In Vivo Delivery of Antisense MORF Oligomer by MORF/Carrier Streptavidin Nanoparticles

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Abstract

Tumor targeting by oligomers is largely limited by the pharmacokinetics and cell-membrane transport obstacles. In this article, we describe the use of a delivery nanoparticle, in which streptavidin served as a convenient bridge between a biotinylated oligomer and a biotinylated cell-membrane-penetrating peptide, to improve the delivery of an antisense phosphorodiamidate morpholino (MORF) oligomer *in vivo*. A biotinylated ^{99m}Tc-radiolabeled MORF oligomer with a base sequence antisense to the RIZ mRNA and its sense control were incorporated separately into nanoparticles, along with biotinylated tat or polyarginine carrier. The streptavidin nanoparticles were administered intravenously to both normal and nude mice bearing SUM149 breast tumor xenografts. The biodistributions showed much higher normal tissue levels for the radiolabeled MORFs, independent of antisense or sense or tat or polyarginine, when administered as the nanoparticles, compared to naked. A statistically significant higher accumulation of both antisense nanoparticles, compared to the respective sense control nanoparticles, was observed, along with much higher tumor accumulations, compared to historical naked controls. This study has provided evidence that the *in vivo* function of an antisense oligomer within the streptavidin nanoparticle is not impeded, and, as such, the MORF/streptavidin/carrier nanoparticles may be suitable for *in vivo* tumor delivery of antisense MORF and other oligomers.

Key words: antisense, nanoparticle, streptavidin, MORF

Introduction

TUMOR TARGETING by antisense oligomers is largely limited to *in vitro* studies due to the pharmacokinetics and cell-membrane transport obstacles of *in vivo* delivery.^{1,2} Our laboratories are developing a nanoparticle designed to improve the delivery of labeled antisense oligomers into tumor tissues *in vivo*. The nanoparticle relies upon streptavidin as a convenient method of binding a biotinylated oligomer to a biotinylated carrier. Recent investigations in our laboratories have demonstrated that the nanoparticles with equimolar constituents can be prepared quickly by simple mixing, and they are suitably stable for both *in vitro* and *in vivo* uses. The delivery nanoparticles appear to be nontoxic, and *in vitro* studies, thus far, have demonstrated no evidence that the functions of the antisense oligomer, the transmembrane car-

rier, and, in the case of the three-component nanoparticle, the antitumor antibody, have been adversely influenced by the presence of the streptavidin.^{3–5} In this article, we report on a further investigation of the nanoparticle properties, in this case their biodistributions in mice. An earlier report from these laboratories on the pharmacokinetics of radiolabeled DNAs, when intravenously (i.v.) administered as nanoparticles, have shown higher liver and lower kidney radioactivity levels for phosphodiester DNA and comparable tissue accumulation levels for phosphorothioate DNA, compared to their respective naked forms.⁵ Since the phosphorodiamidate morpholino (MORF) oligomer exhibits lower nonspecific protein binding, compared to the phosphorothioate DNA,^{6,7} and lower enzymatic instability than both the phosphorothioate and phosphodiester DNAs,^{8–11} it may be a better candidate for *in vivo* antisense tumor targeting.^{12,13} Therefore, it was of interest to

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measure the biodistributions of the nanoparticle with MORF replacing the DNAs.

