ACS Medicinal Chemistry Letters

Letter

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Novel DNA Polymer for Amplification Pretargeting

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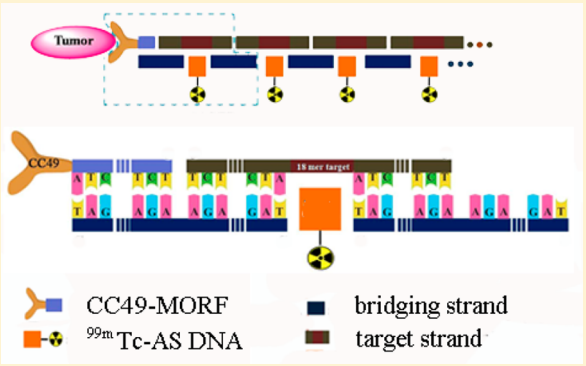
ABSTRACT: In this Letter, different from conventional pretargeting, an additional novel DNA polymer with multiple copies of a target was first designed to be administrated between the antitumor antibody, and the labeled effector served as an amplification pretargeting strategy. Two phosphorothioate DNA strands, a bridging and a target strand, were hybridized to form a polymer. Polymer size, as a function of molar ratios, was then monitored by size exclusion HPLC and electrophoretic mobility shift assay. Moreover, binding efficiency of polymers with the radiolabeled effector and polymer size after hybridization were measured by HPLC as well. As the polymer was expected to produce more binding sites that would be targeted by effectors, amplification pretargeting can greatly improve accumulation of effectors in tumor. This novel proof-of-concept was then well demonstrated by the in vitro test of signal amplification in antibody-binding protein L coated plate and LS174T cells. Compared to conventional pretargeting, significantly increasing radioactive signal was observed in this designed amplification pretargeting, which would serve as a useful paradigm of the potential of oligomer polymers to improve pretargeting and other related approaches.



KEYWORDS: *DNA polymer, pretargeting, amplification, ^{99m}Tc*

Generally, strategies for targeted delivery of therapeutic or imaging radionuclides to tumor sites are divided into two approaches: one where the radionuclide is directly attached to the agent, and the other where a nonradioactive tumor-specific agent bearing a molecular group with a high affinity for a small effector molecule is first administrated, with the radiolabeled effector subsequently given after a suitable interval to allow for tumor targeting and clearance. As a separate injection, radiolabeled effector can be cleared rapidly from the blood and body, potentially improving the signal localization in the tumor and decreasing toxicity. Hence, pretargeting has been widely used as an active strategy to increase tumor to nontumor ratio, so as to improve diagnostic and therapeutic efficiencies.[1−6] Currently, several systems are under investigation for pretargeting, including (strept)avidin/biotin, bispecific antibodies/hapten, and mAbMORF/cMORF.[2,7,8]

An amplification pretargeting approach, where a polymer with multiple copies of a target was administered between the pretargeting antibody and the labeled effector, was thus developed with the potential to greatly increase the absolute accumulation of a radioactive or fluorescence signal in tumor or other lesions.[9−15] By virtue of amplification pretargeting, radioactive signals from focus will be magnified when performing PET or SPECT examinations, benefiting for the localization of small focus, therefore, in favor of early diagnosis and timely therapy. Thus, far, amplification pretargeting studies generally focus on polymers such as polylysine and dendrimers.[10] However, oligomer polymers have not been considered in amplification pretargeting, despite that oligomers possess many advantages over conventional bivalent antibodies or streptavidin/biotin affinity pairs. Flexibility permits manipulation of base sequence or chain length to improve affinity; meanwhile, antioxidant capacity is helpful to inactivate free radicals. Herein, we are developing an amplification pretargeting approach where a novel DNA polymer containing a certain number of two phosphorothioate DNA (PS-DNA) strands, a bridging and a target strand, was first designed (Figure 1). PS-DNA is used as amplification effector here because it is well-known to be more stable in vivo than phosphodiester DNA (PO-DNA).

