HPMA Copolymer CXCR4 Antagonist Conjugates Substantially Inhibited the Migration of Prostate Cancer Cells

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ABSTRACT: A *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer–CXCR4 antagonist (BKT140) conjugate (P-BKT140) was developed and its biological activities were tested. Both free BKT140 and monomer MA-GGPLGLAG-BKT140 (MA is methacryloyl) were prepared by solid phase synthesis. P-BKT140 was prepared by reversible addition–fragmentation chain transfer (RAFT) copolymerization of monomers HPMA and MA-GGPLGLAG-BKT140. The in vitro results show that the free BKT140 and P-BKT140 have similar cytotoxicity against human prostate carcinoma PC-3 cells, indicating that conjugation of BKT140 to HPMA did not significantly impact the cytotoxicity of BKT140. Both BKT140 and P-BKT140 inhibited the CXCL12-induced migration of PC-3 prostate cancer cells, but the P-BKT140 conjugate possessed a substantially higher inhibition activity than free BKT140.
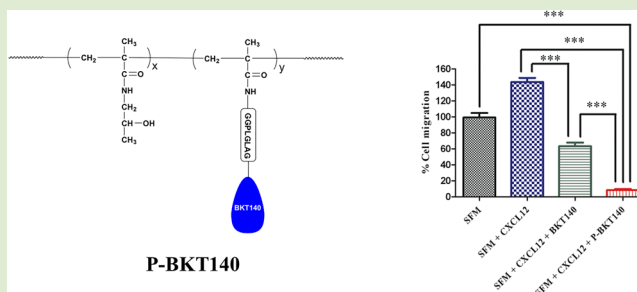
P rostate cancer is the most commonly diagnosed, and has the second leading mortality rate, among men in the United States.[1−3] Most prostate cancer-related deaths are not the result of primary tumor growth but rather due to metastases at distant organs.[4] The five-year survival of metastatic prostate cancer is only about 30%.[1,5] The most common metastatic site of prostate cancer is bone (84% of 74826 patients).[6,7] The interaction between chemokine CXCL12 and its G-protein-coupled receptor CXCR4 appears to play a critical role in the migration of prostate cancer cells from primary prostate tumor to bone.[8−10] CXCR4 was detected in most (94.2%) metastatic prostate cancers.[4,11] The overexpression of CXCR4 accelerates prostate cancer metastasis by promoting the migration and invasion ability of prostate cancer cells toward CXCL12.[12,13] Blocking or inhibiting the CXCL12/CXCR4 interaction by anti-CXCR4 antibody or CXCR4 antagonists impairs the migration and invasion potential of prostate cancer cells.[12,14,15]

Of the several CXCR4 antagonists available, 4F-benzoyl-TN14003 (BKT140; Figure 1A) was chosen for the present study based on two reasons: (i) BKT140 has a high binding affinity (1 nM) toward CXCR4,[16] and (ii) BKT140 is safe in vivo.[17] However, other factors hinder the in vivo application of BKT140. First, BKT140 is sensitive to enzymatic degradation in the circulation because it is a peptide. Second, the renal clearance of BKT140 is fast because the mass of BKT140 is less than 5 kDa.[18] The most common strategy to prolong the plasma residence time of peptides is to conjugate the peptide to a water-soluble polymer.[18−20]

In this letter, HPMA polymer was selected for preparing a polymer–BKT140 conjugate because HPMA is a well-defined hydrophilic, nonimmunogenic, and nontoxic polymer carrier.[21,22] The sequence PLGLAG is susceptible to cleavage by MMP-2.[23] The ability of free BKT140 and HPMA copolymer
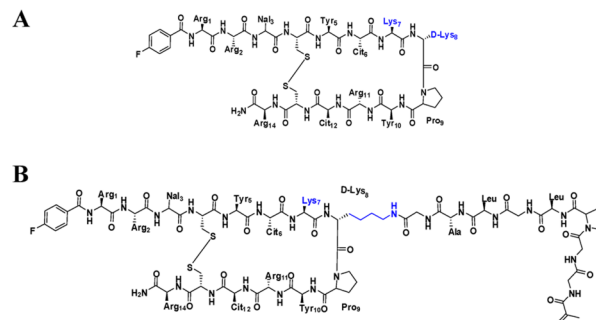

Figure 1. Structures of free BKT140 (A) and monomer MA-GGPLGLAG-BKT140 (B). Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline.

BKT140 conjugates to inhibit prostate cancer cell migration were investigated and compared.

