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Combination therapy of prostate cancer with HPMA copolymer conjugates containing PI3K/mTOR inhibitor and docetaxel

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Keywords

HPMA copolymers; prostate cancer; docetaxel; GDC-0980

1. Introduction

Prostate cancer is the most commonly diagnosed cancer and the second lethal malignancy in men. The therapy-resistant subtypes (such as androgen-independent prostate cancer) frequently emerge and the incidence of relapse or metastasis is very high. To address these therapeutic challenges, combination strategies have been actively investigated. The well-documented advantages of employing combination therapy is to yield synergistic antitumor effect or decrease the possibility of developing resistant cancer cell clones, by using anticancer drugs with different working mechanisms [1, 2]. In particular, as the heterogeneity of cancer cells has been acknowledged, a novel paradigm of combination therapy targeting both cancer stem/progenitor cells and traditional therapy-sensitive bulk tumor cells showed promising results to improve the long-term therapeutic benefit against prostate cancer [3–5]. As the Cancer Stem Cell (CSC) research advances, some anti-cancer therapeutic agents in clinical development have been found to also exhibit potent anti-CSC activities [6–10].

In particular, the combination of PI3K/mTOR inhibitor and chemotherapy has shown superior therapeutic effect over single treatment in prostate cancer models [4, 11]. Activation of the PI3K/mTOR pathway correlates with shorter disease-free survival in cancer patients [12, 13]. In prostate cancer, PTEN (phosphatase and tensin homolog) deletion and the associated PI3K/mTOR pathway up-regulation commonly occur, contributing to tumor initiation, survival and invasion [6, 11, 14, 15]. PI3K/mTOR pathway

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inhibitors show potent cytotoxicity against prostate cancer cells [6, 10, 11]. Noticeably, the PI3K/mTOR signaling network also transit signals for the maintenance and survival of cancer stem/progenitor cells in several cancers [14, 16, 17]. In prostate cancer, inhibiting the PI3K/mTOR pathway activity caused preferential inhibition of prostate cancer stem/progenitor cell growth [4, 14].

Among dual PI3K/mTOR inhibitors, GDC-0980 is potent against both Class I PI3 kinase and mTORC1/2 (Complex 1/2) [6, 18]. Preclinical studies have shown its effective anti-tumor effect in prostate cancer cell lines and in prostate tumor xenografts. It is being evaluated in clinical trials either alone or in combination with chemotherapy in solid tumors and non-Hodgkin's lymphoma [18]. Based on the mechanisms of GDC-0980 action, we hypothesize that it also possesses potent anti-prostate CSC activity.

The main advantages of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers as drug carriers include increasing solubility, prolonging the circulation time, and improving pharmacokinetic/biodistribution profiles of small molecule drugs [19–21]. We previously showed that combination therapy of PC-3 prostate cancer xenografts in nude mice with HPMA copolymer-cyclopamine conjugate and HPMA copolymer-docetaxel conjugate inhibited the tumor growth in long-term [5]. Herein, we employed the HPMA copolymer for the tumor delivery of GDC-0980, which shows more potent anti-tumor activity than cyclopamine [6]. We firstly studied the synthesis of HPMA copolymer-(GDC-0980) conjugate and another conjugate containing a GDC-0980 analog (GDC), and compared the bioactivity of the two conjugates. Then we further investigated the anti-CSC activity of free and conjugated GDC-0980. Finally, the anti-tumor and anti-CSC effects of the combination therapy composed of two conjugates - HPMA copolymer-(GDC-0980) conjugate and HPMA copolymer-docetaxel conjugate were evaluated on PC-3 prostate cancer xenografts in nude mice.

2. Materials and Methods

2.1 Materials

GDC-0980 was purchased from Medi-tech. Docetaxel was purchased from AK Scientific (Union City, CA). 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) and 2,2'-azobis(2,4-dimethyl valeronitrile) (V-65) were from Wako Chemicals (Richmond, VA). Papain and cathepsin B were purchased from Sigma (St. Louis, MO). 4-cyanopentanoic acid dithiobenzoate [22], *N*-(2-hydroxypropyl)methacrylamide (HPMA) [23], *N*-methacryloylglycylphenylalanylleucylglycine (MA-GFLG-OH) [24], and *N*-methacryloylglycylphenylalanylleucylglycine docetaxel (MA-GFLG-DTX) [5] were synthesized as previously described.

2.2 Synthesis and characterization of polymerizable derivative of GDC-0980

The polymerizable derivative of the drug (drug monomer) was synthesized by the reaction of MA-GFLG-OH with GDC-0980 in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as coupling reagent (Figure 1A). Briefly, MA-GFLG-OH (51 mg, 0.11 mmol) was dissolved in 0.4 mL *N,N*-dimethylformamide (DMF), followed by the addition

of DMF solution of EDC (21.1 mg, 0.11 mmol) and HOBt (1-hydroxybenzotriazole) (15.3 mg, 0.11 mmol). GDC-0980 (50 mg, 0.1 mmol) was dissolved in approx. 0.4 mL (DMF) and added into the reaction mixture. The reaction was performed under stirring at r.t. for 24 h. After reaction, the mixture was washed, and precipitated in ether. To confirm the products, the precipitated mixture was redissolved in methanol and run on analytical HPLC (Agilent Technologies 1100 series, Zorbax C8 column 4.6×150 mm) using flow rate 1.0 mL/min and gradient elution from 2% to 90% of buffer B within 30 min (Buffer A: DI H₂O with 0.1% TFA, Buffer B: acetonitrile with 0.1% TFA). Three main peaks were observed (Figure 1B). All peaks were collected, and analyzed by MALDI-TOF mass spectrometry. The analysis confirmed that two monomer products were obtained - MA-GFLG-(GDC-0980) ([M+H]⁺ 941.4) and MA-GFLG-GDC ([M+H]⁺ 869.4) (Figure 1). To purify the two monomer products, the reaction mixture was run on preparative HPLC at the flow rate of 2.5 mL/min. The gradient elution method using buffers A (DI H₂O with 0.1% TFA) and Buffer B (acetonitrile with 0.1% TFA) was as follows: 15% to 28% B in 5 min, 28% B for another 5 min, 28% to 30% B in 10 min, 30% to 40% B in 5 min, 40% to 60% B in 5 min, 60% to 90% B in 5 min and keeping 90% B for another 5 min (total of 40 min). The two monomer peaks can be completely separated on preparative HPLC. Both MAGFLG-(GDC-0980) and MA-GFLG-GDC peaks were collected and the solvent was evaporated. After freeze drying, MA-GFLG-(GDC-0980) 12 mg and MA-GFLG-GDC 10 mg was obtained.

