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Affinity Enhancement Pretargeting: Synthesis and Testing of a ^{99m}Tc-Labeled Bivalent MORF

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Abstract: Pretargeting with bivalent effectors capable of bridging antitumor antibodies (affinity enhancement pretargeting) has been reported to provide superior results by affinity enhancement. Phosphorodiamidate morpholinos (MORFs) and other DNA analogues used for pretargeting are ideally suited as bivalent effectors since they are easily synthesized and the distance between binding regions, a determinant of binding, may be adjusted simply by lengthening the chain. We have shown by surface plasmon resonance that bivalent MORFs will provide superior affinity enhancement provided that suitable spacing exists between the binding regions. The goals of this study were to synthesize a bivalent MORF with a MAG_3 group attached for technetium-99m (^{99m}Tc) radiolabeling, investigate whether the bivalent MORF showed improved cell accumulation in culture compared to its corresponding monovalent MORF and compare biodistributions in normal mice and in pretargeted tumored mice. An excess of an amine derivatized 18 mer MORF with 6 nonbinding bases for spacing was reacted with Fmoc-L-β-homoglutamic acid to form duplexes via their carboxylate groups and, after deprotection, conjugated with NHS-MAG_3 to attach the chelator. The anti-CEA antibody MN14 was conjugated with a 12 mer complementary MORF (i.e., cMORF). The binding behavior between radiolabeled monovalent and bivalent MORFs was compared in LS174T tumor cells at 4 °C pretargeted with MN14−cMORF. Biodistributions of radiolabeled monovalent and bivalent MORFs at 3 h postadministration were measured in normal mice and in tumor mice pretargeted with MN14−cMORF. In the pretargeted cells in culture, the accumulation of the bivalent MORF was significantly higher than the monovalent MORF ($p = 0.002$), thus providing strong evidence for affinity enhancement. In normal mice, whole body clearance of the bivalent and monovalent MORFs was equally rapid. In tumored mice, tumor accumulation of the radiolabeled bivalent MORF was significantly higher than that of the monovalent MORF. In conclusion, a bivalent MAG_3−MORF was successfully synthesized and radiolabeled with ^{99m}Tc. While a pharmacokinetic effect for the higher tumor accumulations in pretargeted mice of the radiolabeled bivalent MORF cannot be excluded, the results may be best explained by affinity enhancement. Thus two monovalent MORFs were covalently conjugated into a bivalent MORF effector to improve tumor targeting by both pharmacokinetics and affinity enhancement influences.

Keywords: Pretargeting; affinity enhancement; bivalent; morpholino; surface plasmon resonance

Introduction

Pretargeting of tumor is becoming a mature, reasonably well understood and successful strategy for radioimmunodiagnosis and therapy that includes systems involving strepavidin/biotin, bispecific antibody/haptens, and recently oligonucleotides and their complements.[1−3] The advantage of pretargeting is usually not in higher absolute tumor

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accumulation but in higher tumor/normal tissue ratios achieved rapidly.[4] The target/nontarget contrast in pretargeting could be further improved by affinity enhancement with bivalent effectors.[5]

