancer activity of camptothecin and doxorubicin combinations

Background: High-molecular-weight (MW) polymers (>50,000 Da) can be conjugated to chemotherapy drugs in order to improve their tumor accumulation, while low MW polymers $\leq 10,000$ Da are often overlooked due to faster plasma clearance. Small polymers, however, may facilitate deeper tumor penetration. **Materials & methods:** Here, we investigate the anticancer efficacy of 10 kDa hyaluronic acid or poly(vinyl alcohol) conjugated to synergistic combinations of camptothecin and doxorubicin, with emphasis on chemical linker impacts. **Results:** Our results emphasize drug hydrolyzability for synergy preservation, and also demonstrate superior cancer cell inhibition with low MW polymer–drug conjugates. **Conclusion:** This study shows the high therapeutic potential of low MW polymer–drug conjugates for polychemotherapy delivery, and provides further insight into the development of polymer–drug therapeutics.

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Polymer–drug conjugates represent a burgeoning area of research in cancer nanotechnology due to their ability to prolong blood circulation, passively deliver chemotherapy drugs to tumors via the enhanced permeation and retention effect, and deliver multiple agents simultaneously. Such systems are also highly versatile, and allow for elegant *ab initio* design of the carrier. Various large [1–5] and small [6–8] polymers have been considered for this application. In addition to molecular weight, properties such as net charge, drug loading and biodegradability can also be finely-tuned [4,9–15]; however, the optimal properties which enable high therapeutic efficacies of anticancer drugs remain unclear, owing to the many variables which can dictate biological activity.

Many literature reports demonstrate the superior tumor accumulation and plasma circulation of high-molecular-weight (~100 kDa)

water-soluble polymers [2–5], and for this reason, most biodegradable polymer–drug conjugates in clinical trials bear molecular weights >50 kDa [16–19]. However, high overall tumor accumulation may not be enough to inhibit cancer cell growth. For example, micelles ranging between 30 and 100 nm have been found to penetrate highly permeable tumors in mice, but only sub-50 nm particles were found to extravasate into tumor tissue with lower vasculature [6,7]. In another study, it was shown that low-molecular-weight polymers were more readily internalized by epithelial cells compared with polymers greater than 80 kDa [8]; thus, high-molecular-weight polymers may impede conjugated drugs from reaching their intracellular targets. It is clear that carrier size governs drug distribution in tumor microenvironments, which further impacts drug activity. Therefore, although low-molecular-weight polymer–drug conjugates

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are less-explored, they may elicit significant therapeutic efficacy.

In this report, low-molecular-weight polymers and varying linker chemistries are investigated in order to understand their impact on the co-delivery of synergistic chemotherapy combinations. We have previously demonstrated the tumor reduction capability of high-molecular-weight 250 kDa hyaluronic acid (HA) conjugated to synergistic chemotherapy drugs camptothecin (CPT) and doxorubicin (DOX) [20]. CPT and DOX represent a large class of highly synergistic combinations, topoisomerase I and II inhibitors [21–23], and thus serve as a model pair for studying the impact of polymeric properties on combination potency. Here, we extend our approach to low-molecular-weight 10 kDa HA and poly(vinyl alcohol) (PVA) drug conjugates with various linker chemistries for the concurrent treatment of CPT and DOX. HA represents a biopolymer with high biocompatibility and active tumor-targeting capabilities [24–27], PVA is an archetypal nonbiodegradable polymer, and both have been found to passively accumulate in tumor tissue via the enhanced permeation and retention effect [3,28].

High anticancer activity of CPT and DOX bound to either of these two distinct polymers provides strong evidence for the potential of low-molecular-weight conjugates for cancer therapies irrespective of polymer type.

Materials & methods

Materials

(S)-(+)-Camptothecin (CPT), 10 kDa PVA, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 4-(dimethylamino)pyridine (DMAP), ethylenediamine, 1,1'-Carbonyldiimidazole (CDI), 1,2-diaminoethane (DAE), succinic anhydride and hydrazine were purchased from Sigma-Aldrich (MO, USA). Doxorubicin hydrochloride salt was obtained from LC laboratories (MA, USA). Hyaluronic acid of molecular weight 10 kDa was purchased from Creative PEGWorks (NC, USA). DRAQ5, Hoechst and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Invitrogen Life Technologies (NY, USA). Mouse metastatic breast cancer cell line 4T1, breast cancer HER2-overexpressing cell line BT-474, Hybri-Care

Figure 1. Chemical reactions utilized for incorporation of DOX and/or CPT onto poly(vinyl alcohol) (PVA) side chains. Reaction (A) activates PVA hydroxyl groups with succinic anhydride to form EDC-reactive carboxylic acid side chains. Reaction (B) shows further conjugation to nucleophilic drugs (designated as R_1 -OH or R_2 -NH₂), with either hydroxyl or amine moieties. This reaction scheme was utilized for the synthesis of CPT-PVA, DOX-PVA and CPT-PVA-DOX F2.