2.3 Synthesis and characterization of HPMA copolymer-(GDC-0980) conjugate (P-(GDC-0980))

P-(GDC-0980) was synthesized by RAFT copolymerization of HPMA and MA-GFLG-(GDC-0980) using VA-044 as initiator and 4-cyanopentanoic acid dithiobenzoate as chain transfer agent (CTA) (Figure 2). HPMA (27.8 mg, 0.194 mmol, 97 mol%) and MA-GFLG-GDC-0980 (5.65 mg, 0.006 mmol, 3 mol%) were added into an ampoule attached to the Schlenk-line. Following three vacuum-nitrogen cycles to remove oxygen, degassed methanol (50 µL), VA-044 solution (0.036 mg in 50 µL) and 4-cyanopentanoic acid dithiobenzoate solution (0.1 mg in 50 µL) in methanol were added via syringe. The mixture was bubbled with nitrogen for 30 min, sealed and polymerized at 40°C for 22 h. The copolymer was then end-modified in the presence of 40× molar excess of V65 (3.5 mg) in methanol at 50°C for 4 h (Figure 2). The final product was obtained by precipitation into acetone and purified by dissolution-precipitation in methanol/acetone twice and dried under vacuum. In another large batch synthesis (batch 2) for *in vivo* experiments, HPMA (139 mg) and MA-GFLG-(GDC-0980) (28 mg) were copolymerized to yield the conjugate.

P-GDC was synthesized from HPMA (27.8 mg, 0.194 mmol, 97 mol%) and MA-GFLGGDC (5.2 mg, 0.006 mmol, 3 mol%) following the same procedure.

The molecular weight and molecular weight distribution of the conjugates was determined by size-exclusion chromatography (SEC) on an AKTA FPLC system (Pharmacia) equipped with miniDAWN and OptilabEX detectors with acetate/30% acetonitrile as mobile phase. Superose 6 HR10/30 column (Pharmacia) was used. The yield of P-(GDC-0980) was about 15 mg (45%, Mw 47.9 kDa, PDI 1.06) and 100 mg (60%, Mw 94.4 kDa, PDI 1.04),

respectively. The yield of P-GDC was about 13 mg (40%, Mw 54.4 kDa, PDI 1.05). GDC-0980 and GDC contents in the conjugates were determined by enzymatic cleavage of GFLG with excess of papain. The cleaved sample was run on HPLC and the amount of drug was calculated based on the AUC of the elution peak and the calibration curve of free GDC-0980 with known concentrations.

2.4 Synthesis of HPMA copolymer-docetaxel conjugate (P-DTX)

P-DTX was synthesized as previously described [5]. Briefly, RAFT copolymerization of HPMA (277.8 mg, 1.94 mmol, 97 mol%) and MA-GFLG-DTX (75 mg, 0.06 mmol, 3 mol%) was performed using V65 (8×10^{-4} mmol) as initiator and 4-cyanopentanoic acid dithiobenzoate (3.2×10^{-3} mmol) as chain transfer agent. The reaction yielded white solid 178 mg (50.5%). The copolymer was end-modified in the presence of 40× molar excess of V65 (29.3 mg) in methanol at 50°C for 4 h. The yield of the final end-modified P-DTX (Mw 38.6 kDa, PDI 1.06) was 165 mg. DTX content in P-DTX was determined by enzymatic cleavage of GFLG in the presence of excess of papain. The cleaved sample was run on HPLC and the amount of DTX was calculated based on the AUC of the DTX elution peak and the calibration curve of free DTX with known concentrations.

2.5 Enzymatic cleavage of HPMA copolymer-(GDC-0980) conjugate and HPMA copolymer-GDC conjugate

To compare the enzymatic release kinetics of GDC-0980 from P-(GDC-0980) conjugate and GDC from P-GDC conjugate, the two conjugates were exposed to cathepsin B (an enzyme present in the lysosomes) for cleavage and the cleaved drug contents were monitored at different time points. The cleavage reaction was performed in McIlvaine's buffer (50 mM citrate/0.1 M phosphate) at pH 5.0, 37 °C. Briefly, cathepsin B stock solution was prepared by dissolving cathepsin B in McIlvaine's buffer and the exact concentration was determined by measuring UV absorbance at 278 nm using $A_{1\%} = 20$ (0.195 mg/mL). The enzyme activity was measured by incubation of a standard substrate Bz-PheValArg-NAP with cathepsin B (cathepsin B activity 6.53 min^{-1}). For the cleavage experiments of P-(GDC-0980) and P-GDC, the conjugate stock solutions were prepared at the final concentration of 25 mg/mL in DMSO. For each sample of the cleavage mixture, cathepsin B solution (20 µL) and McIlvaine's buffer containing 5 mM glutathione (70 µL) was preincubated for 5 min at 37 °C, followed by addition of conjugate stock solution (10 µL). The mixture with cathepsin B (final concentration of $1.5 \times 10^{-6} \text{ M}$) was incubated at 37 °C for 0.5, 1, 2, 4, 8, 20 h. At different predetermined time points, samples were run on HPLC (Agilent Technologies 1100 series, Zorbax C8 column $4.6 \times 150 \text{ mm}$) using flow rate 1.0 mL/min and gradient elution from 2% to 90% of buffer B within 30 min (Buffer A: DI H₂O with 0.1% TFA, Buffer B: acetonitrile with 0.1% TFA). The % release was calculated based on the AUCs of the drug elution peak.

In another experimental setting, in order to evaluate whether cathepsin B was capable to cleave the GFLG linker in P-GDC conjugate, high concentration of cathepsin B ($1 \times 10^{-5} \text{ M}$) was used to perform the cleavage experiment. The experimental procedures were the same as described above.