Materials and Methods

The base sequence for the antisense MORF was 5'-GCGTGCCTCCTCACTGGC-3', targeting the NH₂-terminal 8–13 codons of human protein kinase (PKA).¹⁴ The oligomers were commercially obtained (Gene Tools, Philomath, OR) with a biotin on the 3'-end through an aminohexanoic acid as the linker and a primary amine on the 5'-end through a 3-aminopropionic acid as the linker. N-hydroxysuccinimidyl S-acetylmercaptoacetyltriglycinate (NHS-MAG3) was synthesized in-house and characterized by nuclear magnetic resonance (NMR), mass spectrometry (MS), and element analysis.^{15,16} The tat (G-R-K-K-R-R-Q-R-R) and polyarginine (poly R) peptide were both 10 mer and were purchased as high-performance liquid chromatography (HPLC)-purified native L isomers with a biotin group attached to the N-terminal also via an aminohexanoic acid linker (21st Century Biochemicals, Marlboro, MA). The human inflammatory breast cancer cell line, SUM149, was obtained from Asterand (Asterand, Inc., Detroit, MI) and maintained and cultured in Ham's F-12 medium supplied with 5 μ g/mL of insulin, 1 μ g/mL of hydrocortisone, and 5% fetal bovine serum (FBS), according to the supplier's instructions. The ^{99m}Tc-pertechnetate was eluted from a ⁹⁹Mo–^{99m}Tc generator (Bristol-Myers Squibb Medical Imaging, Inc., North Billerica, MA). Other chemicals were purchased as analytical reagent grade or above from various suppliers and used without further purification.

Oligomer conjugation and radiolabeling

Both the antisense and sense MORF oligomers were conjugated with NHS-MAG3 via the primary amine group on the 5'-end, as previously described.¹⁷ The NHS-MAG3 has been extensively used in our laboratories to introduce this chelator into biomolecules for radiolabeling with ^{99m}Tc.¹⁷ Radiochemical purity was evaluated by size-exclusion HPLC on a Superose 12 (Amersham Pharmacia Biotech, Piscataway, NJ) installed on a Waters 515 solvent delivery system (Waters, Milford, MA), equipped with an in-line radioactivity detector and a Waters UV2487 dual-wavelength absorbance detector. The running solution was 20% acetonitrile in 0.1 M of TRIS-HCl (pH 8.0) at a flow rate of 0.6 mL/min. Radioactivity recoveries were routinely measured in all HPLC analyses and were, in all cases, 90% or better.

Nanoparticle preparation

All additions to streptavidin of biotinylated MORF and peptides were equimolar with respect to streptavidin. The preparation and quality control of ^{99m}Tc-labeled MORF/streptavidin/carrier nanoparticles were described previously.³ However, in this investigation, modifications were necessary when both the commercial antisense and sense MORF oligomers were shown to be only about 50% biotinylated, by size-exclusion HPLC analyses before and after the addition of an excess of streptavidin (see below). Although the nanoparticles could be radiolabeled after construction via the MAG3 group attached to the MORFs, labeling efficiency would have been less than 90%, since heating must be avoided to protect the streptavidin. For this reason, in this investigation, the MORF

oligomers were radiolabeled before construction of the nanoparticles.

The ^{99m}Tc-labeled MORF (16 μ g) was added very slowly and with continuously vigorous agitation to 75 μ g of streptavidin (Sigma-Aldrich, St. Louis, MO), dissolved in 80 μ L of normal saline, and the solution was incubated at room temperature for 1 hour before purification over a 1 $\times$ 20 cm G75 open column, using phosphate-buffered saline (PBS) as an eluant. The early fractions with peak radioactivity were combined and the concentration of streptavidin determined colorimetrically, using a bicinchoninic acid (BCA) reagent (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). Absorbance at 280 nm could not be used for this determination, since the MORF oligomer also absorbs in this range. With continuous stirring, the biotinylated tat or polyarginine was added slowly at a streptavidin-to-carrier molar ratio of 1:1, and the preparation was incubated at room temperature for 30 minutes. The absence of 2:1 or higher order additions of MORFs or carriers at interfering levels was confirmed both by size-exclusion HPLC, in which the appearance of a single peak in the radioactivity profile was taken as evidence of a 1:1 construct, and by adding trace ^{99m}Tc-labeled phosphodiester DNA complementary to each unlabeled MORF/streptavidin/carrier, followed by HPLC analysis, as previously described.³ In this case, the appearance of a single peak in the ultraviolet (UV) traces at both 265 (MORF) and 280 nm (streptavidin), and a single peak in the radioactivity trace was evidence of a successful preparation. Both assays are capable of resolving 2:1 and higher order nanoparticles.³

Animal studies

Human inflammatory breast cancer SUM149 cells were trypsinized from 75 cm² culture flasks and collected. Each female NIH SWISS nude mouse received subcutaneously (s.c.) in one thigh 10⁶ cells in 100- μ L serum-free medium. Animals were used about 28 days later when tumors were 0.7 cm or less in any dimension.