Affinity enhancement was first introduced to explain improved tumor accumulations with radiolabeled bivalent effectors capable of bridging the pretargeting antibody at the tumor site.[6] Subsequent studies have confirmed that use of a bivalent effector can improve tumor pretargeting by what has been referred to as the affinity enhancement system.[5] The enhancement is explained by assuming increased affinity of a bivalent compared to a monovalent effector because of the ability only of the former to bridge two antibodies on the tumor cell surface. At the same time, untethered free antibody, such as that in circulation, is said to be unable to participate in bivalent binding at least to the same degree.[7] The result is thought to be preferential binding to tumor. The bivalent effectors used successfully in bispecific antibody pretargeting include Ac-Lys(In-DTPA)-Tyr-Lys(In-DTPA)-Lys(TscG-Cys-)-NH_2,[7,8] Janus bivalent hapten,[9] pGlu-Leu-Tyr-Glu-Asn-Lys(DTPA)-Pro-Arg-Arg-Pro-Tyr-Ile-Leu and DTPA-Gly-Glu-Leu-Tyr-Glu-Asn-Lys(Ac)-Pro-Arg-Arg-Pro-Tyr-Ile-Leu (DTPA-Gly-NT);[10] and Ac-Phe-Lys(-DTPA)-Tyr-Lys(DTPA)-NH_2[11] and have been variously radiolabled with ^{99m}Tc, ^{188}Re, ^{125}I, ^{131}I, ^{111}In and most recently ^{68}Ga for radioimmunodetection and radiotherapy of tumor models.[12−15] One important observation from these investigations has been the importance of molecular spacing between binding sites. This laboratory has previously reported encouraging results in tumored mice with pretargeting using monovalent DNA and its analogues (collectively: oligomers) in which a radiolabeled phosphorodiamide morpholino (MORF) effector is administered subsequent to the administration of an antitumor antibody conjugated with the complementary MORF (cMORF) as the first injection.[16−19]

One advantage of using oligomers for affinity enhancement pretargeting is the ease with which bivalent effectors may be constructed and the ease with which the molecular dimension separating the binding sites may be shortened or

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elongated. In previous studies,[20,21] we reported significant affinity enhancement of a bivalent MORF over its monovalent equivalent by surface plasmon resonance (SPR). The bivalent MORFs used in those investigations were not derivatized with a chelator and therefore were incapable of being radiolabeled. We now describe the successful synthesis of a bivalent morpholino derivatized with MAG_3 for ^{99m}Tc radiolabeling and its evaluations in vitro in cell binding studies and in vivo with normal mice and pretargeted tumored mice.

An 18 mer MORF oligomer was selected to construct the bivalent MORF for affinity enhancement in this study based on the our previous SPR results that showed the largest affinity enhancement (i.e., ratio of affinity constants of the bivalent over the monovalent MORF) of almost 3 orders of magnitude for an 18 mer bivalent MORF hybridizing to a 12 mer cMORF presumably because use of a shorter oligomer for hybridization lowers the equilibrium constant for monomolecular binding. Furthermore, after bridging two MORF conjugated antibodies, the bivalent MORF will have a flexible single strand six bases long on either side furthest from the antibodies that should favor bimolecular binding:

5'-TAGTTGTGAAGTAGAAGA-HGA-AGAAGATGAAGTGTTGAT-5'

Ab-linker-ATCAACACTTCA-5' 5'-ACTTCACAACTA-linker-Ab

Experimental Procedures

Materials and Methods. The 18 mer MORF and 12 mer cMORF were obtained with a $-C(O)-CH_2-CH_2-NH_2$ linker on the 3′ equivalent end (Gene-Tools, Corvallis, OR) and were used as received. The 18 mer MORF (5′-TAGTTGTGAAGTAGAAGA-linker-amine) and the 12 mer cMORF (5′-ACTTCACAACTA-linker-amine) were both used by us previously in pretargeting studies.[20,21] The 12 mer cMORF and its 12 mer MORF complement were also obtained with a $-C(O)-CH_2-CH_2$-biotin linker on the 3′ equivalent end for the SPR measurements. The tBu ester of Fmoc-L-β-homoglutamic acid (Fmoc-HGA-OtBu) was purchased from New England Peptides, Inc. (Gardner, MA). Anhydrous dimethylformamide (DMF), *N*-methyl-2-pyrrodinone (NMP), hydroxy-7-azabenzotriazole (HOAt), *N*,*N*′-dicyclohexylcarbodiimide (DCC) and diisopropylethylamine (DIEA) were from Sigma-Aldrich (St. Louis, MO). The *S*-acetyl NHS-MAG_3 was synthesized in house[22] and the structure confirmed by elemental analysis, proton NMR and mass spectroscopy. All other chemicals were reagent grade and were used without purification. The LS174T (CEA+) human colorectal cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). The high affinity murine anti-CEA antibody MN14 (IgG 1 subtype, MW 160 kDa) was a gift from Immunomedics (Morris Plains, NJ).