Figure 2. Cell growth inhibition of human breast cancer cell line BT-474 in the presence of (A) DOX-PVA or (B) CPT-PVA. Cells were incubated with each polymer–drug conjugate for 72 h, then subsequently analyzed via MTT assay. Dashed lines represent dose-effect curves fit to experimental data using Chou and Talalay's median-effect model. D_{50} concentrations determined from the median-effect model are $1.5 \pm 0.9 \mu\text{M}$ DOX and $14 \pm 0.9 \mu\text{M}$ CPT for DOX-PVA and CPT-PVA, respectively. (C) Toxicity of S-PVA. Data expressed as mean $\pm$ SD ($n \geq 12$). For all studies, S-PVA concentrations $< 0.6 \text{ mg/ml}$ were utilized to avoid compounding polymer toxicity with drug effects.

medium and cell culture grade water were purchased from ATCC (VA, USA). Cell culture reagents FBS, RPMI-1640 media, phosphate buffered saline (PBS), 0.25% trypsin, Nunc Lab-Tek 8-chambered coverglass, as well as Slide-A-Lyzer MINI dialysis devices were obtained from Thermo Scientific (MA, USA). All cell culture flasks and microplates were purchased from Corning (NY, USA), Sephadex G-25 PD-10 columns were obtained from GE Healthcare Life Sciences (NJ, USA), Amicon Ultra micro-centrifugal filters were purchased from EMD Millipore (MA, USA), and Sep-Pack C18 columns were obtained from Waters (MA, USA). All reagents used for polymer–drug conjugate synthesis were ACS grade. All other chemicals were obtained from Fisher Scientific and were the highest commercial grade available.

Synthesis of conjugates

To synthesize ester-conjugated drugs to PVA, reactions in Figure 1 were utilized. PVA was partially succinated (5%) by reaction with succinic anhydride using DMAP as catalyst in dry DMSO; this introduced a carboxylic acid which could be further conjugated to nucleophilic drugs. The reaction was allowed to proceed overnight, under moderate heating and stirring. The succinated PVA (S-PVA) was precipitated and rinsed multiple times in acetone, and finally redissolved in DMSO. The carboxyl groups on S-PVA were activated with EDC and for the coupling of CPT and DOX, respectively through ester and amide bonds.

The resulting drug-polymer conjugate was purified using size exclusion Sephadex G-25 PD-10 columns (MWCO 10 kDa) and concentrated with Amicon® Ultra centrifugal filters (MWCO 3kDa, Millipore). To determine the grafting efficiency of succinate on PVA, weight gain was utilized. S-PVA was dried completely via rotary evaporation at reduced pressure and high temperature. After solvent removal, the product weight was compared with the initial polymer weight in order to determine the molar ratio of succinate to PVA.

CPT-PVA and DOX-PVA are the designations utilized for CPT- and DOX- conjugated PVA, respectively, via reactions in Figure 1. In the case of CPT- and DOX- co-conjugated to PVA, designated as CPT-DOX-PVA, CPT was first made to react with PVA for 2 h, followed by the addition of fresh EDC and DOX for 30 min at 40°C . Drugs were added at 5 mol% relative to the polymer concentration and 30% excess. CPT and DOX concentrations on all polymer–drug conjugates were determined via absorbance at 366 and 480 nm, respectively, of vehicle serial dilutions using a Tecan Infinite M200 Pro plate reader.

Reactions in Figure 3 were adopted to prepare amide-linked DOX to PVA. Briefly, PVA was partially aminated (5%) by reaction with CDI and DAE in DMSO. Briefly, the hydroxyl groups on PVA were activated with a stoichiometric amount of CDI for 5 min and subsequently reacted with DAE in large excess at room temperature. The aminated PVA (A-PVA) was precipi-

tated and rinsed multiple times in acetone, and finally redissolved in DMSO. A-PVA grafting efficiency was determined similar to S-PVA, through weight change after complete solvent removal. In parallel, both CPT and DOX were succinated by reaction with succinic anhydride and DMAP in dry DMSO. The reaction was allowed to proceed overnight, at 40°C while stirring. The succinated DOX and CPT (S-DOX and S-CPT) were purified by reverse phase C-18 chromatography using Sep-Pack C18 columns. Finally, S-DOX and S-CMT were activated with EDC in DMSO and coupled onto A-PVA overnight, at 40°C while stirring. Drugs were added at 5 mol% relative to the polymer concentration and 30% excess. The resulting polymer–drug conjugate was purified as explained above. DOX-aPVA and CPT-aPVA is utilized to designate amide-linked drug conjugates.