The specific ^{99m}Tc radioactivity of the MORF/streptavidin/carrier nanoparticles was adjusted to about 0.55 MBq per μ g of MORF oligomer for the normal mouse studies and, because of the longer sacrifice time, at 3.7 MBq per μ g MORF for the tumored mouse studies. Each CD-1 normal mouse and NIH Swiss tumor-bearing mouse received (in 150 μ L) a dosage of nanoparticles containing 1 μ g of MORF. At either 3 (normal mice) or 21 hours (tumored mice) postinjection, mice were sacrificed by cervical dislocation after anesthesia with inhalation of isoflurane. The radioactivity accumulated in tissues/organs of interest was measured by removing the tissues/organs, followed by counting on an automatic gamma-counter. The tumor and tissue/organ accumulations of radioactivity were reported as percent injected dose per gram (%ID/g).

One of the normal mice was also imaged on a Nano-SPECT/CT (single-photon emission computed tomography/computed tomography small-animal camera (Bioscan, Washington, DC) at 30, 60, and 180 minutes postadministration under anesthesia by inhalation of oxygen-isoflurane.

Statistical test

In the tumored mouse study, a paired one-tailed distribution Student's *t*-test was used to evaluate whether a statisti-

FIG. 1. Radioactivity profiles by size-exclusion high-performance liquid chromatography. (A) ^{99m}Tc -labeled antisense phosphorodiamidate morpholino (MORF). (B) ^{99m}Tc -labeled antisense MORF/streptavidin nanoparticle before purification. (C) ^{99m}Tc -labeled antisense MORF/streptavidin nanoparticle after purification. (D) ^{99m}Tc -labeled antisense MORF/streptavidin/tat nanoparticle.

cally significant difference existed between accumulations of antisense and sense nanoparticles in each tissue/organ.

Results

Nanoparticle preparation

As shown in Figure 1A, the HPLC radiochromatogram of the naked ^{99m}Tc -labeled antisense MORF consisted of a single peak at 26.0 minutes. Figure 1 also presents HPLC profiles of the antisense MORF/streptavidin nanoparticle before (Fig. 1B) and after (Fig. 1C) purification on the open G75 column and that of the antisense MORF/streptavidin/tat nanoparticle (Fig. 1D). Analysis of the labeled antisense MORF/streptavidin nanoparticle preparation before purification (Fig. 1B) provided one peak at 26.0 minutes due to the radiolabeled free MORF without its biotin group and

one peak at 21.5 minutes due to the radiolabeled antisense MORF/streptavidin nanoparticle. After purification, the radiolabeled free MORF oligomer was almost completely removed (Fig. 1C). Under the HPLC elution conditions of this study, the retention time of the antisense MORF/streptavidin/tat nanoparticle was only slightly shortened to 20.8 minutes due to the addition of the tat peptide (Fig. 1D). The slight shoulder at 18.4 minutes in the profile of the antisense MORF/streptavidin/tat nanoparticle (Fig. 1D) was probably due to the presence of a higher order tat nanoparticle (possibly a result of experimental uncertainties in the streptavidin concentration measurement by the BCA protein assay). The slightly higher background following the peak, in this case, is consistent with a slightly lower recovery and our observation that this nanoparticle tends to stick to surfaces. Similarly, elevated backgrounds were also observed in the analyses of the sense nanoparticle with tat and both the antisense and sense nanoparticles with polyarginine (data not shown).

Animal study

Table 1 presents the biodistributions at 3 hours in normal male CD-1 mice of ^{99m}Tc -labeled sense and antisense MORFs as the free oligomers and as both the MORF/streptavidin/tat and MORF/streptavidin/polyarginine nanoparticles. There were no statistically significant differences in sampled tissues between antisense and sense for either the tat or the polyarginine nanoparticles, except for the liver and spleen. However, the biodistributions of both naked MORFs showed much less accumulation in all organs other than kidneys. For example, while only 0.06–0.11 ID%/g of the naked MORFs were present in blood at this time, this values were almost 10% for each nanoparticle.