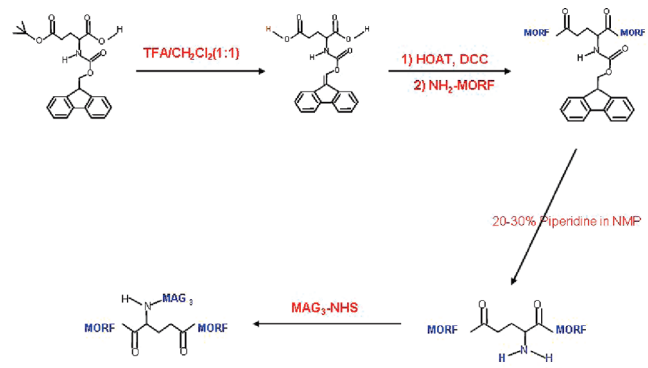

Figure 1. Synthesis of MAG_3 conjugated bivalent MORF.

Chromatography. Ion exchange chromatography was used to purify the bivalent from unreacted monovalent MORF as previously described[20] taking advantage of the negative charge at pH 12. Ion exchange (IE) HPLC was performed using a HiTrapTM Q HP column (Amersham Biosciences, Uppsala, Sweden) with 0.01 N NaOH (solvent A) and 0.01 N NaOH in 2 N NaCl (solvent B) at a flow rate of 1.0 mL/min and going from 100% A to 70% A over 30 min. Before each analysis, the column was stabilized with 0.01 N NaOH and after each analysis the column was cleaned with solvent B. For small scale preparative purifications, samples were loaded on the column by several injections in running solvent A but without exceeding the column's maximum binding capability. The bivalent MORF was collected in fractions during the gradient elution.

Size-exclusion (SE) HPLC analysis was performed on a Superose-12 HR10/30 column with an optimal separation range between 1×10^3 and 3×10^5 Da (Amersham Pharmacia Biotech, Piscataway, NJ) using 0.10 M pH 7.2 phosphate buffer as eluant at a flow rate of 0.6 mL/min. The HPLC was equipped with both an in-line radioactivity detector and a Waters 2487 dual wavelength absorbance detector (Milford, MA). Recovery of radioactivity was routinely measured and was always greater than 90%.

Synthesis of Bivalent MORF−MAG_3. The synthesis of the monovalent MORF−MAG_3 and radiolabeling with ^{99m}Tc was as previously described.[23]

The synthesis of the bivalent MORF−MAG_3 is presented schematically in Figure 1. The tBu ester of Fmoc-L-β-homoglutamic acid (OtBu) (i.e., Fmoc-HGA-OtBu) was first hydrolyzed in TFA/CH_2Cl_2 (1:1) for 2.5 h. The solvent was removed under reduced pressure and the deprotected product

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dried by Speedvac SC110 (Savant Instruments, Inc., Farmingdale, NY). The crude brown residue (Fmoc-HGA) was dissolved in NMP at 1.0 mg/mL and neutralized by adding DIEA. Both free carboxylate groups were then activated and each conjugated to the free amino group on the MORF in the presence of DCC and HOAT. This reaction mixture was passed through the PD-10 column using water as eluant to separate the MORF species from small molecules. The mixed MORF fractions were pooled and adjusted to pH 12, and the bivalent MORF ($(MORF)_2$-Fmoc-HGA) was separated from monovalent forms (MORF and MORF−Fmoc-HGA) by IE HPLC. The bivalent fractions were pooled and passed through PD-10 again to exchange the solvent into water, and the product was dried again. The clear bivalent intermediate was dissolved in NMP and the Fmoc group was removed in 50% piperidine. After deprotection, the mixture was passed through PD-10 again using water as eluant to collect the bivalent MORF with a free amine group for conjugation with NHS-MAG_3. After drying again, the bivalent product was characterized by MALDI TOF MS for molecular weight and SPR for affinity enhancement. To permit radiolabeling, the product was dissolved in HEPES pH 8.0 and reacted with NHS-MAG_3 followed by purification via PD-10 in pH 5.2 NH_4OAc buffer. The peak fractions were pooled and adjusted from pH 5.2 to pH 7.6 with a pH 9.2 buffer (0.5 M Na_2HCO_3, 0.25 M NH_4OAc, 0.175 M NH_3). After heating for 20 min, the solution was again purified via PD-10 using the pH 5.2 NH_4OAc buffer as eluant.[16] The peak fractions were pooled as before, and the concentration was quantitated by UV absorbance at 265 nm.