HPMA copolymer drug conjugates can be prepared via copolymerization or polymer-analogous attachment.[21] In this study, we used reversible addition–fragmentation chain transfer (RAFT) polymerization to prepare the HPMA copolymer–BKT140 conjugate because this strategy can avoid the production of side products in the postpolymerization modifications with functionalized oligopeptides. The challenge was to prepare a polymerizable form of BKT140: (1) the conjugation should not significantly impact the activity of BKT140; (2) the conjugation should selectively occur only at one lysine group, as there are two lysine residues in BKT140 (a

double-substituted BKT140 would act as a cross-linking agent). The D-Lys at position 8 was chosen for BKT140 conjugation because the modification of this site does not interfere with the binding affinity of BKT140 toward CXCR4.[24−26] To be sure that the reaction is selective at the D-Lys[8] rather than at the L-Lys[7] position, we selected Fmoc-D-Lys(Dde)-OH and Fmoc-L-Lys(Boc)-OH for the synthesis of monomer MA-GGPLGLAG-BKT140 (Figure 1B) because 1-(4,4-dimethyl-2,6-dioxocyclo-hexylidene)-3-ethyl (Dde) protecting group can be selectively removed with hydrazine.

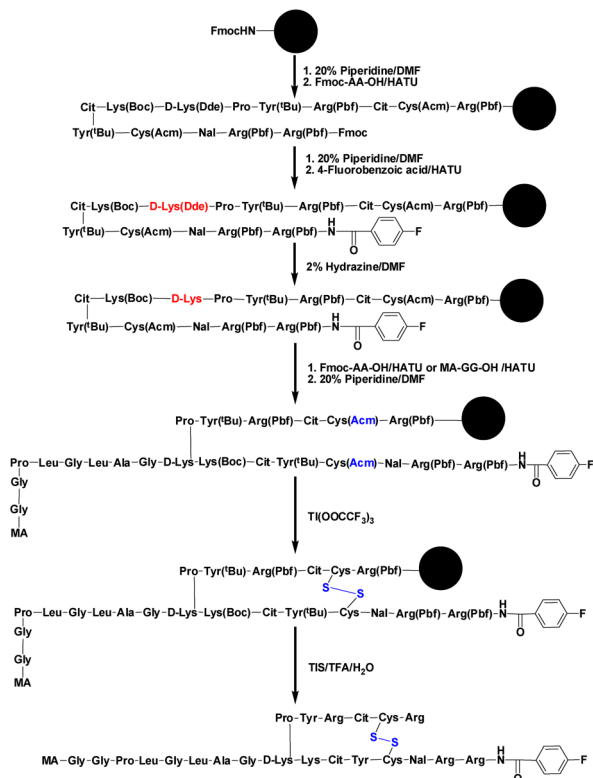As shown in Figure 2, monomer MA-GGPLGLAG-BKT140 was prepared by conventional solid phase synthesis. The

Figure 2. Synthetic scheme of monomer MA-GGPLGLAG-BKT140. Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline.

synthesis of monomer MA-GGPLGLAG-BKT140 started with the deprotection of Fmoc group on the resin by mixing the Rink amide MBHA resin (300 mg, 0.52 mmol/g, 156 μmol) with 20% piperidine in DMF. Then, protected amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Cit-OH (Cit = L-citrulline), Fmoc-Arg(Pbf)-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Pro-OH, Fmoc-D-Lys(Dde)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cit-OH, Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Nal-OH (Nal = L-3-(2-naphthyl)alanine), Fmoc-Arg-(Pbf)-OH, Fmoc-Arg(Pbf)-OH) (468 μmol), and 4-fluoroben-zoic acid (468 μmol) were sequentially attached to the resin in the presence of 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b] pyridinium 3-oxid hexafluoro-phosphate (HATU; 169 mg, 444.6 μmol). The following step was to selectively remove the Dde protecting group from the D-Lys[8] amino acid residue by mixing the Rink amide MBHA bound protected linear BKT140 peptide with 2% hydrazine in DMF. The next step was to sequentially attach protected amino acids (Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Pro-OH; 468 μmol) and MA-GG-

OH (468 μmol) to resin. Then the resin-bound linear peptide was cyclized by mixing the resin bound linear BKT 140 peptide with a solution of thallium(III) trifluoroacetate (Tl(OOCF$_3$)$_3$; 254 mg, 468 μmol) in DMF.[27] The resin-bound peptides were washed with methanol and DMF twice. The final step was to cleave the peptide from the resin with a mixture of trifluoroacetic acid (9.5 mL), triisopropylsilane (0.25 mL) and H$_2$O (0.25 mL) for 3 h. The acid labile protecting groups were also removed in this step. The monomer MA-GGPLGLAG-BKT140 was purified by RP-HPLC (Agilent 1100 series) and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The calculated mass of monomer MA-GGPLGLAG-BKT140 [M + H]$^+$ was 2849.45, and the found mass was 2849.30 (Figure 3). The synthesis of free BKT140 was similar as the procedure for preparing monomer MA-GGPLGLAG-BKT140.
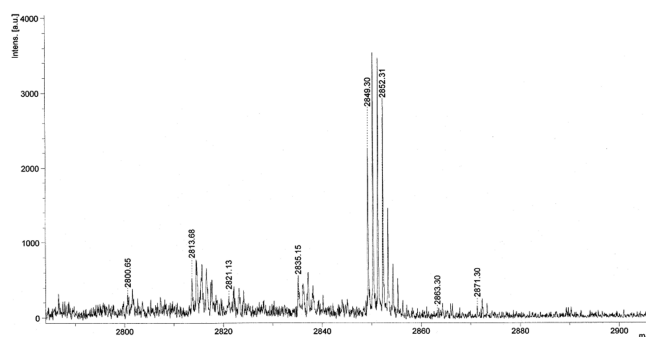