Hydrazine-linked DOX to PVA was synthesized using reactions in Figure 4, and is designated as DOX-aPVA. PVA activated with hydrazide groups (H-PVA) was prepared by activating S-PVA, obtained as explained above, with EDC followed by reaction with hydrazine in DMSO. The reaction was allowed to proceed overnight, under moderate heating and stirring. DOX was added at 5 mol% relative to the polymer concentration and 30% excess. The H-PVA was precipitated and rinsed in acetone, redissolved in DMSO and contacted with DOX overnight at 60°C while stirring. The resulting DOX-aPVA conjugate was purified as explained above.

CPT and DOX co-conjugated to 10 kDa HA was synthesized as previously reported [20]. Briefly, HA carboxylic acid groups were activated with EDC and DMAP, both in a molar ratio of 0.75:1 with respect to HA, in a 1:1 mixture of DMSO:water under slight heating and stirring for 1 h. CPT was slowly added to the reaction mixture at a molar ratio of 0.4:1 CPT:HA, and allowed to react for 3 days. DOX was subsequently added in a molar ratio of 0.2:1 DOX:HA with fresh EDC and DMAP, and also allowed to react for 3 days. Separation of conjugated polymer from unreacted reagents was achieved via size exclusion chromatography in Sephadex G-25 PD-10 columns. Polymers were subsequently concentrated using microcentrifuge filter tubes.

Cell culture

4T1 and BT-474 cells were cultured in a humidified incubator maintained at 5% CO₂ and 37°C. Cells were grown in media recommended by ATCC; 4T1 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, while BT-474 cells were cultured in Hybri-Care medium supplemented with 10% FBS.

Cell viability studies

To assess the effect of polymer–drug conjugates on cancer cell proliferation, MTT cytotoxicity assays were utilized. BT-474 cells or 4T1 cells were seeded at 10,000 or 1000 cells in 100 µl per well in a 96-well cell culture plate, and were allowed to adhere overnight. Media was then replaced with fresh media containing polymer–drug conjugates. BT-474 cells were treated with conjugates for 72 h, while 4T1 cells were treated for 48 h. Seeding density and incubation times for each cell line were optimized such that control cells reached 70% confluency by the end of the drug incubation period. Post drug-incubation, media was replaced with fresh media containing 0.5 mg/ml MTT, and allowed to incubate with cells for 4 h. Media was then aspirated and replaced with DMSO to facilitate dissolution of formazan crystals (intracellularly reduced MTT). Absorbance was measured at 570 nm using a Tecan Infinite M200 Pro plate reader (Männedorf, Switzerland). Control wells containing only free drug were also analyzed to ensure negligible drug interactions with MTT absorbance readings. The fractional cell inhibition was calculated by subtracting absorbance of cells in experimental wells from that of untreated control cells and normalizing against control cells.

Drug release

Drug release studies of DOX from DOXePVA were conducted utilizing Slide-A-Lyzer MINI Dialysis Devices of 10 kDa MWCO (Life Technologies). polymer–drug conjugates were placed in dialysis devices with fresh PBS buffer of indicated pH, and were further inserted into microcentrifuge tubes containing PBS. Tubes were allowed to shake at 100 RPM and 37°C. DOX fluorescence of surrounding media was measured at specified intervals in order to determine percent of drug released.

Cellular uptake of free drugs or conjugates

To assess the impact of polymer-conjugation and linker-chemistry on drug internalization, confocal laser scanning microscopy was utilized. BT-474 cells were seeded at 80,000 cells per 300 µl media in an 8-well chambered borosilicate coverglass (Nunc Lab-Tek). After adhering overnight, the cells were exposed to either free drug or polymer-bound drugs for 24 h, at drug-equivalent concentrations of 1 µM DOX or 28 µM CPT. Post drug-incubation, cells were washed twice with warm PBS and subsequently stained with nuclear dyes. For the visualization of cell nuclei which were exposed to DOX formulations, Hoechst nuclear dye was utilized at a concentration of 25 µg/ml for 30 min. For labeling cell nuclei which were exposed to CPT formulations, DRAQ5 was used at a concentra-