As demonstrated in Figure 1, a monoclonal antibody CC49 was chosen as the pretargeting antibody because it could recognize a unique Sialyl-Tn antigen present on tumor-associated mucin, TAG-72, overexpressed on tumor types[16,17] including LS174T (human colon cancer cell line) tumor.[18,19] For convenience, our standard pretargeting antiTag72 antibody

Received: January 27, 2015
Accepted: July 26, 2015
Published: July 27, 2015

DOI: 10.1021/acsmedchemlett.5b00265
ACS Med. Chem. Lett. 2015, 6, 972−976

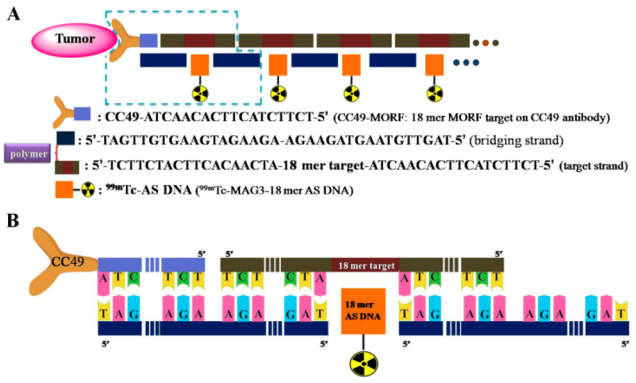


Figure 1. (A) Design of amplification pretargeting bearing DNA polymers. (B) Zoom in on section of blue frame in panel A.

CC49 was used already conjugated with our standard affinity enhancement system, i.e., 18-mer MORF[20] (phosphodiamidate morpholino, a DNA analogue oligomer) "antibody target", employing a commercial Hydralink linker following the instruction of the manufacturer and experiences in this laboratory.[21]

DNA polymers were designed as an alternation of bridging strands and target strands. Both bridging strand and target strand have been designed to avoid self-hybridization and hairpins, and to elongate depending upon molar ratios. The bridging strand consisted of two identical DNA sequences against the standard 18-mer MORF on the antibody (i.e., cDNA18/cDNA18). The 5′-to-3′ direction of the DNA polymerization was changed to 3′ to 5′ in the middle. The target strand consisted of two DNAs on either end of a unique "polymer target", in this case an 18-mer DNA (tDNA) was inserted, i.e., DNA18/tDNA18/DNA18. When mixed in solution, the bridging strands would alternate with the target strands to produce a polymer chain, which would hybridize to the tethered antibody and provide as expected as many exposed binding sites (tDNA) that were ready to be reacted with radiolabeled effector.

The unique "polymer target" on the target strand should be distinguished from the "antibody target" sequence (i.e., 18-mer MORF here) on the antibody. In general, any chain length or any polymer target sequence that does not hybridize to the above sequences may be selected. For convenience, an 18-mer sense PS-DNA was selected. To permit radiolabeling of the "polymer target", an 18-mer AS DNA complementary to the 18-mer "polymer target" was conjugated with NHS-MAG_3 (mercapto-acetylglycyl-glycyl-glycine) for ^{99m}Tc radiolabeling to afford ^{99m}Tc-AS DNA (i.e., ^{99m}Tc-MAG_3-18-mer AS DNA) following the similar method as previously described.[22] ^{99m}Tc-cDNA (i.e., ^{99m}Tc-MAG_3-cDNA18, on which DNA sequences was paired with the standard 18-mer MORF on the antibody) applied in conventional pretargeting was selected as the reference control. The typical HPLC radiochromatograms of ^{99m}Tc-cDNA and ^{99m}Tc-AS DNA determined by size exclusion HPLC showed that labeling efficiency in both cases was about 95% (Figure 2).