2.6 Cell culture

PC-3 prostate cancer cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown and maintained at 37 °C, 5% CO₂ in RPMI1640 containing 10% FBS (fetal bovine serum) or in a keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (BPE) and recombinant epidermal growth factor (EGF) for prostasphere growth (Life Technologies, Gaithersburg, MD).

2.7 Cytotoxicity assay

Cytotoxic effects of free GDC-0980, GDC, P-(GDC-0980) and P-GDC on PC-3 cells at different drug or drug equivalent concentrations were determined using Cell Counting Kit-8 (CCK-8, Dojindo, Japan). The stock solutions of free GDC-0980 and GDC were prepared in DMSO and the stock solutions of P-(GDC-0980) and P-GDC were prepared by dissolving the conjugate in sterile H₂O. PC-3 cells were seeded in 96-well plates, grew for 24 h in RPMI1640 medium (10% FBS) and were incubated with different concentrations of GDC-0980, GDC, P-(GDC-0980) and P-GDC, respectively, for another 72 h. After treatment, cells were washed and incubated with CCK-8 solution (15× dilution in fresh growth medium) at 37°C, 5% CO₂ for about 1 h. The absorbance in each well was measured by a microplate reader at 450 nm (630 nm as reference). The experiments were done in triplicate. Untreated cells were set as 100% viable.

The cytotoxic effects of single and combination of GDC-0980 and DTX, or P-(GDC-0980) and P-DTX on PC-3 cells were also determined using the same method as described above.

2.8 CD133 cell surface marker expression by flow cytometry

The effects of GDC-0980, DTX, P-(GDC-0980), P-DTX, as well as the combination of GDC-0980 and DTX, or P-(GDC-0980) and P-DTX on the relative amount of CD133+ prostate cancer stem/progenitor cells in PC-3 cells were evaluated. PC-3 cells growing in log phase were trypsinized and seeded in 6-well plates at the initial density of 4×10⁴ cells/well. Following 24 h growth, the cells were exposed to different concentrations of drug/conjugate-containing medium (free GDC-0980 (20 nM, 50 nM, 100 nM), P-(GDC-0980) (drug equivalent 100 nM, 500 nM, 1000 nM), P-DTX or DTX (drug or drug equivalent 10 nM), as well as the combination of GDC-0980 50 nM + DTX 10 nM, and P-(GDC-0980) 500 nM + P-DTX 10 nM). After 72 h incubation, drug or conjugate solutions were discarded and cells were washed and collected. For flow cytometry, cells were harvested and stained with mouse antihuman allophycocyanin (APC)- labeled antiCD133/2 (293C3) (Miltenyi Biotec, MA) using similar method as previously described [20]. Briefly, cells were suspended in cold staining buffer (phosphate buffered saline pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA), preblocked with 20 µL 3% bovine serum albumin in PBS and stained with antibody solution for 30 min at 4°C. Cells were washed twice after staining and Hoechst 33258 was added 10 min before flow cytometry analysis to exclude dead cells. Samples were run on FACSCanto flow cytometer (Becton Dickinson, NJ) and data were analyzed by FlowJo (Tree Star, OR). The data were collected from three independent experiments. For each batch of experiments, the CD133 expression level in untreated control cells was set as 100%; the percentage of CD133+ cells in each batch following different treatments was normalized to the untreated control.

2.9 Prostatosphere culture

Single cell suspensions of PC-3 cells were prepared in sphere growth serum-free medium (KSFM supplemented with 10 ng/mL EGF, 10 ng/mL basic fibroblast growth factor and B27, Invitrogen). Cells were seeded in cell culture 24-well plates (precoated with 0.5% agar) at the density of 1000 cells/well and treated with GDC-0980 or P-(GDC-0980) at the final concentrations of GDC0980 (20 nM, 50 nM, 100 nM) and P-GDC0980 (drug equivalent 100 nM, 500 nM, 1000 nM). Cells were cultured for 8 days to monitor the sphere formation. The number of spheres with the diameter between 50 to 200 μ m was counted under the microscope.

2.10 In vivo anti-tumor activity

Animal studies were carried out according to the University of Utah IACUC guidelines under approved protocols. To establish subcutaneous xenograft tumor model in nude mice, viable PC-3 cells (3×10^6 /mouse) suspended in 200 μ L phosphate buffered saline were inoculated subcutaneously into the right flank region of 6–7 week old male nude mice. When the tumor sizes reached an average of 55–60 mm³, treatments were initiated. Treatment groups included saline control, GDC-0980, P-(GDC-0980) (batch 2), P-DTX, combination of DTX and GDC-0980, and combination of P-DTX and P-(GDC-0980), at the dose and frequency described below. Mice in GDC-0980 or P-(GDC-0980) groups received six doses of drug or conjugate starting from day 0 (at the drug or drug equivalent concentration of 5 mg/kg) twice per week via intravenous injections (n=4); P-DTX was given as a single intravenous injection (DTX equivalent 10 mg/kg on day 0) (n=4); For free drug or conjugate combination treatment group, mice received a single dose of DTX or P-DTX (DTX or equivalent 10 mg/kg) on day 0 and six doses of GDC-0980 or P-(GDC-0980) (GDC-0980 or equivalent 5 mg/kg) twice per week starting on day 1 (n=4). Tumor growth and body weight were monitored every two or three days from day 0 for 95 days. During the monitoring period, mice were sacrificed if the body weight loss exceeded 20% due to tumor growth or the tumor weight exceeded 10% of the body weight. The mice survival curve was constructed based on the mice sacrifice criteria.

2.11 Characterizations of residual tumor cells

Immediately after sacrificing the mice, tumors were harvested and single cell suspensions were prepared as previously described [5, 25]. Briefly, the tumor mass was chopped into small pieces and digested in collagenase IV solution (3 mg/mL) for 30 min, followed by filtration through 70 μ m mesh. Dead cells and red blood cells were discarded from the cell suspension by gradient centrifugation. Half of the cancer cell suspensions were sampled to evaluate the percentage of CD133+ cells by flow cytometry using the staining protocol described in 2.7. The average percentage of CD133+ cells in saline group was set as 100% and the average percentage of CD133+ cells in the other treatment groups were all normalized to the saline group. The rest of the cells were tested for clonogenic ability. Briefly, the cells were counted and seeded in 6- well plates (at least three wells/mouse tumor cell sample) at the density of 400 cells/well in 1 mL growth medium. The cells were allowed to grow for 20 days. On day 20, the number of colonies formed was counted after fixation (methanol:acetic acid 1:1) and crystal violet staining.