Figure 3. Chemical reactions utilized for incorporation of DOX and/or CPT onto poly(vinyl alcohol) (PVA) side chains. Reaction 3a activates nucleophilic drugs (designated as $R_1\text{-OH}$ or $R_2\text{-NH}_2$) with succinic anhydride to form EDC-reactive drugs. Reaction (B) shows amination of PVA side chains, which is further reacted with products of 2a in reaction 3c. CPT-PVA-DOX F1 and DOXaPVA were synthesized via this reaction scheme.

tion of $5\text{ }\mu\text{M}$ for 60 min. All incubations occurred at $5\%\text{ CO}_2$ and 37°C . After nuclei staining, cells were washed twice more with warmed PBS before adding fresh media. Cells were immediately imaged live in a 37°C temperature-controlled imaging chamber with an Olympus Fluoview 1000 spectral confocal and a 60X silicon oil objective. DOX, Hoechst, CPT and DRAQ5 were excited with the following lasers: 488 nm 10 mW Argon gas (DOX), 405 nm 50 mW diode (CPT or Hoechst) and 635 nm 20 mW diode (DRAQ5). Images were collected as $10\text{ }\mu\text{m}$ z-stacks with an average of 25 cells in each field view, and each stack was collapsed into an averaged image utilizing ImageJ 1.47h software (NIH). Fluorescence intensity per cell was calculated as the raw integrated density divided by the number of cells.

Combination studies

The Combination Index (CI) method was adopted to characterize chemotherapy combination potencies and to quantify synergistic effects [29]. First, fractional cell inhibition dependence on drug concentration obtained from cell viability studies was fit to the median-effect model developed by Chou and Talalay [29]. Various

ratios of CPT-PVA and DOX-PVA were combined and exposed to cancer cells, their fractional cell inhibition was quantified and the Combination Index (CI) was calculated from the previously-determined median-effect models. $\text{CI} < 1$, $\text{CI} = 1$ and $\text{CI} > 1$ corresponded to synergistic, additive and antagonistic effects, respectively.

Results & discussion

CPA-PVA & DOX-PVA cancer cell inhibition

polymer–drug conjugates provide a controlled method of delivering precise combinations of chemotherapy drugs specifically to tumors. However, the optimal vehicle properties, such as molecular weight, conjugation efficiency and drug linkers which will facilitate significant tumor reduction or even complete eradication are not clear. Our focus in this study was to elucidate the potential of low-molecular-weight polymers as well as the effect of chemical linkers on combination chemotherapy anticancer activity. The conjugation of CPT and DOX to a low-molecular-weight 10 kDa polymer was first studied in a characteristic nonbio-degradable polymer, poly(vinyl alcohol) (PVA) with one of the most commonly-used hydrolyzable tethers,

Figure 4. Chemical reactions utilized for incorporation of DOX (designated as R_2 -COCH₂OH) onto poly(vinyl alcohol) (PVA) side chains. Reaction (A) activates PVA hydroxyl groups with succinic anhydride to form EDC-reactive carboxylic acid side chains. Reaction (B) shows hydrazine conjugation to activated PVA side chains. Hydrazine-conjugated PVA is further reacted with a ketone-containing drug (DOX) to form a hydrazine-linked PVA-drug conjugate (DOXhPVA).

esters. Each drug was conjugated to PVA utilizing reactions in Figure 1, which resulted in 0.39 mol% CPT or 0.16 mol% DOX relative to PVA. These conjugation efficiencies are expected, since both reaction steps in Figure 1, the succinic anhydride modification of PVA and the esterification of succinic acid, are known to be thermodynamically limited [30,31]. It has been previously shown that higher CPT conjugation efficiencies, as high as 30 mol%, can be achieved if synthesis does not require prior polymer modifications and if CPT is modified to contain a more reactive amine functionality [32].