The formations of polymer, such as relative size and purity, were estimated by size exclusion HPLC using UV detection at 265 nm, and further analyzed via electrophoretic mobility shift assay (EMSA). The polymer was prepared *in vitro* by mixing bridging and target strands at different molar ratios (1:1, 1:2, 2:1, 5:1, 1:5, 1:10, 10:1). As displayed in Figure 3A, when the

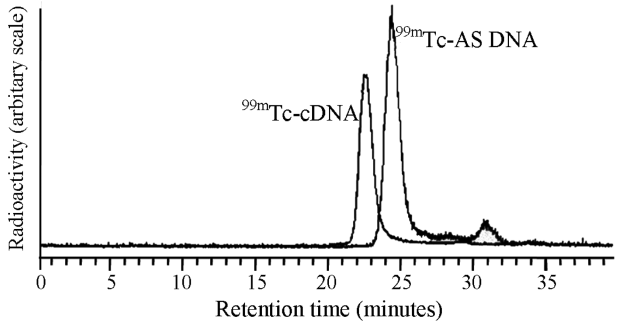


Figure 2. Size-exclusion HPLC radiochromatograms of ^{99m}Tc labeled cDNA and AS DNA.

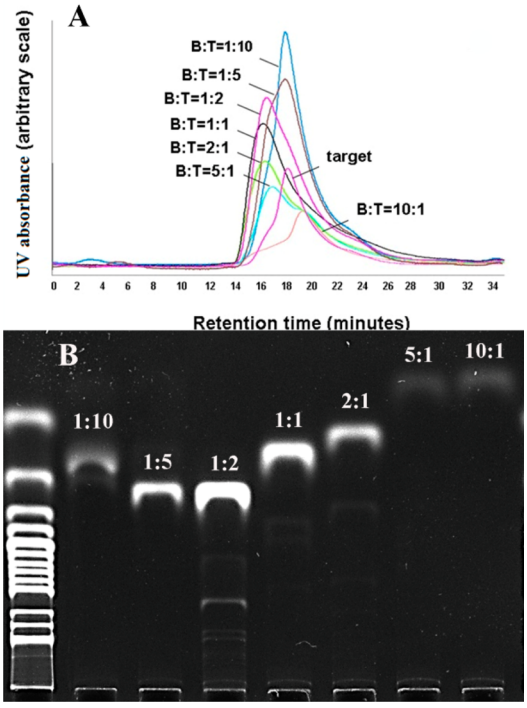


Figure 3. Size-exclusion HPLC chromatograms of polymers prepared at different molar ratios of bridging to target strand with UV detection at 265 nm (A) and electrophoretic mobility shift assay results (B) with references on the left (50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, and 800 bp, from top to bottom).

ratio of target strand increased greatly (B/T = 1/5 or 1/10), the retention time is almost the same as that of solo target strand. As shown in Figure 3B, polymer size was largest (about 125 bp) when B/T = 1/2. However, considering that remaining strands might self-hybridize or form hairpin structures in the case of unequal ratios, which would influence the formation, polymers in condition of equal molar ratio (1:1) are still the optimum choice and used in subsequent *in vitro* binding assay.

Binding efficiency was measured via radioactivity detection following addition of trace ^{99m}Tc-AS DNA. Total binding to polymers was observed, no matter the molar ratio of bridging strand and target strand. Furthermore, in Figure 4, single radioactivity peak was observed for each polymer after hybridizing with the radiolabeled effector, which also indicated the uniform size formed when different individual molar ratio was applied.

Thereafter, the proof-of-concept was carried out *in vitro* on a Protein L coated 96-well flat-bottomed plate using a polymer

DOI: 10.1021/acsmedchemlett.5b00265
ACS Med. Chem. Lett. 2015, 6, 972−976

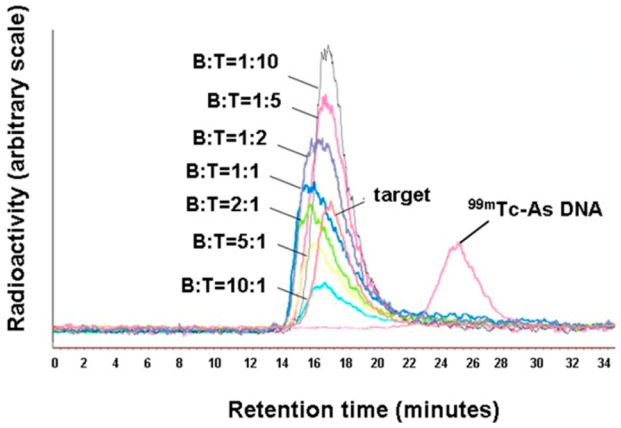


Figure 4. Size-exclusion HPLC chromatograms of polymers prepared at different molar ratios of bridging to target strand with radioactivity detection following addition of trace ^{99m}Tc-AS DNA.