Surface Plasmon Resonance. Surface plasmon resonance was performed on a BIAcore 2000 (BIAcore, Piscataway, NJ) instrument operating at room temperature as previously described.[21] As before, the biotinylated 12 mer cMORF and its 12 mer MORF complement were added at 20 nM in 5−10 μL aliquots to the first and second flow cells respectively on a fresh streptavidin dextran coated SA sensor chip at a flow rate of 20 μL/min only until a response of about 100 (±10) RUs was reached. The absence of mass transfer effects was confirmed by running separately one concentration of 18 mer MORF at three different flow rates (10, 30, and 75 μL/min) and demonstrating identical response and curve shape for all three sensorgrams. Solutions of the amine derivatized bivalent MORF were prepared at 5−100 nM in the same running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM Na_2EDTA, 0.005% P20, pH 7.4) and injected over the active (biotinylated cMORF) or control (biotinylated MORF) surfaces at a flow rate of 30 μL/min. The chip surface was regenerated with 100 mM HCl. To correct for nonspecific binding and refractive index changes, the responses from the control surface were subtracted from those obtained from the active surface. A minor baseline drift resulting from a slow dissociation of the complex on the active and control surfaces was eliminated by also subtracting responses obtained following the injection of running buffer alone.[24]

Radiolabeling of Bivalent MORF−MAG_3. The radiolabeling of both the bivalent and monovalent MORFs was achieved as is routine in this laboratory.[23] The ^{99m}Tc-pertechnetate generator eluate (5 mCi, 50 μL) was added to a combined solution of MAG_3-conjugated bivalent MORF (50 μL, 3.0 μg) in the pH 5.2 NH_4OAc buffer (10 μL of 50 μg/μL Na_2tartrate·$2H_2O$ in the pH 9.2 buffer, and 3 μL of 4 μg/μL $SnCl_2$·$2H_2O$ in 1 μg/μL sodium ascorbate in 10 mM HCl). The final pH was 7.8. After vortexing and then heating for 20 min in boiling water, the radiolabeled cMORF was purified by SE HPLC with 50 mM pH 7.2 phosphate buffer as eluant and the fractions between 26−27 min were pooled. The quality of the radiolabeled bivalent MORF was assured by the SE HPLC radioactivity profile of the purified product that showed only a single peak.

MN14−cMORF. The MN14−cMORF was synthesized using a commercial Hydralink kit (Solulink Biosciences, San Diego, CA). The MN14 was first conjugated with succinimidyl 4-hydrazinonicotinate acetone hydrazone while the 12 mer cMORF was conjugated with succinimidyl 4-formylbenzoate according to the instructions of the manufacturer. After purification, combining hydrazine modified MN14 and benzaldehyde modified cMORF resulted in hydrazone formation as previously described.[25] The average groups per molecule of MN14−cMORF was 1.2 when measured as previously described.[25]