Conjugation of chemotherapy drugs to polymers typically alters drug activity, as was evident in our previous study of 250 kDa hyaluronic acid conjugated to CPT and DOX [20]. However, drug activity should not be compromised so much as to entirely prevent its anticancer effects. Therefore, initial studies evaluated the *in vitro* cancer cell growth inhibition of 10 kDa PVA-conjugated CPT or DOX. As seen in Figure 2, both PVA-drug conjugates were active at inhibiting BT-474 cancer cell growth at polymer concentrations which provide negligible anticancer effects. The concentrations required to inhibit 50% cell growth (D_{50}) of DOX-PVA and CPT-PVA were 1.5 μ M DOX and 14 μ M CPT, respectively. In comparison, previous studies in our lab demonstrated D_{50} concentrations of

free DOX and CPT to be 0.3 μ M and 100 μ M, respectively [20]; hence, conjugation to 10 kDa PVA reduced activity of DOX, whereas it enhanced activity of CPT. The enhanced anticancer activity of PVA-bound CPT may be attributed to its enhanced water solubility upon conjugation to PVA. These results are in agreement with 250 kDa HA-conjugates previously reported. It is also noteworthy that the D_{50} concentrations of 10 kDa PVA-conjugates are lower than those of 250 kDa HA-conjugates (96 μ M DOX and 400 μ M CPT), which suggest the superiority of 10 kDa conjugates. Previous studies have also shown that smaller particles are better internalized by cancer cells [33]. However, different polymers were utilized for these studies, albeit with the same conjugation chemistry, and thus further investigation may be required to verify the significance of molecular weight.

Polymer-drug linker affects drug activity

Chemical linkage between the drug and polymer is another variable which can greatly impact drug activity. Depending on the chemical linker, the drug may be released from the polymer carrier at various rates or may not hydrolyze at all. In order to determine the effect of hydrolysis on drug activity, DOX was conjugated to 10 kDa PVA via ester, amide or hydrazone linkers using reaction schemes from Figures 1, 3 or 4,

respectively, and the cancer cell growth inhibition was evaluated *in vitro* for all conjugates. The conjugation achieved for the ester, amide and hydrazone-linked DOX were 0.25 mol%, 0.81 mol% and 0.03 mol% DOX relative to PVA, respectively. Low conjugation efficiencies were expected as multiple polymer and drug modifications were required to achieve the final products. Ester bonds are the most commonly used in literature due to ease of synthesis, and also represent hydrolytically labile bonds; amide-linked conjugates represent hydrolytically stable bonds, whereas hydrazone linkers are hydrolytically degraded at endosomal pH 5.0 but stable at physiological pH 7.4 [11,34]. *In vitro* cancer cell cytotoxicity studies in **Figure 5A** show that the cleavable conjugates, ester-linked DOX (DOXePVA) and hydrazone-linked DOX (DOXhPVA), are active against cancer cells, whereas the noncleavable amide-linked DOX (DOXaPVA) is not. Further, drug

release from DOXePVA in **Figure 5B** demonstrates pH-dependent drug release, which can be attributed to the acid-labile ester bond.

Confocal studies were performed in order to assess the impact of chemical linkage on drug internalization and potential correlations with anticancer activity. As seen in **Figure 5C & D**, cell internalization of DOX in the form of DOXePVA and DOXaPVA is much lower than that of free DOX, which suggests that polymer–drug conjugates require active internalization, whereas free DOX can diffuse through the cell membrane. Furthermore, the internalization of polymer-bound DOX is dictated by the chemical linker, as indicated by the significant decline of drug internalization when introduced as DOXaPVA compared with DOXePVA. One possible reason for this sharp contrast is that drug hydrolysis may occur prior to cell entry in the case of the ester-linked conjugate,

Figure 5. The impact of various PVA-drug linkers on drug activity and internalization. DOXePVA refers to ester-linked drug, which was synthesized via reaction schemes in **Figure 1**; DOXaPVA refers to amide-linked drug, which was synthesized via reactions in **Figure 3**, and DOXhPVA refers to hydrazone-linked drug, which was synthesized via reactions in **Figure 4**. **(A)** Comparison of the *in vitro* BT-474 cancer cell growth inhibition of the various linkers. DOXePVA is indicated by circles, DOXaPVA is represented by squares and DOXhPVA is indicated by triangles. **(B)** Release of DOX from DOXePVA in PBS at pH 5.6 (circles) or pH 7.4 (squares) at 37°C under constant stirring. **(C)** Fluorescence of DOX in free solution, DOXePVA or DOXaPVA present in BT-474 cells after 24 h of incubation at 37°C and 5% CO_2 . Initial drug loadings were 1 μM DOX-equivalents. Data expressed as mean $\pm$ SD ($n = 3$). **(D)** Representative images of DOX (red) internalization from free solution (left), DOXePVA (middle) or DOXaPVA (right). Cells were labeled with Hoechst nuclear dye. Images are averages of 10 μm z-stacks.