prepared in a 1:1 bridging/target strand ratio, where CC49 can be recognized by immunoglobulin-binding bacterial protein L. Figure 5 presents the percentage of radioactivity for

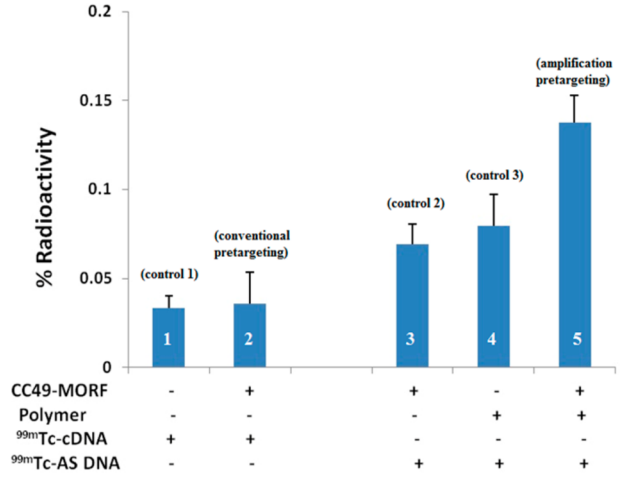


Figure 5. Histograms presenting the percentage on radioactivity obtained in a Protein L coated plate for amplification pretargeting and conventional pretargeting. Results were averages, error bars present one SD (N = 6).

amplification pretargeting and conventional pretargeting. Probably because of high background in this study, the result for conventional pretargeting (bar 2) is not statistically different from that of control 1 (bar 1). The addition of ^{99m}Tc-AS DNA generally improves the targeting efficiency (bars 3−5); moreover, the radioactivities increase more when adding both polymer and ^{99m}Tc-AS DNA (bars 4 and 5). Compared to conventional pretargeting (bar 2) and other controls (bars 1, 3, and 4), complete amplification pretargeting (bar 5) would significantly enhance the radioactive signal, which is about twice higher than conventional pretargeting, suggesting that DNA polymer plays an important role in the accumulation of radioactivity in this case.

In addition, the effect of amplification pretargeting was also confirmed in a cell signal amplification study using a polymer prepared in a 1:1 bridging/target strand ratio. As shown in Figure 6, the trend of signal amplification on LS174T is similar to that on Protein L coated plate (Figure 5). Radioactivity

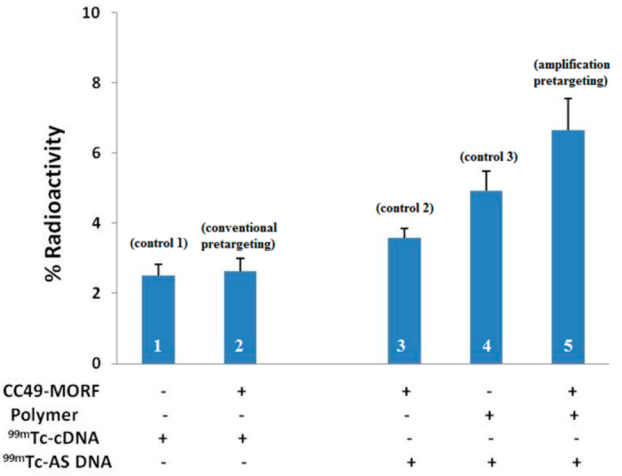


Figure 6. Histograms presenting the percentage of radioactivity accumulated in LS174T cells for amplification pretargeting and conventional pretargeting. Results were averages; error bars present one SD (N = 4).

accumulation of amplification pretargeting (bar 5) is almost 2-fold more than that of the conventional one without polymer (bar 2), which is the direct reflection of polymer formation.