Figure 2 presents projections from the SPECT/CT acquisition of a normal mouse taken at 30, 60, and 90 minutes after i.v. administration of the ^{99m}Tc -MORF/streptavidin/tat nanoparticles. The images clearly show high levels of radioactivity in the liver, kidneys, and heart.

Table 2 presents biodistributions in tumored mice of the four nanoparticles with sacrifice at 21 hours selected with the

TABLE 1. BIODISTRIBUTIONS AT 3 HOURS IN NORMAL MICE OF ^{99m}Tc -LABELED SENSE AND ANTISENSE MORFs AS THE FREE OLIGOMERS AND AS BOTH THE STREPTAVIDIN (SA)/TAT AND STREPTAVIDIN/POLYARGININE (POLYR) NANOPARTICLES

Organs	%ID/g					
	Antisense MORF			Sense MORF		
	Naked	MORF-SA-PolyR	MORF-SA-tat	Naked	MORF-SA-PolyR	MORF-SA-tat
Liver	0.97 ± 0.08	4.53 ± 0.47	4.50 ± 0.36	1.33 ± 0.08	3.42 ± 0.39	2.67 ± 0.38
Heart	0.10 ± 0.01	2.92 ± 0.44	2.51 ± 0.45	0.10 ± 0.01	2.48 ± 0.09	1.96 ± 0.25
Kidney	42.56 ± 8.59	9.69 ± 3.12	9.54 ± 1.91	16.48 ± 3.77	8.40 ± 1.09	8.43 ± 0.60
Lung	0.11 ± 0.01	3.06 ± 0.46	3.15 ± 0.35	0.15 ± 0.00	2.77 ± 0.10	2.53 ± 0.25
Spleen	0.45 ± 0.08	3.30 ± 0.39	3.17 ± 0.64	0.48 ± 0.06	2.58 ± 0.33	1.89 ± 0.35
Stomach	0.56 ± 0.17	1.69 ± 0.29	1.16 ± 0.24	0.49 ± 0.11	1.20 ± 0.28	1.19 ± 0.39
Small intestine	0.21 ± 0.07	1.11 ± 0.15	0.99 ± 0.16	0.16 ± 0.02	1.04 ± 0.14	1.00 ± 0.18
Large intestine	0.65 ± 0.07	1.30 ± 0.13	1.13 ± 0.08	0.94 ± 0.04	1.18 ± 0.09	1.31 ± 0.12
Muscle	0.03 ± 0.01	0.51 ± 0.04	0.55 ± 0.05	0.05 ± 0.01	0.43 ± 0.10	0.42 ± 0.08
Blood	0.06 ± 0.00	9.97 ± 0.91	9.98 ± 1.01	0.11 ± 0.01	9.60 ± 0.31	9.23 ± 0.46

Each animal received 1 μg of MORF regardless of chemical form. Data are presented as mean ± standard deviation ($N = 4$). %ID/g, percent injected dose per gram.

FIG. 2. Single-photon emission computed tomography/computed tomography images of a normal mouse at 30, 60, and 90 minutes post-administration of the ^{99m}Tc -MORF/streptavidin/tat nanoparticle.

expectation that background blood levels would be reduced from that observed in the normal mice study with sacrifice at 3 hours.

Discussion

In a continuing effort to improve antisense tumor imaging *in vivo* by i.v. administration, our laboratories have considered radiolabeled DNAs and other oligomers administered to tumored mice as naked oligomers,^{14,18,19} complexed electrostatically with jetPEITM (Qbiogene, Irvine, CA), ChariotTM (Active Motif, Carlsbad, CA), and NeophectinTM²⁰ (Neo Pharm, Waukegan, IL) or as duplexes.²¹ These studies helped

to confirm that two critical properties of any radiolabeled antisense agent are pharmacokinetic and cell-membrane transportation.²² As a means of improving cell-membrane delivery of radiolabeled antisense oligomer, we considered attaching carriers, such as tat, cholesterol, or polyarginine, to the oligomer but by using streptavidin as a linker, in part, to avoid covalent conjugation.^{3-5,23}