Tumor Cell Pretargeting Studies. The LS174T cells were grown in RPMI medium, supplemented with FCS and penicillin/streptomycin (Life Technologies, Grand Island, NY). For cell binding studies, the tumor cells were washed with 0.9% NaCl, suspended first in 0.25% trypsin and resuspended in PBS to a concentration of 10^6 cells/100 μL. Four groups were investigated in quadruplicate. The two study groups received 30 μg of MN14−cMORF followed 1 h later by either 1.0 μg of ^{99m}Tc radiolabeled bivalent MORF or 0.5 μg of monovalent MORF (and therefore roughly equimolar in MORF) while the two control groups did not receive the MN14−cMORF but were otherwise treated identically. To avoid any complications due to internalization, incubations were at 4 °C and for only 1 h before the cells were harvested and counted for radioactivity in a NaI(Tl) well counter (Cobra II automatic gamma counter, Packard Instrument Company, CT). In both the bivalent and monovalent MORF cell studies, a correction was applied for nonspecific binding by subtracting counts from cells not

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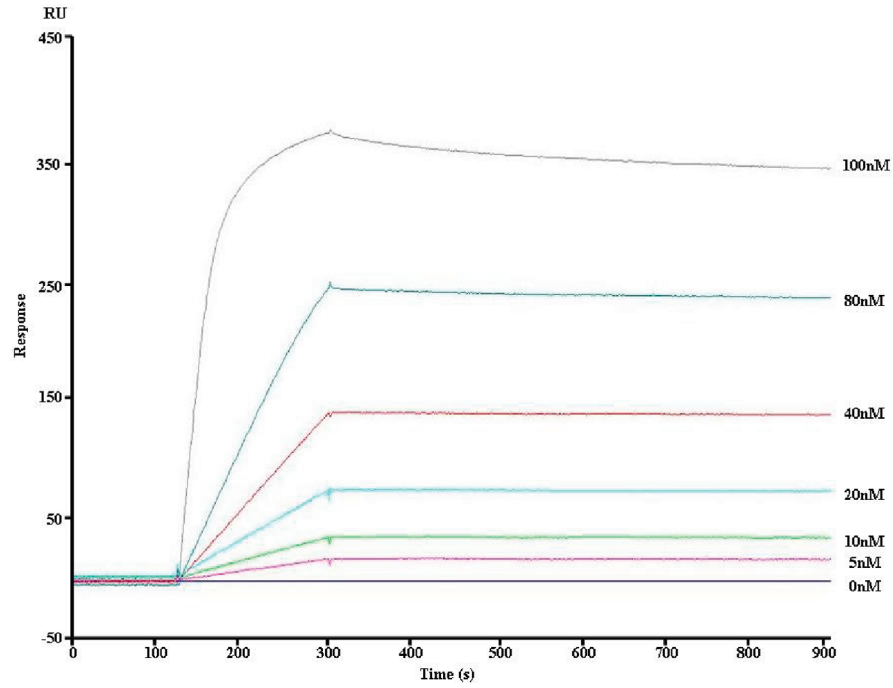

Figure 2. Surface plasmon resonance sensorgrams showing rapid association and slow dissociation obtained by passing the bivalent MORF over a cMORF coated cell surface at different concentrations.

receiving the antibody but incubated identically with the bivalent or monovalent MORF respectively.

Animal Studies. *Biodistribution in Normal Mice.* All animal studies were performed with the approval of the UMMS Institutional Animal Care and Use Committee. Eight normal CD-1 male mice were injected via a tail vein with 0.1 mL of normal saline containing about 0.1 μg (20 μCi) of either ^{99m}Tc radiolabeled monovalent or bivalent MORF. Animals were sacrificed at 3 h. Major organs and blood were removed, weighed and the radioactivity measured in a NaI (Tl) well counter. The biodistributions are reported as the percentage of the injected dose per gram (% ID/g) of tissue, and values are corrected for background radioactivity, physical decay during counting and for retention of radioactivity in the tail. Tissues were always counted against a standard of the injectate. The biodistribution of the bivalent MORF was compared to the monovalent MORF.