Figure 6. (A) Fluorescence of CPT in free solution or as a PVA-conjugate form present in BT-474 cells after 24 h of incubation at 37°C and 5% CO₂. Formulations were incubated at CPT-equivalent concentrations of 28 μM. Data expressed as mean ± SD (n = 3). (B) Representative images of CPT (cyan) internalization when incubated as a free solution (left) or as a PVA-conjugate (right). Cells were labeled with DRAQ5 nuclear dye. Images are averages of 10 μm z-stacks.

which then allows DOX to become internalized via passive, non-energy intensive mechanisms. Also noteworthy is the difference in nuclear drug accumulation between the different conjugates. Since DOX inhibits topoisomerase II enzymes, drug co-localization within the nucleus may be directly correlated to its ability to inhibit proliferation. As a free drug, nuclear accumulation of DOX is evident through the purple nuclear hue (red and blue co-localization) in Figure 5D. Less nuclear DOX accumulation is seen in cancer cells incubated with DOX-PVA, and barely any is observed in cancer cells incubated with DOX-PVA. Therefore, it seems that non-cleavable conjugates prevent DOX from escaping endosomes and reaching the nucleus, which in turn hinders its anticancer activity. Collectively, internalization studies and cancer cell growth inhibition results suggest that DOX must be cleaved from the polymer at physiological conditions in order to remain active at inhibiting cancer cell growth, and the polymer-bound drug is not active prior to drug hydrolysis.

Polymer conjugation improves CPT internalization

With the ultimate goal of conjugating both DOX and CPT to PVA, further studies were conducted to assess the impact of PVA-conjugation on CPT internalization, as well. CPT was conjugated via esterification to PVA in order to allow drug hydrolysis from the polymer, and internalization studies were performed. As seen in Figure 6A, 100-fold more CPT fluorescence was found in cells exposed to CPT-PVA compared with those incubated with free CPT. This is not surprising as conjugation of CPT to water soluble polymers has been found to improve CPT water solubility up to 50-fold [35], and hence can improve cell interactions. It is also noteworthy that CPT-PVA was internalized as elongated fibrous-like structures (Figure 6B), which may be attributed to aggregates of CPT that form when multiple moieties are bound to the same polymer. Therefore, prior to eliciting its anticancer effects on the cell, CPT must both hydrolyze from the polymer and dissociate from any aggregates. This small enhancement in free intracellular CPT when PVA-bound can explain the slightly improved cancer cell cytotoxicity seen in Figure 2B, and also verifies that ester-bound CPT is still highly active against cancer cells.

Identification of optimal polymer-bound ratio (R) CPT:DOX

After establishing that CPT or DOX conjugated to PVA via esterification are still able to inhibit cancer cell growth, CPT-PVA and DOX-PVA were simultaneously exposed to cancer cells at various ratios to identify optimal combinations of polymer-bound drugs which synergistically inhibit cancer cell growth. Combination interactions were evaluated and quantified utilizing the Combination Index (CI) method [29], where CI < 1 indicates synergy and CI > 1 suggests antagonism. Previous studies conducted by our lab demonstrated that free CPT and DOX synergistically inhibit BT-474 cancer cell growth at ratios (R) greater than 0.1 M:M CPT:DOX, with the greatest synergy occurring at R > 2 [20]. Figure 7B shows that the same synergistic ratios hold true for the 10 kDa PVA-bound drugs; for R > 2.2, CI values were found to be <<1 and significantly more synergistic than combinations at R < 2.2. The anticancer activity of single drug-conjugates which comprise the combinations is seen in Figure 7A, and verifies that the combination of CPT-PVA and DOX-PVA is indeed more potent at inhibiting cancer cell proliferation than either of the individual constituents. These results are consistent with our previous findings of CPT and DOX individually conjugated to 250 kDa HA. From both of these studies, it seems that CPT and DOX synergy can generally be conserved upon

polymer conjugation, regardless of molecular weight or polymer choice, provided that the polymer does not significantly compromise either drug's activity and that the two drugs are combined in optimal ratios.

CPT & DOX co-conjugated to PVA

Finally, both CPT and DOX were conjugated to the same PVA polymer carrier to determine whether drug synergy could be conserved. Two different reaction schemes were utilized for drug conjugation in order to further clarify the effect of linker on drug activity. CPT-PVA-DOX F1 utilized reactions in Figure 3, which conjugated DOX and CPT via amide and ester bonds, respectively. CPT-PVA-DOX F2 was synthesized with reactions in Figure 1, which conjugated both drugs via ester bonds. In the case of CPT-PVA-DOX F1, only one drug was hydrolytically labile and capable of releasing from the polymer under physiological conditions; on the contrary, CPT-PVA-DOX F2 can release both drugs since ester linkages were used to conjugate CPT and DOX. Both schemes resulted in the conjugation of CPT and DOX in ratios at which the free drugs exhibited synergistic cancer cell kill, $R > 0.1$ (Figure 8A). Also noteworthy is the poor conjugation efficiency of Figure 1 reactions compared with those in Figure 3. Whereas Figure 3 reactions achieved conjugations of 0.10 and 0.28 mol% CPT and DOX, respectively, Figure 1 reactions only yielded 0.01 mol% for both drugs. This can be attributed to the overall

less favorable nature of hydroxyl esterification compared with amide formations.