We have evidence, by HPLC and gel electrophoresis,³ that the streptavidin nanoparticles are essentially free of higher order constructs, and the nanoparticles are stable in 37°C PBS and 60% mouse serum.⁴ We have also shown that the nanoparticles are not cytotoxic, and the presence of the streptavidin apparently does not interfere with the function of the

TABLE 2. BIODISTRIBUTIONS AT 21 HOURS IN SUM149 TUMORED NIH SWISS FEMALE MICE OF ^{99m}Tc -LABELED SENSE AND ANTISENSE MORFs AS BOTH THE STREPTAVIDIN (SA)/TAT AND STREPTAVIDIN/POLYARGININE (POLYR) NANOPARTICLES

Organs	%ID/g					
	MORF-SA-PolyR			MORF-SA-tat		
	R1 α antisense	R1 α sense	p-value	R1 α antisense	R1 α sense	p-value
Liver	11.08 $\pm$ 0.41	13.08 $\pm$ 1.11	0.021	11.90 $\pm$ 0.82	13.51 $\pm$ 1.30	0.090
Heart	1.89 $\pm$ 0.09	1.99 $\pm$ 0.28	0.203	1.98 $\pm$ 0.14	2.01 $\pm$ 0.33	0.451
Kidney	31.74 $\pm$ 2.42	37.10 $\pm$ 7.77	0.112	33.95 $\pm$ 5.20	38.17 $\pm$ 8.56	0.224
Lung	2.28 $\pm$ 0.20	2.32 $\pm$ 0.29	0.388	2.41 $\pm$ 0.11	2.34 $\pm$ 0.31	0.366
Spleen	3.32 $\pm$ 0.33	4.13 $\pm$ 0.33	0.040	3.47 $\pm$ 0.53	4.19 $\pm$ 0.37	0.077
Stomach	0.64 $\pm$ 0.13	0.52 $\pm$ 0.09	0.153	0.66 $\pm$ 0.14	0.53 $\pm$ 0.10	0.148
Small intestine	0.84 $\pm$ 0.09	0.79 $\pm$ 0.09	0.298	0.89 $\pm$ 0.15	0.80 $\pm$ 0.10	0.258
Large intestine	1.56 $\pm$ 0.39	1.15 $\pm$ 0.27	0.144	1.65 $\pm$ 0.51	1.16 $\pm$ 0.28	0.147
Muscle	0.58 $\pm$ 0.05	0.72 $\pm$ 0.28	0.193	0.62 $\pm$ 0.10	0.74 $\pm$ 0.29	0.262
Tumor	3.22 $\pm$ 0.16	2.35 $\pm$ 0.80	0.037	3.40 $\pm$ 0.42	2.36 $\pm$ 0.80	0.020
Blood	2.94 $\pm$ 0.10	2.95 $\pm$ 0.65	0.487	3.10 $\pm$ 0.34	3.01 $\pm$ 0.71	0.430

Each animal received 1 μg of MORF regardless of chemical form. Data are presented as mean $\pm$ standard deviation ($N=4$).

%ID/g, percent injected dose per gram.

tat, polyarginine, or cholesterol carriers, at least in culture studies.^{3,4}

These two-component nanoparticles are also under investigation after the addition of an biotinylated antitumor antibody to provide tissue specificity as a three-component nanoparticle and again observed encouraging results, in tumor cells in culture, that streptavidin apparently does not interfere with the antisense function of the oligomer.⁴

The conclusion from these investigations is that streptavidin provides a convenient method of combining antibodies, peptides, and oligomers or other components and without any apparent adverse influence on the functions of these components. Accordingly, it next became important to determine whether the presence of streptavidin adversely influenced the pharmacokinetic properties.