2.12. Ultrasound imaging of tumor vasculature

Before and at two weeks after treatment initiation, the tumors were evaluated by the VisualSonics VEVO2100 ultrasound platform with the MS700 probe and Power Doppler. Briefly, mice were anesthetized in the chamber containing 2% isoflurane in oxygen and then placed on the imaging stage. The same acquisition parameters (Doppler gain 33dB, 2D gain 30 dB) were used for all the 2D and 3D measurements. Blue and red signals represent the blood flow from different directions. To quantify the density of blood flow signal in 3D images, the tumor region of each mouse was firstly defined based on the B-mode image. At least 10× 2D slides (with no background signal interference) were randomly chosen from the 3D stacks and both the blue and red signals were quantified in ImageJ. The average signal in saline group was set as 1 and the signals in other treatment groups were normalized to the saline group.

2.13 Statistical analysis

Student's t test was used for comparing two groups and one-way analysis of variance coupled with Tukey's post-hoc analysis was used to compare three or more groups, with p values of <0.05 indicating statistically significant differences. Mice survival data was compared using the log-rank test, with p value <0.05 indicating statistically significant difference.

3. Results and discussion

3.1 Synthesis and characterization of polymerizable MA-GFLG-(GDC-0980) monomer

The reaction of MA-GFLG-OH with GDC-0980 in the presence of coupling reagent resulted in two monomer products as shown in the analytical HPLC profile of the reaction mixture (Figure 1B). The elution peak at 10.5 min corresponds to the free drug GDC-0980. As confirmed by mass spectrometry, the product eluted at 16.3 min is MA-GFLG-(GDC-0980) ($[M+H]^+$ 941.4), which results from the reaction of the amino group on 2-aminopyrimidine in GDC-0980 with the carboxyl group in MA-GFLG-OH. The mass of another peak at 15.8 min matched with the monomer MA-GFLG-GDC ($[M+H]^+$ 869.4) (Figure 1B); it is formed via the reaction of carboxyl group in MA-GFLG-OH with the nitrogen on piperazine moiety. Following enzymatic cleavage, the monomer MA-GFLG-GDC would not release the original GDC-0980. However, previous medicinal chemistry studies of GDC-0980 showed that the 2-aminopyrimidine is the main functional group that confers the bioactivity, whereas the modification of the piperazine ring by lactic amide group is mainly for increasing the solubility/stability/pharmacokinetics of the bioactive compound [18, 26]. Considering that the GDC-0980 analog (GDC) still retains the PI3K pathway inhibitory activity [17], the two conjugates P-(GDC-0980) and P-GDC were both synthesized for further biological evaluations.

3.2 Synthesis and characterization of HPMA copolymer-drug conjugates

All the HPMA copolymer-drug conjugates, P-(GDC-0980), P-GDC and P-DTX, were synthesized successfully via RAFT copolymerization of HPMA and relevant polymerizable drug derivatives. The characterization of all the conjugates is shown in Table 1. The

molecular weights of all the conjugates are well-controlled. The molecular weight distributions of all the conjugates are very narrow, lower than 1.1.

3.3 Cytotoxicity of free and HPMA copolymer-conjugated GDC-0980 and GDC on PC-3 cells

GDC-0980 is a potent anticancer drug against prostate cancer cells with the *in vitro* IC₅₀ values in the nanomolar range [6, 18]. Here the cytotoxic effects were evaluated and compared between GDC-0980 and GDC, as well as between free and conjugated drugs, in order to investigate the structure-potency relationship. To facilitate comparison, the molecular weight and drug content of P-(GDC-0980) and P-GDC were similar. As shown in Figure 3, the IC₅₀ values of GDC-0980, P-(GDC-0980), GDC and P-GDC are around 45 nM, 550 nM, 410 nM and 110 μ M, respectively. The results demonstrate that the GDC-0980 analog (GDC) with no lactic amide on the piperazine ring is still cytotoxic towards PC-3 cells, but the IC₅₀ value of GDC is around nine times higher than that of GDC-0980. When comparing the potency of free and polymer-bound drugs, however, different phenomena were observed between GDC-0980 and GDC. A 10-time difference in the IC₅₀ values of P-(GDC-0980) and GDC-0980 was observed. The different cellular uptake rates between free diffusion of free drug and macropinocytosis/endocytosis of polymer-bound drugs account for the higher IC₅₀ of the polymer-bound drug than the free drug [27]. However, the IC₅₀ value of P-GDC is dramatically higher (>260 times) than that of GDC, which indicates that other factors than the differential uptake mechanisms participate in the observed change of activity.

The GFLG tetrapeptide side chain that chemically links the drug (or drug analog) to the HPMA copolymer backbone has been designed to allow the cleavage by a mixture of lysosomal enzymes, of which one major component is the cysteine proteinase cathepsin B [24, 28, 29]. We compared the *in vitro* drug release properties of the two conjugates in the presence of cathepsin B under the same experimental condition [30]. More than 80% of the total GDC-0980 in the P-(GDC-0980) conjugate was cleaved from the conjugate rapidly within 30 min of exposure to cathepsin B; however, no release of GDC from P-GDC conjugate was observed until 20 h incubation (Figure 4A). The differential cleavage efficiency between the two conjugates could be due to the different conditions of the enzyme-substrate complex formation. In the case of P-GDC, GFLG side chains are linked to GDC via the secondary amine group in GDC, instead of the primary amine in the case of P-(GDC-0980). This structure might not favor the S₁-P₁ and S'₁-P'₁ interactions within the enzyme-substrate complex (nomenclature of Schechter and Berger [31]). But when cathepsin B concentration was high enough, the GFLG linker in P-GDC could still be cleaved (Figure 4B). However, the major cleaved product was glycine-GDC ([M+H]⁺ 484.2), with only a small portion of GDC ([M+H]⁺ 427.2) (Figure 4B). Therefore, for P-GDC, cathepsin B prefers to cleave the tetrapeptide linker at the site between leucine and glycine residues (F in the P2 position, nomenclature of Schechter and Berger [31]) despite of the steric hindrance of the HPMA copolymer backbone.