The activity of these two CPT-PVA-DOX conjugates against BT-474 cancer cell growth was evaluated via MTT cytotoxicity assays, and compared with the anticancer activities of the single ester-linked PVA-conjugates. CPT-PVA-DOX F1 showed similar efficacy as CPT-PVA and DOX-PVA, with no clear advantage over either single drug conjugate (Figure 8B & C). On the other hand, CPT-PVA-DOX F2 exhibited more than double the potency of either CPT-PVA or DOX-PVA alone. While the two formulations bear different drug ratios, both ratios of 0.36 and 1.50 are expected to exhibit synergy in the polymer form, as demonstrated in Figure 7. Thus, the differences in cancer cell growth inhibition can be clearly attributed to differences in linker chemistry. These results further emphasize the necessity of drug hydrolysis from the polymer in order to preserve both drug activity and combination synergy.

CPT & DOX co-conjugated to HA

While the ester-linked CPT and DOX conjugates to PVA showed promising efficacy, PVA is not biodegradable *in vivo*. Hence, to assess the therapeutic potential of low-molecular-weight polymers for CPT and DOX delivery with a degradable polymer, the drugs were conjugated to another 10 kDa polymer, one which is biocompatible, biodegradable [24,36–38], and possesses tumor-targeting abilities; that is, hyaluronic acid

Figure 7. (A) Cell inhibition of human breast cancer cell line BT-474 treated with combinations of CPT-PVA and DOX-PVA at various molar ratios (black bars). Single PVA-conjugate treatments of DOX-PVA (white) and CPT-PVA (hatched) at concentrations which make-up the combination are juxtaposed for direct comparison. Drug concentrations (μM) of CPT and DOX respectively corresponding to each R ratio were: 0.1 (0.53, 5.34), 0.3 (0.53, 1.89), 0.5 (0.53, 1.07), 2.2 (4.27, 1.90), 4.5 (4.27, 0.95) and 9.0 (8.54, 0.95). Cells were incubated with PVA-conjugates for 72 h, and subsequently analyzed via MTT cytotoxicity assays. Data are represented as mean $\pm$ SD ($n = 6$). **(B)** Combination Index (CI) values calculated for the CPT-PVA and DOX-PVA combinations seen in (A). Significance was calculated from Student's *t*-test. $**p < 0.01$.

Figure 8. (A) Formulations which utilized reactions in Figure 3 or Figure 1 to conjugate both CPT and DOX to the same polymer. Cell inhibition of BT-474 cells in the presence of CPT-PVA-DOX conjugates F1 (triangles) and F2 (squares) are compared to the cell inhibition of cells treated with (B) CPT-PVA alone (circles) or (C) DOX-PVA alone (circles). CPT-PVA and DOX-PVA were synthesized utilizing esterification (reaction Scheme 1). Cells were incubated with formulations for 72 h, and assessed for cell viability utilizing the MTT cytotoxicity assay. Data are reported as mean $\pm$ SD ($n \geq 6$).

(HA). We have previously reported that the combination's synergy can be retained through conjugation to high-molecular-weight 250 kDa HA, and we applied

the same reaction schemes here to synthesize 10 kDa CPT-HA-DOX [20]. Through nucleophilic acyl substitution, we achieved drug loadings of 1.9 mol% DOX

Figure 9. Cell inhibition of 4T1 cells in the presence of 10 kDa CPT-HA-DOX reported with respect to (A) CPT concentration and (B) DOX concentration. CPT-HA-DOX was synthesized as previously reported, via nucleophilic acyl substitution. Cells were incubated with HA-conjugates for 72 h, and assessed for cell viability utilizing the MTT cytotoxicity assay. Data are reported as mean $\pm$ SD ($n = 6$). D_{50} concentrations determined by fits to the median-effect model were $0.50 \pm 0.04 \mu\text{M}$ CPT and $0.07 \pm 0.01 \mu\text{M}$ DOX.