Biodistribution in Tumor Bearing Mice. For tumor induction, 10^6 LS174T colon cancer cells were implanted into the left thigh of each Swiss NIH nude mouse (Taconic Farms, Germantown, NY). Half the animals each received 30 μg of MN14−cMORF via a tail vein on the 12th day when tumors were about 1 g. Three days later, all mice received about 20 μCi of ^{99m}Tc on either 0.5 μg of monovalent or 1.0 μg of bivalent MORF (and therefore equimolar with respect to MORF) via a tail vein. Biodistribution was determined 3 h later. The tumor-bearing thigh was also excised for counting but after the skin and as much as possible of the muscle and bone had been removed. The radioactivity was attributed to the tumor since the radioactivity in bone and muscle was negligible. Thereafter the tumor was dissected away so that the remaining bone and muscle could be weighed, and this weight was subtracted to provide the net tumor weight used in the calculation of tumor accumulation. Radioactivity remaining in the carcass was measured in a dose calibrator. Summation of radioactivity in all organs sampled and in the remaining carcass was taken as the whole-body radioactivity.

Results

Synthesis and Radiolabeling of Bivalent MORF. The molecular weights of both the intermediate bivalent MORF before MAG_3 conjugation and the final MAG_3 conjugated bivalent MORF were confirmed by MALDI-TOF as 12926 $\pm$ 29 (calculated: 12955) and 13150 $\pm$ 33 (calculated: 13183) daltons respectively. A high affinity of this bivalent MORF for its complement (K_{eq}: 5.21 $\times$ 10^9 M^{-1}) was confirmed by SPR. Figure 2 shows slow release of the bivalent MORF compared to the monovalent MORF similar to that previously reported for a different bivalent effector.[21] The labeling efficiency of monovalent MORF was greater than 90% while that of bivalent MORF was 50−70%, but the radiochemical purity of both was greater than 90% after purification by SE HPLC. Both radiochromatography profiles of the ^{99m}Tc-bivalent MORF and the ^{99m}Tc monovalent MORF showed essentially a single peak with the bivalent MORF appearing at 26 min and therefore slightly before that of the monovalent MORF at 28 min.

Tumor Cell Pretargeting Studies. The two study groups received 30 μg of MN14−cMORF followed 1 h later by either 1.0 μg of radiolabeled bivalent MORF or 0.5 μg of monovalent MORF and were incubated at 4 °C for 1 h. Cell counts were corrected for nonspecific binding by counting the control cells that did not receive the antibody. Cell binding of the bivalent MORF was significantly higher than that of monovalent MORF (p = 0.0002) at 23 $\pm$ 3% vs 15 $\pm$ 2% (Figure 3).

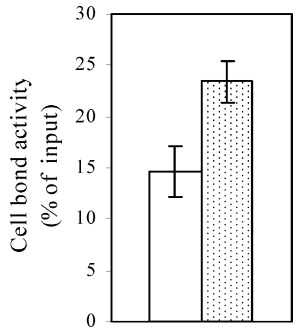

Figure 3. Accumulations at 4 °C in LS174T cells pretargeted with MN14−cMORF after 1 h of incubation with ^{99m}Tc labeled bivalent MORF (closed bar) or monovalent MORF (opened bar). Error bars signify one standard deviation of the mean. ($N = 3$)

Table 1. The Biodistributions of ^{99m}Tc Labeled Monovalent and Bivalent MORFs in Normal Mice at 3 h Postadministration, Presented As Percent Injected Dose per Gram, Mean and 1 sd ($N = 4$)

	monovalent		bivalent		
organ	mean	sd	mean	sd	*p* value[a]
liver	0.37	0.07	0.41	0.14	ns
heart	0.05	0.00	0.12	0.03	0.005
kidney	2.11	0.24	5.55	1.61	0.01
lung	0.16	0.01	0.28	0.02	0.001
spleen	0.12	0.04	0.22	0.07	ns
muscle	0.08	0.03	0.09	0.02	ns
blood	0.11	0.01	0.25	0.11	0.01

[a] Student's *t* test; NS = Not Significant ($P < 0.05$).