In summary, the cytotoxicity of P-GDC is suboptimal possibly due to inefficient intracellular drug release. In contrast, the biological activity of GDC-0980 is retained after

conjugating to the HPMA copolymer backbone via GFLG linker. Therefore, P-(GDC-0980) was chosen for further *in vitro* and *in vivo* evaluations of the anti-CSC effects.

3.4 Evaluation of the anti-prostate CSC effects of HPMA copolymer-(GDC-0980) conjugate *in vitro*

Recent investigations have revealed the preferential CSC inhibitory effect of some PI3K/mTOR dual inhibitors in prostate cancer and other cancer models [14, 16, 17]. Here we evaluated whether P-(GDC-0980) possesses inhibitory effect against the CD133+ prostate stem/progenitor cells in PC-3 cell line. The relative proportion of CD133+ cells and prostasphere forming ability were tested following treatment with GDC-0980 and P-(GDC-0980) at concentrations lower than, close to and higher than their IC₅₀ values. As shown in Figure 5A, both GDC-0980 20 nM and P-(GDC-0980) (drug equivalent 100 nM) were able to decrease the percentage of CD133+ cells by about 50% within the surviving prostate cancer cells. To be noted, drug or conjugate at these concentrations showed minimal or very low cytotoxic effect towards the whole cell line (Figure 3). In addition, the number of prostaspheres formed following P-(GDC-0980) (drug equivalent 100 nM) treatment was also decreased when compared to the untreated control (Figure 5B). Taken together, GDC-0980 and P-(GDC-0980) at low concentrations were effective against CD133+ prostate cancer stem/progenitor cells, even though the general cytotoxic effect was weak.

Furthermore, GDC-0980 and P-(GDC-0980) at concentrations close to or higher than the IC₅₀ values (GDC-0980 50 and 100 nM, and P-(GDC-0980) at drug equivalent concentrations 500 and 1000 nM) still significantly decreased the relative proportion of CD133+ cells (Figure 5A). The relative percentages of CD133+ cells did not show dose-dependent decrease across different concentrations of GDC-0980 and P-(GDC-0980). However, the absolute amount of surviving CD133+ cells most likely decreased progressively, because the total number of surviving prostate cancer cells was reduced progressively as the concentration of the drug or conjugate increased.

These findings may be of valuable therapeutic significance: 1) low dose P-(GDC-0980) (or other drugs of similar properties) can be employed to inhibit CSCs even at the concentration that does not inhibit the bulk tumor growth; 2) P-(GDC-0980) at relatively higher dose may efficiently inhibit both bulk tumor cells and CSCs; 3) combination of P-(GDC-0980) and a traditional chemotherapy drug may not only effectively kill CSCs, but also show enhanced killing of bulk tumor cells due to the combinational effect of both drugs.

3.5 Therapeutic effect of the combination of HPMA copolymer-(GDC-0980) conjugate and HPMA copolymer-docetaxel conjugate *in vitro*

The general tumor cell growth inhibitory effects of single and combination of P-(GDC-0980) and P-DTX were evaluated using the cytotoxicity assay. In this study, the concentrations of drugs or conjugates around their IC₅₀ values were chosen. As shown in Figure 6A, the combination of P-(GDC-0980) and P-DTX (or GDC-0980 and DTX) led to the strongest tumor cell growth inhibition comparing to either of the single treatments. To determine whether the combinational effect of the two conjugates is synergistic, more evidence such as comprehensive combination index studies may be needed.

Another important evaluation parameter is the anti-CSC effect after combining the two conjugates. P-(GDC-0980) (or GDC-0980) effectively decreased the relative proportion of CD133+ cells, while P-DTX (or DTX) failed to decrease the relative proportion of CD133+ cells. Importantly, the combination of P-(GDC-0980) and P-DTX (or GDC-0980 and DTX) also decreased the proportion of CD133+ cells effectively (Figure 6B). Therefore, combination therapy provides additional therapeutic benefit than either of the single conjugate by effectively inhibiting both CD133+ and CD133- cells.

In our previous studies, we evaluated the anti-CSC and anti-bulk tumor effect of a HPMA copolymer-cycloamine conjugate (P-CYP) and its combination with P-DTX [5]. One of the obvious differences from P-(GDC-0980) is that P-CYP was preferentially active against CD133+ cells, but not effective against bulk tumor cells even when the drug equivalent concentration was in micromolar range *in vitro* [5, 32]. Under the *in vitro* culture condition, combination of P-DTX and P-CYP did not show immediate advantage on tumor growth inhibition over P-DTX single therapy. Considering the relatively high potency of GDC-0980 against both CSCs and bulk tumor cells and based on relevant studies of PI3K/mTOR inhibitors, we employed a much lower dose of GDC-0980 (or drug equivalent in the conjugate) for *in vivo* evaluation.

3.6 Mice survival following single and combination therapy

PC-3 tumors grew aggressively in the mice xenograft model. In all the experimental groups, mice were sacrificed mostly due to the excessive body weight loss caused by tumor burden (Figure S1), except one mouse in the free drug combination group (due to development of a big cyst around the tumor area). In saline control group with no therapeutic intervention, tumors grew the fastest among all groups and the mice started to lose weight significantly within 20 days from the treatment initiation (Figure S1). All the mice in the saline group were sacrificed by day 40 (Figure 7). Mice in GDC-0980 treatment group were sacrificed starting from relatively early time points due to the excessive body weight loss (Figure 7). The median survival of mice in P-(GDC-0980), P-DTX single treatment groups and free drug combination (DTX+GDC-0980) were slightly longer than in GDC-0980 and saline group, however, the difference was not statistically significant. All the single treatment and free drug combination strategies did not show superior survival benefit over saline treatment, possibly due to the relative low dose or reduced dose frequency of the therapeutic agent administered. The dosing regimen could be further optimized in future based on the safety and efficacy evaluation. On the other hand, the combination treatment of P-DTX and P-(GDC-0980) led to significantly prolonged mice survival over the saline control ($p<0.01$), P-(GDC-0980) ($p<0.01$) and P-DTX ($p<0.05$). This result supports that the combination therapy of two conjugates showed superior anti-tumor effect than either of the single therapy, in accordance with the *in vitro* cytotoxicity study. The macromolecular combination offered better survival results than the free drug combination, probably due to prolonged circulation and increased tumor accumulation of the two drugs.