In conclusion, a novel oligomer (bridging and target strands included) was designed to form a polymer with variable lengths. After first determining the appropriate molar ratio (1:1) of bridging and target strand by size exclusion HPLC and EMSA, the polymer was utilized to produce more binding sites (targets) that would be recognized by the effector. This hypothesis was thus well verified by the *in vitro* experiment of signal amplification in antibody-binding protein L coated plate and LS174T cells. Both studies of this new pretargeting approach displayed two times higher radioactive signal increase than conventional technology, demonstrating that radioactive signal could be amplified by importing more radioactive isotope labeled DNA by virtue of hybridization with more targets on the polymer. Therefore, this investigation manifested a novel proof-of-concept thus far, which serves as a useful paradigm of the potential of oligomer polymers to improve pretargeting and other related approaches.

EXPERIMENTAL PROCEDURES

Materials. Two uniform phosphorothioate DNA strands and phosphorothioate oligomers were purchased from Integrated DNA Technologies (Coralville, IA). Eighteen-mer MORF was purchased from Gene-Tools (Philomath, OR) with 3′-amine modification and with the same base sequence used in this laboratory. *N*-Hydroxysuccinimidyl *S*-acetylmercaptoacetyl-triglycine was synthesized from a previous procedure.[23] Protein L coated plate was obtained from Pierce (Rockford, IL). CC49 antibody was prepared by Strategic Biosolutions (Newark, DE) from CC49 murine hybridoma cell line, a gift from Dr. Jeffery Schlom (Laboratory of Tumor Immunology and Biology, Center for Cancer Research, NCI, NIH, Bethesda, MD). The human colon cancer cell line (LS174T) was obtained from American Type Culture Collection (ATCC, Manassas, VA). ^{99m}Tc-pertechnetate was eluted from a ^{99}Mo-^{99m}Tc generator (Bristol-Myers Squibb Medical Imaging Inc., North Billerica, MA). All other chemicals were reagent grade and were used without purification.

Preparation of CC49-MORF. The CC49 antibody was conjugated with MORF using a commercial Hydralink linker following the instruction of the manufacturer and experiences in this laboratory.[21]

DOI: 10.1021/acsmedchemlett.5b00265
ACS Med. Chem. Lett. 2015, 6, 972−976

Preparation of Polymers. The polymers were prepared by mixing bridging and target strands at molar ratios of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10 in 0.1 M PBS. The mixtures were vortexed for 10 s, heated to 95 °C for 5 min, and then slowly cooled to room temperature.

Preparation of ^{99m}Tc-Labeled Phosphothioate DNAs. Briefly, DNAs were first conjugated with *S*-acetyl NHS-MAG_3 via the 3′-derivatized amine in HEPES buffer (pH = 8.0). The reaction mixtures were then incubated at room temperature for 2 h before purification on a 0.7 × 20 cm P4 column with 0.1 M PBS as eluant. The peak fractions were collected and quantitated by UV spectrophotometry. For radiolabeling, 10 μL of ^{99m}Tc-pertechnetate generator elute was introduced into a solution consisting of 20 μL (7 μg) of MAG3-DNA in PBS, 15 μL of 50 μg/μL sodium tartrate in a buffer (pH = 9.2), and 4 μL of 5 μg/μL $SnCl_2 \cdot 2H_2O$ in ascorbate-HCl solution. The solution was heated to 100 °C for 20 min. The labeling efficiency was determined by size exclusion radio-HPLC.

Characteristics of Polymers. Size-exclusion high-performance liquid chromatography was performed on a Superose 12 column (HR10/30; Amersham Pharmacia Biotech) with 0.10 mol/L phosphate buffer (pH = 7.0) as eluent at a flow rate of 0.8 mL/min. A radioactivity detector (Flow-Count, Bioscan, DC) was used for radiochemical purity test and binding efficiency measurement. Each reaction sample and a mixture of DNA markers were loaded onto a 6% polyacrylamide gel in 0.5× TBE (45 mmol/L Tris borate, 1 mmol/L EDTA) and electrophoresed at 100 V for 2 h, then developed using BIO-RAD gel imaging system (Gel Doc XR+, MA).