Animal Studies. The biodistributions of the ^{99m}Tc radiolabeled bivalent and monovalent MORFs in normal mice are presented in Table 1. The results show slightly higher radioactivity levels for the bivalent MORF in most tissues but rapid blood clearance in both cases. For example, assuming that blood constitutes 7% of body weight, only 0.9% of the injected radiolabeled bivalent MORF was in circulation at 3 h compared to 0.3% for the monvalent MORF.

The pretargeting results in tumor bearing nude mice of the radiolabeled bivalent and monovalent MORFs are shown in Table 2. Compared to normal mice, the blood level for the bivalent MORF in tumor mice has increased almost 10-fold compared to only 7-fold for the monovalent MORF. However, the increase in accumulation in tumored compared to normal mice in other normal organs/tissues between both MORFs is less striking. For example, the increase in liver accumulation is roughly equal between MORFs and about 3-fold. As is common in pretargeting studies, unless a clearing step is used, the background radioactivity levels are generally higher in animals previously receiving the antibody, even 3 days earlier as in this case, due to binding to residual antibody in blood and tissues. As in the normal mice (Table 1), the radioactivity accumulation in most tissues was higher for the bivalent MORF. The tumor/normal tissue ratios are moderately favorable for the bivalent in most tissues. As shown, tumor levels were also significantly higher for the bivalent MORF.

Table 2. The Biodistribution at 3 h of ^{99m}Tc Labeled Monovalent and Bivalent MORFs in LS174T Tumor Bearing Nude Mice Pretargeted with MN14−cMORF 3 Days Earlier, Presented as Percent Injected Dose per Gram, Mean and 1 sd ($N = 4$)[a]

	monovalent			bivalent			
organ	mean	sd	T/NT	mean	sd	T/NT	*p* value[b]
liver	1.46	0.13	1.13	1.35	0.12	2.70	ns
heart	0.24	0.03	6.88	0.60	0.19	6.07	0.01
kidney	3.12	0.21	0.53	6.34	1.31	0.57	0.003
lung	0.47	0.06	3.51	0.87	0.14	4.18	0.002
spleen	1.00	0.20	1.65	0.73	0.07	4.99	ns
muscle	0.14	0.02	11.8	0.30	0.02	12.1	0.000
tumor	1.65	0.40		3.64	0.77		0.0004
blood	0.78	0.15	2.36	2.32	0.31	1.57	0.000

[a] Also presented are the tumor/normal tissue ratios (T/NT). [b] Student's *t* test, NS = Not Significant ($P < 0.05$).

Discussion

The promise of affinity enhancement pretargeting is the combination of improved target/nontarget ratios by conventional pretargeting with the higher binding affinities provided by affinity enhancement. We previously confirmed by SPR that the phenomenon of affinity enhancement is real and can be achieved with bivalent MORF effectors.[20,21] We have also demonstrated with MORF effectors that MORFs with different molecular spacings may be readily synthesized and that spacing is a critical variable.[21] The objective of this investigation was to synthesize a bivalent MORF capable of being radiolabeled and compare its properties to the radiolabeled monovalent version in cell culture and in normal and pretargeted tumored mice.

In this investigation, our standard 18 mer MORF sequence was again used, now for the synthesis of a bivalent MORF capable of chelating ^{99m}Tc and capable of hybridizing with a 12 mer cMORF conjugated to the monoclonal antibody MN14. The 12 mer cMORF chain length was selected based on our previous SPR results showing the largest affinity enhancement (i.e., ratio of affinity constants of the bivalent over the monovalent MORF) of almost 3 orders of magnitude for an 18 mer bivalent MORF hybridizing with a 12 mer cMORF presumably because the shorter cMORF lowered the equilibrium constant for monomolecular binding. The SPR results also showed that using bivalent effectors can greatly improve MORF pretargeting and that bivalent MORFs with reduced equilibrium constants may actually provide higher affinity enhancement.[20,21] The cMORF sequence was complementary to only 12 of the 18 bases of MORF. Thus after bridging two cMORF conjugated antibodies, the bivalent MORF will have a flexible single strand six bases long on either side furthest from the antibodies that should favor bimolecular binding.