and 14.4 mol% CPT, corresponding to a synergistic ratio of 7.5. It is noteworthy that the same chemistry and initial starting materials resulted in less than half as much CPT conjugation and hence a less synergistic ratio ($R = 3.2$) in higher molecular weight 250 kDa HA; one possible reasoning is the reduced steric hindrance in smaller polymeric chains. The anticancer activity of 10 kDa CPT-HA-DOX is presented in **Figure 9**. D_{50} concentrations of the 10 kDa conjugates (0.50 μ M CPT, 0.07 μ M DOX) were determined by fitting experimental data to the median-effect model, and are lower than those corresponding to the 250 kDa conjugates (5.8 μ M CPT, 1.8 μ M DOX) [20]. The D_{50} concentration of CPT is reduced 12-times, and the DOX D_{50} dose is halved when the pair is used as a 10 kDa HA conjugate as opposed to a 250 kDa conjugate. This finding may be attributed to either the higher, more synergistic drug loading capabilities of the 10 kDa polymer, or due to a possible enhancement of cellular uptake with lower molecular weight polymers [39]. In either case, our studies indicate that low-molecular-weight polymers are capable of maintaining combination chemotherapy synergy, and are more effective than higher molecular weight polymers at inhibiting cancer cell growth *in vitro*. To the best of our knowledge, one other report has previously demonstrated potent anticancer efficacy of low-molecular-weight polymer–drug conjugates for combination chemotherapy. Specifically, N-(2-hydroxypropyl)methacrylamide co-conjugated to gemcitabine and DOX with cumulative molecular weight of 23.5 kDa was shown

to reduce free DOX IC_{50} by 150-fold [40]. While this report did not include comparisons to higher molecular weight polymers, it provides further evidence to the potency of low molecular combination conjugates. Collectively, our findings encourage investigations of low-molecular-weight polymers for polychemotherapy delivery.

Conclusion

In this study, low-molecular-weight polymers and various linker chemistries were investigated for the co-delivery of synergistic chemotherapy pair CPT and DOX. It was found that physiologically labile linkers such as esters and hydrazones enhance intracellular concentrations of drugs and thus maintain drug activity, whereas hydrolytically stable amide bonds significantly reduce drug activity as well as combination potency. We also demonstrated a higher conjugation efficiency in low-molecular-weight HA compared with previously reported high-molecular-weight HA conjugates, and hence higher *in vitro* anticancer activity of the low-molecular-weight conjugates. Our results uncover the high therapeutic potential of low-molecular-weight polymers for combination chemotherapy delivery, and warrants further investigation to determine the translatability and possible superiority of low-molecular-weight conjugates. Our findings also emphasize the vast implications each physical and chemical polymeric property can inflict on drug activity, and underscores the importance of thorough investigations into other vehicle properties in order to

Executive summary

CPA-PVA & DOX-PVA cancer cell inhibition

- Esterification via reactions in **Figure 1** resulted in 0.16 mol% DOX and 0.39 mol% CPT conjugation to PVA.
- CPT-PVA enhances free CPT anticancer activity, whereas DOX-PVA reduces free DOX activity.

Polymer-drug linker affects drug activity

- DOXaPVA exhibits reduced drug activity compared with DOXePVA and DOXhPVA, and is attributed to poor hydrolyzability of amide bonds.
- DOXaPVA is internalized to a significantly lesser extent compared with DOXePVA and DOXhPVA.
- Release of DOX from DOXePVA is pH-dependent.

Polymer conjugation improves CPT internalization

- CPT-PVA gets internalized to a greater extent relative to free CPT.

Identification of optimal polymer-bound ratio (R) CPT:DOX

- Synergistic anticancer activity of the pair CPT and DOX depends on drug ratio.
- $R > 0.1$ CPT-PVA:DOX-PVA provide synergistic cancer cell kill ($CI < 1$), although $R > 2.2$ provides the greatest synergy.

CPT & DOX co-conjugated to PVA

- Both reaction schemes in **Figures 1 & 3** are able to co-conjugate CPT and DOX at synergistic drug ratios.
- Nonhydrolyzable CPT-PVA-DOX F1 does not retain drug synergy, whereas hydrolyzable CPT-PVA-DOX F2 does retain CPT and DOX synergy.

CPT & DOX co-conjugated to HA

- CPT and DOX co-conjugated to low MW HA at $R = 7.5$ retains their synergistic anticancer activity.
- Low MW CPT-HA-DOX exhibits greater *in vitro* cancer cell inhibition than previously reported high MW CPT-HA-DOX.

further the potential of polymer–drug conjugates as a whole.

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