Figure 3. Mass spectrum of monomer MA-GGPLGLAG-BKT140.

The HPMA copolymer BKT140 conjugate P-BKT140 was prepared by RAFT copolymerization of monomers HPMA[28] and MA-GGPLGLAG-BKT140 (Figure 4). This polymer-
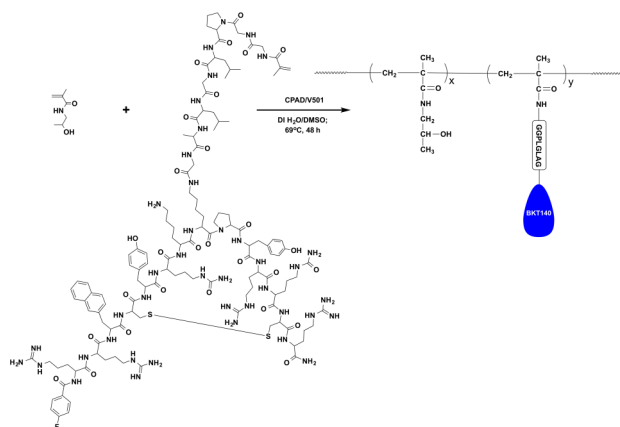

Figure 4. Synthetic scheme of polymer conjugate P-BKT140.

ization was conducted in a mixture of dimethyl sulfoxide (DMSO) and deionized (DI) water at 69 °C for 48 h. 4-Cyanopentanoic acid dithiobenzoate (CPAD) and 4,4′-azobis-(4-cyanopentanoic acid) (V-501) were used as the chain transfer agent and initiator, respectively. The molecular weight and molecular weight distribution of P-BKT140 were analyzed by size exclusion chromatography using a Superose 6 HR10/30 analytical column on an AKTA FPLC system (Pharmacia) equipped with refractive index (RI) and UltraViolet (UV) detectors. HPMA homopolymer fractions were used for molecular weight calibration. The number-average molecular

weight (M_n), weight-average molecular weight (M_w), and polydispersity (M_w/M_n) of P-BKT140 were 100 kDa, 123 kDa, and 1.23, respectively. The content of BKT140 in the polymer conjugate P-BKT140 was measured by amino acid analysis. Alanine and tyrosine were used for calibration. The measured content was 68.6 nmol BKT140/mg polymer, and the weight percentage was 14.8% (BKT140/polymer).

The biological activity of P-BKT140 and BKT140 was tested on human prostate carcinoma PC-3 cells (ATCC), which were established from bone metastasis of a prostate cancer patient.[29] In particular, the efficacy to inhibit CXCL12 induced migration of PC-3 cells was evaluated. To rule out the possibility that the inhibition of cell motility is due to nonspecific cytotoxic effects, we first conducted an in vitro cytotoxicity assay to find the appropriate concentrations of BKT140 or P-BKT140 for migration study. Five different concentrations (0.05, 0.5, 5, 10, and 25 μM) of BKT140 or BKT140 equiv of P-BKT140 were tested against PC-3 cells, and the cell cytotoxicity results are summarized in Figure 5. P-BKT140 has similar cytotoxicity
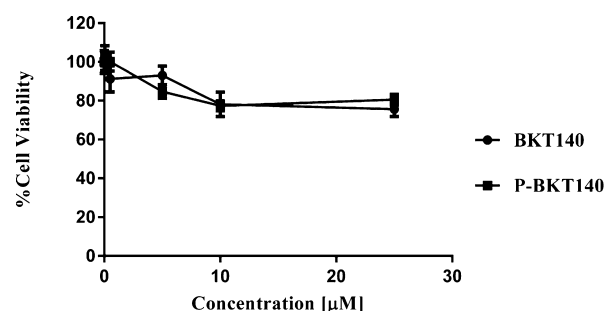

Figure 5. In vitro cytotoxicity of BKT140 and P-BKT140 toward PC-3 prostate cancer cells.

as free BKT140, indicating the conjugation of BKT140 to HPMA copolymer did not result in the loss of BKT140 cytotoxicity. The results also show that both BKT140 and P-BKT140 had only minor cytotoxicity against PC-3 cells when the concentration is less than 10 μM. Thus, BKT140 or BKT140 equiv of P-BKT140 with concentrations lower than 10 μM was used for migration studies.