To facilitate the synthesis of a MAG_3 conjugated bivalent MORF, the Fmoc-HGA-OtBu with two carboxylate groups

and one protected amine was selected. After deprotection with TFA, the tBu protected carboxylate groups were readily conjugated with the amine derivatized MORFs without affecting the Fmoc protected amine that was subsequently deprotected before reacting with MAG_3-NHS.

Table 2 presents the results of the tumor animal pretargeting study and shows that tumor accumulation was significantly higher in animals receiving the same MORF dosage as the bivalent compared to the monovalent MORF effector. However, a fraction of the higher tumor accumulation may be the result of pharmacokinetic influences since the accumulation of the bivalent MORF in muscle and blood was also higher than that of the monovalent MORF both in normal mice (Table 1) and in tumored mice (Table 2). The differences in size and/or base sequence between the two MORFs may explain the observed differences in pharmacokinetics. Nevertheless, the tumor to normal tissue ratios are moderately in favor of the bivalent MORF as evidence of affinity enhancement pretargeting. The evidence for affinity enhancement is more definitive in the cell binding studies shown in Figure 2 where the radiolabeled bivalent MORF showed enhanced tumor cell binding compared to the monovalent MORF added at the same MORF dosage.

It is important to emphasize that the tumored mouse pretargeting study of this investigation was intentionally designed with a dosage of the radiolabeled bivalent MORF effector much lower than that required to saturate the accessible cMORF in tumor and therefore much lower than optimum.[23] In principle, the pretargeting results for the bivalent compared to the monovalent MORFs should have been compared with both at their optimum dosages (i.e., at their maximum percent tumor accumulations).[4]

The blood accumulations suggest that the lower binding affinity of bivalent effectors to the untethered MN14−cMORF in circulation may not have been observed in this case as was expected.[7] The large difference in blood levels between the bivalent and monovalent MORFs in the tumored animals with circulating MN14−cMORF (2.38% vs 0.78%) would be expected to be smaller than the difference in the normal mice without circulating MN14−cMORF (0.25% vs 0.11%). Instead the opposite was observed. Although different pharmacokinetics certainly contributed, the cell binding results suggest that affinity enhancement is a likely explanation for the higher tumor uptake for bivalent MORF in vivo.

As previously reported, there are many factors such as target density that contribute to affinity enhancement.[21] While our SPR experiments have provided clear evidence of affinity enhancement, unfortunately, the cMORF target density that is easily defined and controlled on SPR microchips cannot be accurately defined or reproduced in living cells in vitro and even less so in xenografts in which pharmacokinetic factors are involved. Thus while the dosage of MN14−cMORF may be a critical parameter since this can define the concentration of the available cMORF in the tumor, it may be difficult or even impossible to predict the number and spacing of the pretargeting antibody following administration. Accordingly, affinity enhancement may prove to be of more value in connection with amplification pretargeting in which polymeric cMORF as the pretargeted entity is used since, in that case, the number and spacing of the cMORFs on the polymer can be more easily controlled especially in patients.

In conclusion, a bivalent MAG_3−MORF was successfully synthesized and radiolabeled with ^{99m}Tc. The bivalent MORF behaved similarly in vivo to the monovalent MORF in that both showed the rapid clearance required for pretargeting applications. Although it is not possible to attribute the higher tumor accumulation in mice entirely to affinity enhancement because of differences in the pharmacokinetics between the bivalent and monovalent MORF, both the in vitro and in vivo studies showed evidence of improved binding of the bivalent compared to the monovalent MORF certainly in part due to affinity enhancement. As an obvious advantage of affinity enhancement pretargeting with oligomers, the sequences and length of the MORF of this investigation can be optimized to minimize unfavorable pharmacokinetics and to more definitively favor affinity enhancement pretargeting. Thus bivalent MORFs merit further attention as radiolabeled effectors in MORF pretargeting studies.

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