In previous *in vivo* evaluation of P-CYP and P-DTX, single and combination treatments all achieved immediate tumor growth inhibition significantly during treatment period, but the differences between single and combination therapy were only observed at later time points

after the stop of treatments [5]. In this study, GDC-0980 and P-(GDC-0980) did not significantly inhibit tumor growth during the treatment period, possibly due to the relative low dose; while the combination of P-DTX and P-(GDC-0980) offered immediate tumor growth inhibitory effect. However, the three of four tumors in the conjugate combination treatment group still grew back after the stop of the treatment. Therefore, besides inhibiting CSCs, the adequate initial tumor cell killing and growth inhibition might be important for achieving long-term therapeutic benefit.

To be noted, efficacy evaluation of anti-cancer therapeutics in the animal model is more complicated than in the *in vitro* culture of cancer cells, due to the complex tumor microenvironment that may affect the tumor growth and drug delivery. Besides harnessing cancer cell growth, PI3K/mTOR pathway inhibitors have been reported to also act on tumor vasculature in different ways in the tumor models [33–35]. We observed that the tumor blood flow was different following different treatments (Figure S2). Comparing to the detectable tumor blood vessel signal in the saline control group, GDC-0980 and P-(GDC-0980) dramatically increased the tumor blood flow two weeks after treatment initiation. In contrast, P-DTX did not change the tumor vasculature comparing to the saline control. The combination of free drugs and conjugates also increased tumor blood flow significantly during treatment period. This effect may be beneficial in terms of enhancing the therapeutics delivery into the tumor tissue. But on the other hand, whether the increased blood supply stimulates tumor cell growth still needs to be investigated.

3.7 Ex vivo characterization of residual tumor cells

In order to evaluate the infinite proliferative potential of residual tumor cells, clonogenic cell survival assay of the harvested tumor cells following different treatment strategies was performed. As shown in Figure 8A, the clonogenic ability of tumor cells after P-DTX treatment (29/400 cells) did not change significantly comparing to saline treated cells (30/400 cells). But the colony numbers decreased significantly following P-(GDC-0980) treatment (16/400 cells), or following combination therapy of free (17/400 cells) and conjugated (14/400 cells) drugs. Insult by chemotherapy such as docetaxel can impair the clonogenic ability of cancer cells under some experimental settings [36]. However, under the experimental condition in our study, the residual tumor cells were analyzed not during the treatment period when the tumor growth was effectively inhibited, but at the time when the tumor grew back rapidly and mice needed to be sacrificed. The proliferative potential of tumor cells at this time point was high. In summary, results from the clonogenic assay indicate that the tumor cells surviving from combination therapy of drugs or conjugates may be less aggressive and did not proliferate actively than the surviving cancer cells following P-DTX treatment in long-term.

The analysis of the CD133 expression in residual tumor cells suggests the impact of treatment strategies on CD133+ cancer stem/progenitor cells. Tumor cells in P-DTX group still showed relatively high levels of CD133 expression. However, free and conjugated GDC-0980, as well as the combination of free drugs or conjugates decreased CD133 expression (statistically significant differences from saline control were observed in GDC-0980, free drug combination and conjugate combination groups) (Figure 8B).

Therefore, GDC-0980 or P-(GDC-0980) via systemic administration to tumor-bearing mice retains the ability to inhibit CD133+ tumor cells. As discussed in 3.6, the dose of drug or conjugate was not high enough to arrest the aggressive tumor growth in long-term, but they still showed inhibitory effect against CD133+ cells. This observation met with the *in vitro* evaluation of the dose-efficacy relationship shown in 3.4.

4. Conclusions

Increasing number of preclinical/clinical studies have identified anti-cancer agents with CSC inhibitory activities and endeavored to evaluate the therapeutic effect and cancer prognosis by inhibiting CSCs. Combination of anticancer agents which fail to kill CSCs with those that exhibit anti-CSC activities may provide superior therapeutic benefits by targeting CSCs and synergistically inhibiting bulk tumor cells. Macromolecular combination therapeutics based on CSC theory may not only improve the drug solubility and circulation time, but also offer unique combinational anti-tumor effect.

We synthesized HPMA copolymer-(GDC-0980) conjugate with controlled molecular weight and narrow molecular weight distribution via RAFT copolymerization of HPMA and MAGFLG-(GDC-0980) monomer. P-(GDC-0980) effectively inhibited CD133+ prostate cancer stem/progenitor cells not only at the dose which is cytotoxic against bulk tumor cells, but also at low concentrations which do not cause significant bulk tumor cell death. In parallel, a conjugate containing GDC-0980 analog (GDC) was also synthesized via the same synthetic method used for P-(GDC-0980). However, it is not potent against PC-3 cells at least partially due to inefficient drug release. The combination of P-(GDC-0980) and P-DTX showed enhanced tumor growth inhibition than either single conjugate, while still retaining effective anti-CSC activity *in vitro*. In PC-3 xenografts, combination of P-(GDC-0980) and P-DTX significantly delayed tumor progression and prolonged the mice survival. At the particular dose tested in this study, tumor regression was not observed in all treatment strategies. But both P-(GDC-0980) and combination therapy decreased the clonogenic ability and relative amount of CD133+ tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hurwitz M, Petrylak DP. Sequencing of agents for castration-resistant prostate cancer. *Oncology*. 2013; 27(11):1144–1149. [PubMed: 24575543]
2. Gallardo E, Arranz JA, Maroto JP, León LA, Bellmunt J. Expert opinion on chemotherapy use in castration-resistant prostate cancer progressing after docetaxel. *Crit. Rev. Oncol. Hematol*. 2013; 88(2):357–367. [PubMed: 23867574]

3. Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C, Lander ES. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell*. 2011; 146(4): 633–644. [PubMed: 21854987]
4. Dubrovskaya A, Elliott J, Salamone RJ, Kim S, Aimone LJ, Walker JR, Watson J, Sauveur-Michel M, Garcia-Echeverria C, Cho CY, Reddy VA, Schultz PG. Combination therapy targeting both tumor-initiating and differentiated cell populations in prostate carcinoma. *Clin. Cancer Res*. 2010; 16(23): 5692–5702. [PubMed: 21138868]
5. Zhou Y, Yang J, Rhim JS, Kopeček J. HPMA copolymer-based combination therapy toxic to both prostate cancer stem/progenitor cells and differentiated cells induces durable antitumor effects. *J. Control. Release*. 2013; 172(3):946–953. [PubMed: 24041709]
6. Wallin JJ, Edgar KA, Guan J, Berry M, Prior WW, Lee L, Lesnick JD, Lewis C, Nonomiya J, Pang J, Salphati L, Olivero AG, Sutherlin DP, O'Brien C, Spoerke JM, Patel S, Lensun L, Kassees R, Ross L, Lackner MR, Sampath D, Belvin M, Friedman LS. GDC-0980 is a novel class I PI3K/mTOR kinase inhibitor with robust activity in cancer models driven by the PI3K pathway. *Mol. Cancer Ther*. 2011; 10:2426–2436. [PubMed: 21998291]
7. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES. Identification of selective inhibitors of cancer stem cell by high-throughput screening. *Cell*. 2009; 138(4):645–659. [PubMed: 19682730]
8. Hirsch HA, Iliopoulos D, Tschlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res*. 2009; 69(19):7507–7511. [PubMed: 19752085]
9. Dlugosz A, Agrawal S, Kirkpatrick P. Vismodegib. *Nat. Rev. Drug Discov*. 2012; 11(6):437–438. [PubMed: 22653209]
10. Maira SM, Stauffer F, Brueggen J, Furet P, Schnell C, Fritsch C, Brachmann S, Chène P, De Pover A, Schoemaker K, Fabbro D, Gabriel D, Simonen M, Murphy L, Finan P, Sellers W, García-Echeverría C. Identification and characterization of NVPBEZ235, a new orally available dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor with potent *in vivo* antitumor activity. *Mol. Cancer Ther*. 2008; 7(7):1851–1863. [PubMed: 18606717]
11. Emerling BM, Akcakanat A. Targeting PI3K/mTOR signaling in cancer. *Cancer Res*. 2011; 71(24):7351–7359. [PubMed: 21987725]
12. Martelli AM, Evangelisti C, Chiarini F, McCubrey JA. The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. *Oncotarget*. 2010; 1(2):89–103. [PubMed: 20671809]
13. Gallay N, Dos Santos C, Cuzin L, Bousquet M, Simmonet Gouy V, Chaussade C, Attal M, Payrastra B, Demur C, Recher C. The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia*. 2009; 23(6):1029–1038. [PubMed: 19158829]
14. Dubrovskaya A, Kim S, Salamone RJ, Walker JR, Maira S-M, García-Echeverría C, Schultz PG, Reddy VA. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. *Proc. Natl. Acad. Sci. U.S.A.* 2009; 106(1):268–273. [PubMed: 19116269]
15. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012; 149(2):274–293. [PubMed: 22500797]
16. Park S, Chappuis N, Bardet V, Tamburini J, Gallay N, Willems L, Knight ZA, Shokat KM, Azar N, Viguie F, Ifrah N, Dreyfus F, Mayeux P, Lacombe C, Bouscary D. PI-103, A dual inhibitor of class IA phosphatidylinositide 3-kinase and mTOR, has antileukemic activity in AML. *Leukemia*. 2008; 22(9):1698–1706. [PubMed: 18548104]
17. Zhou J, Wulfkühle J, Zhang H, Gu P, Yang Y, Deng J, Margolick JB, Liotta LA, Petricoin E, Zhang Y. Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. *Proc. Natl. Acad. Sci. U.S.A.* 2007; 104(41):16158–16163. [PubMed: 17911267]
18. Sutherlin DP, Bao L, Berry M, Castaneda G, Chuckowree I, Dotson J, Folks A, Friedman L, Goldsmith R, Gunzner J, Heffron T, Lesnick J, Lewis C, Mathieu S, Murray J, Nonomiya J, Pang J, Pegg N, Wei Prior W, Rouge L, Salphati L, Sampath D, Tian Q, Tsui V, Chi Wan N, Wang S, Wei BQ, Wiesmann C, Wu P, Zhu B-Y, Olivero A. Discovery of a potent, selective, and orally

available class I phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) kinase inhibitor (GDC-0980) for the treatment of cancer. *J. Med. Chem.* 2011; 54(21):7579–7587. [PubMed: 21981714]