Signal Amplification on Plates. Measurement was performed on a protein L coated 96-well flat-bottomed plate using a polymer prepared at a 1:1 bridging/target strand ratio. Five different studies, consisting of three controls (1, 3, and 4), a conventional pretargeting (2), and an amplification pretargeting (5) were performed in six wells. Figure 5 lists the detailed additions for each study. Wells for studies 1 and 4 received only PBS, while wells for studies 2, 3, and 5 each received 0.33 μg of CC49-MORF in 100 μL. The plate was then incubated for 1 h at room temperature before rinsing with 3 × 200 μL washing buffer. Thereafter, 50 μL of a polymer solution was added to wells for studies 4 and 5. Fifty microliters of PBS was added to other studies. The plate was incubated for an additional 1 h at room temperature before rinsing with 3 × 200 μL of washing buffer. Next, 50 μL of ^{99m}Tc-cDNA was added to wells for studies 1 and 2. The sequence of the ^{99m}Tc-cDNA was complementary to the MORF antibody target. Finally, wells for studies 3, 4, and 5 received 50 μL of ^{99m}Tc-AS DNA. After an additional 1 h of incubation at room temperature, the wells were rinsed again with 3 × 200 μL of washing buffer. The radioactivity bound to the plate was removed with 2 × 200 μL of 1% sodium dodecyl sulfate (SDS) in 0.2 M NaOH and 200 μL of 2 M hydrochloride for counting in a Na(Tl) well counter.

Signal Amplification on Cells. Cells were suspended in minimum essential medium (MEM) with 10% FBS and were seeded in 24-well plates at 1 mL/well. The cells were used when 80% confluence was reached, and the medium was changed to MEM with 1% FBS. A 4% milk blocking buffer was added to all wells, and then incubated for 1 h at 37 °C before rinsing with 3 × 0.5 mL of MEM with 1% FBS. Thereafter, wells for studies 1 and 4 received only PBS, while wells for studies 2, 3, and 5 each received 0.33 μg of CC49-MORF in 50 μL. Figure 6 lists the additions for each study. The plate was then incubated for 1 h at 37 °C before rinsing with 3 × 0.5 mL of MEM with 1% FBS. Then 50 μL of polymer solution were added to wells for studies 4 and 5, while 50 μL of PBS was added to the remaining wells. The plate was incubated for 1 h at 37 °C before rinsing with 3 × 0.5 mL of MEM with 1% FBS. To studies 1 and 2, 100 μL of ^{99m}Tc-cDNA were added. Wells for studies 3, 4, and 5 received 100 μL of ^{99m}Tc-AS DNA complementary to the polymer antibody target. After an additional 30 min of incubation at 37 °C, the cell medium was then carefully removed, and the cells were rinsed with 3 × 0.5 mL of cold PBS. The cells were then lysed with 1% SDS in 0.2 M NaOH, and the radioactivity in the medium and cells was measured in the well gamma counter.

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Author Contributions

⊥These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This study is supported in part by The National Nature Science Foundation of China (81201130 and 81271608), Shanghai Pujiang Programme (13PJ1401400), and Shanghai Municipal Commission of Health and Family Planning (XYQ2013106).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Most spectroscopic analysis of new molecules was conducted in the Small Molecule NMR Facility and Mass Spectrometry Research Center at the Vanderbilt Institute of Chemical Biology. Electrophoretic mobility shift assay was conducted in the Laboratory of Physical Biology at the Shanghai Institute of Applied Physics. The authors would like to thank professor Donald Hnatowich (University of Massachusetts Medical School) who contributed efficiently to the study design and the manuscript editing.

ABBREVIATIONS

PS-DNA, phosphorothioate DNA; PO-DNA, phosphodiester DNA; NHS-MAG3, mercapto-acetylglycyl-glycyl-glycine; SDS, sodium dodecyl sulfate; MEM, minimum essential medium

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DOI: 10.1021/acsmedchemlett.5b00265
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