The migration assay was done by using Transwell (Corning, Corning, NY) with 6.5 mm insert and 8 μm pore polycarbonate membrane. CXCL12 was added to the lower chamber to induce the PC-3 cell migration. Based on cytotoxicity results, we evaluated the capacity of BKT140 (5 μM) and P-BKT140 (5 μM BKT140 equiv) to inhibit the CXCL12 induced PC-3 prostate cancer cells migration. In the migration experiments, the following steps were performed: (i) to the bottom chambers of Transwell were added RPMI-1640 serum-free media (SFM) with or without CXCL12 (400 ng/mL); (ii) to the top chamber of Transwell was added PC-3 cells following incubation with SFM, BKT140 in SFM, or P-BKT140 in SFM; (iii) loaded Transwell was then incubated at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) for 24 h; (iv) the nonmigratory cells on the upper membrane surface were removed; (v) the cells that migrated through to the lower membrane surface were stained with 4′,6-diamidono-2-phenyl-indole (DAPI, 1 μg/mL, Sigma) and quantified by manually counting 20 representative fields using an inverted fluorescence microscope.

The average cell number in one representative field in the SFM group was set as 100%. The (cell migration) % was

calculated by dividing the migrated cell number in the treated group by the average migrated cell number in the SFM group. The results are expressed as (mean $\pm$ SEM) %. The results are summarized in Figure 6: SFM (99.26 $\pm$ 5.56)%, SFM +
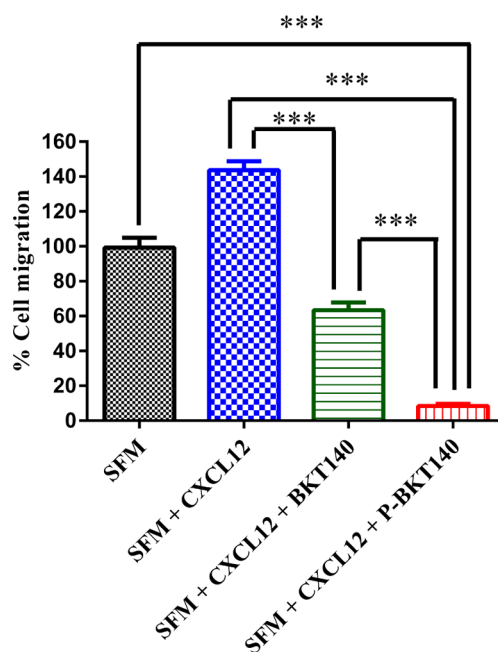

Figure 6. Effects of BKT140 and P-BKT140 on CXCL12 induced PC-3 prostate cancer cells migration. Statistics: One Way ANOVA plus Tukey's post-hoc test (P < 0.001 = ***).

CXCL12 (143.68 $\pm$ 5.01)%, SFM + CXCL12 + BKT140 (63.38 $\pm$ 4.43)%, SFM + CXCL12 + P-BKT140 (8.53 $\pm$ 1.15)%. As shown in Figure 6, CXCL12 enhanced the migration of PC-3 prostate cancer cells. Free BKT140 inhibited the CXCL12 induced migration of PC-3 prostate cancer cells at some extent, and the conjugate P-BKT140 inhibited almost all migration of PC-3 prostate cancer cells.

In summary, a HPMA copolymer CXCR-4 antagonist conjugate (P-BKT140) was successfully prepared via RAFT copolymerization of monomers HPMA and MA-GGPLGLAG-BKT140. We designed a concise scheme to prepare the monomer MA-GGPLGLAG-BKT140. For easy handling, all synthetic steps of MA-GGPLGLAG-BKT140 were conducted on MBHA resin. Use of the two different orthogonal protecting groups at the two lysine residues enabled regioselective modification of the D-Lys with the polymerizable group. The synthesis methodology for P-BKT140 can be used to design other formulation of BKT140 conjugates. Both free BKT140 and P-BKT140 have shown similar cytotoxicity against prostate cancer cells, indicating that the conjugation of BKT140 to HPMA copolymer does not significantly alter the cytotoxicity of free BKT140. Although, both BKT140 and P-BKT140 inhibited the CXCL12 induced migration of PC-3 prostate cancer cells, the HPMA copolymer—BKT140 conjugate (P-BKT140) possessed a substantially higher inhibition activity than free BKT140. In this respect, our work may provide a new method for inhibiting prostate cancer metastasis.

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Notes

■ ACKNOWLEDGMENTS

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