19. Kopeček J, Kopecková P. HPMA copolymers: origins, early developments, present, and future. *Adv. Drug Deliv. Rev.* 2010; 62(2):122–149. [PubMed: 19919846]
20. Zhou Y, Kopeček J. Biological rationale for the design of polymeric anti-cancer nanomedicines. *J. Drug Targeting.* 2013; 21(1):1–26.
21. Tucker BS, Sumerlin BS. Poly(*N*-(2-hydroxypropyl)methacrylamide)-based nanotherapeutics. *Polym. Chem.* 2014; 5:1566–1572.
22. Mitsukami Y, Donovan MS, Lowe AB, McCormick CL. Water-soluble polymers. 81. Direct synthesis of hydrophilic styrenic-based homopolymers and block copolymers in aqueous solution via RAFT. *Macromolecules.* 2001; 34(7):2248–2256.
23. Kopeček J, Bažilová H. Poly[*N*-(2-hydroxypropyl)methacrylamide]. 1. Radical polymerization and copolymerization. *Eur. Polym. J.* 1973; 9(1):7–14.
24. Kopeček J.; Rejmanová, P.; Strohalm, J.; Říhová, B.; Chytrý, V.; Lloyd, JB.; Duncan, R. Synthetic polymeric drugs. US Pat. 5,037,883. 1991.
25. Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, Reilly JG, Chandra D, Zhou J, Claypool K, Coghlan L, Tang DG. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene.* 2006; 25(12):1696–1708. [PubMed: 16449977]
26. Salphati L, Pang J, Plise EG, Lee LB, Olivero AG, Wei Prior W, Sampath D, Wong S, Zhang X. Preclinical assessment of the absorption and disposition of the phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor GDC-0980 and prediction of its pharmacokinetics and efficacy in human. *Drug Metab. Dispos.* 2012; 40(9):1785–1796. [PubMed: 22696419]
27. Demoy M, Minko T, Kopecková P, Kopeček J. Time- and concentration-dependent apoptosis and necrosis induced by free and HPMA copolymer-bound doxorubicin in human ovarian carcinoma cells. *J. Controlled Release.* 2000; 69(1):185–196.
28. Rejmanová P, Pohl J, Baudyš M, Kostka V, Kopeček J. Polymers containing enzymatically degradable bonds. 8. Degradation of oligopeptide sequences in *N*-(2-hydroxypropyl)methacrylamide copolymers by bovine spleen cathepsin B. *Makromol. Chem.* 1983; 184:2009–2020.
29. Duncan R, Cable HC, Lloyd JB, Rejmanová P, Kopeček J. Polymers containing enzymatically degradable bonds. 7. Design of oligopeptide side-chains in poly[*N*-(2-hydroxypropyl)methacrylamide] copolymers to promote efficient degradation by lysosomal enzymes. *Makromol. Chem.* 1983; 184:1997–2008.
30. Dvořák M, Kopecková P, Kopeček J. High-molecular weight HPMA copolymer-adriamycin conjugates. *J. Control. Release.* 1999; 60(2–3):321–332. [PubMed: 10425337]
31. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* 1967; 27(2):157–162. [PubMed: 6035483]
32. Zhou Y, Yang J, Kopeček J. Selective inhibitory effect of HPMA copolymer-cyclopamine conjugate on prostate cancer stem cells. *Biomaterials.* 2012; 33:1863–1872. [PubMed: 22138033]
33. Fokas E, Im JH, Hill S, Yameen S, Stratford M, Beech J, Hacki W, Maria S-M, Bernhard EJ, McKenna WG, Muschel RJ. Dual inhibition of the PI3K/mTOR pathway increases tumor radiosensitivity by normalizing tumor vasculature. *Cancer. Res.* 2012; 72(1):239–248. [PubMed: 22108822]
34. Dormond-Meuwly A, Dufour M, Demartines N, Dormond O. mTOR inhibition and the tumor vasculature. *Curr. Angiogenesis.* 2012; 1(1):11–19.
35. Sampath D, Oeh J, Wyatt SK, Cao TC, Koeppen H, Eastham-Anderson J, Robillard L, Ho CC, Ross J, Zhuang G, Reslan HB, Vitorino P, Barck KH, Ungersma SE, Vernes JM, Caunt M, Van Bruggen N, Ye W, Vijapurkar U, Meng YJ, Ferrara N, Friedman LS, Carano RA. Multimodal microvascular imaging reveals that selective inhibition of class I PI3K is sufficient to induce an antivascular response. *Neoplasia.* 2013; 15(7):694–711. [PubMed: 23814482]
36. Zhao L, Lee BY, Brown DA, Molloy MP, Marx GM, Pavlakis N, Boyer MJ, Stockler MR, Kaplan W, Breit SN, Sutherland RL, Henshall SM, Horvath LG. Identification of candidate biomarkers of

therapeutic response to docetaxel by proteomic profiling. *Cancer Res.* 2009; 69(19):7696–7703.
[PubMed: 19773444]

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Figure 1.

Synthesis of drug monomer MA-GFLG-(GDC-0980) and MA-GFLG-GDC. A) Synthetic route for MAGFLG-(GDC-0980) and MA-GFLG-GDC; B) Analytical HPLC profile of the reaction products; GDC-0980, MAGFLG- GDC and MA-GFLG-(GDC-0980) elute at 10.5 min, 15.8 min and 16.3 min, respectively (gradient elution method: 2% to 90% buffer B in 30 min).

Figure 2. Synthetic scheme of HPMA copolymer-(GDC-0980) conjugate and HPMA copolymer-GDC conjugate via RAFT copolymerization.

Figure 3. Cytotoxicity of different drugs or conjugates on PC-3 cells. A) GDC-0980 and P-(GDC-0980); B) GDC and P-GDC. The error bars represent the standard deviation of the data points (n=3).

Figure 4.

Enzymatic cleavage of P-(GDC-0980) and P-GDC. A) Drug release profile of P-(GDC-0980) and P-GDC following incubation with cathepsin B; B) HPLC profiles of free GDC-0980 (upper), cleavage mixture of P-(GDC-0980) (middle) and cleavage mixture of P-GDC (in the presence of high concentration of cathepsin B) (lower).

Figure 5. CD133 expression and prostasphere formation following GDC-0980 and P-(GDC-0980) treatment. A) Change in the relative percentage of CD133+ cells; B) sphere formation. All data are presented as mean $\pm$ SD from three independent experiments. * represents a significant difference from the control group, $p < 0.05$.

Figure 6. Growth inhibition and CD133 expression following single and combination of drug or conjugate treatments. A) Growth inhibition; B) change in the relative percentage of CD133+ cells. All data are presented as mean $\pm$ SD from three batches of experiments. * represents a significant difference from the control group, $p < 0.05$.

Figure 7.

Mice survival following single and combination treatment of drugs or conjugates. Mice were treated with saline, GDC-0980 (5 mg/kg, twice per week, six doses), P-(GDC-0980) (drug equivalent 5 mg/kg, twice per week, six doses), P-DTX (drug equivalent 10 mg/kg, single dose), as well as the combination of two free drugs or two drug conjugates (n=4 for all groups). Mice exited the group when body weight loss exceeded 20% or tumor weight exceeded 10% of body weight. The statistically significant differences in the mice survival between groups are shown. *, $p < 0.05$; **, $p < 0.01$.

Figure 8.

A) Clonogenic ability and B) the relative percentage of CD133+ cells within residual tumor cells following single or combination treatment of drugs or conjugates. The data are presented as mean $\pm$ SD. The error bars represent the standard deviation of the data obtained from 3–4 mice/group. *, $p < 0.05$.

Table 1

Characterization of HPMA copolymer-drug conjugates.

Conjugate	Mn, kDa	Mw, kDa	Mw/Mn	Drug content (wt%)
P-GDC	51.9	54.4	1.05	3.0
P-(GDC-0980) (batch 1)	45.2	47.9	1.06	4.4
P-(GDC-0980) (batch 2)	94.4	98.8	1.04	5.3
P-DTX	36.4	38.6	1.06	7.2