Before proceeding to tumored mice, the biodistributions of the nanoparticles were first compared to that of the free MORFs in normal mice. The differences shown in Table 1 in biodistributions of the antisense and sense MORFs, when administered as free oligomers, is due to the influence of the base sequence. As we reported previously, the free MORF oligomers are cleared from circulation, mainly through the kidneys, and their retention in this organ increases in proportion to the number of cytosine base in their sequences.²⁴ Thus, the higher kidney accumulations of the free antisense MORF (43% ID/g), compared to the sense MORF (17% ID/g), is a reflection of the eight cytosines in the base sequence of the former, compared to only five cytosines in the latter, oligomer. That this difference disappears when the MORFs are administered as nanoparticles indicates that this phenomenon is masked in the presence of the streptavidin.

As is also apparent in the results presented in the Table 1, the accumulations in all tissues other than kidneys were lower for both naked MORFs, in some cases by more than 2 orders of magnitude, compared to either nanoparticle. In our previous study of phosphorothioate DNA (PS DNA) nanoparticles, the accumulations of PS DNA was also lower when administered naked but by no more than a factor of 4.⁵ We believe that these observations may be explained by the assumption that the pharmacokinetics of the PS DNA, whether naked or as nanoparticles, is dominated by the protein-binding affinity of this DNA, while the pharmacokinetics of the MORF oligomers, by not displaying a similar affinity for proteins, are dominated within the nanoparticle by the streptavidin. The fact that the biodistributions in normal mice are generally identical regardless of whether the nanoparticle contains the tat or polyarginine carrier or, in most cases, identical regardless of whether the nanoparticle contains the antisense or sense MORF, supports the suggestion that the biodistributions are dominated by the streptavidin. This explanation is logical, since enlarging a radiolabeled agent from approximately 6 to more than 66 KDa must be expected to slow its pharmacokinetics, in particular by preventing clearance by glomerular filtration, since the molecular-weight threshold limiting glomerular filtration is between about 30 and 45 KDa.²⁵ Further, except for muscle, the tissue-to-blood ratios in these normal mice are greater than 1 in the case of both naked MORFs, suggesting that these small oligomers have largely diffused into tissues within the 3-hours sacrifice time. In contrast, these ratios for all nanoparticles are all less than 1, suggesting that their larger size restricts this diffusion.

Based on the observations in normal mice of relatively slow clearance of the nanoparticles, compared to the naked MORFs, sacrifice of the tumored mice was delayed until 21 hours. As shown in Table 2, the blood levels of the radiolabeled nanoparticles in the tumored mice at 21 hours were lower than that observed in normal mice at 3 hours, as expected. However, accumulations in most tissues were only slightly lower in the tumored animals, compared to normal animals, despite the longer sacrifice time and, in the case of the liver and kidneys, were substantially higher. As an example, the liver accumulations at 21 hours increased in the tumored mice (11–13 ID%/g), compared to that in normal mice at 3 hours (2.7–4.5 ID%/g). A similar increase was apparent in the kidneys. Ignoring the possibility that these differences may have been species related or may have been related to the health of the tumored animals, the tissue-to-blood ratios suggest that diffusion of the radiolabel, in some chemical form, was occurring only in the liver and in kidneys and, possibly, in tumor, but only in the case of the two antisense nanoparticles.

The essentially identical biodistributions between the tat and polyarginine nanoparticles were again apparent in the tumored mice as in the normal mice. Also apparent were the essentially identical biodistributions between the antisense and sense nanoparticles. Among the normal tissues, there were no significant differences in the case of the tat nanoparticles, and only the liver and spleen were significantly different in the case of the polyarginine nanoparticles, in both cases with the sense nanoparticle showing higher accumulations. It is gratifying, however, that for both the tat and polyarginine nanoparticles, the accumulation in tumor was significantly higher for the antisense, compared to the sense control, nanoparticles. This observation is consistent with our previous studies of the nanoparticle, that the presence of the streptavidin has apparently not interfered with the antisense function of the oligomers.^{3,4}

No naked MORF control was used in the tumored mouse study, because previous studies of radiolabeled free MORFs in tumored mice have shown rapid clearance from blood and normal tissues other than kidneys, which was similar to that reported in Table 1 for normal mice, and extremely low levels of accumulation in tumor,²⁶ contrary to that reported in Table 2.

Conclusions

By replacing DNA with MORF as the oligomer, we have been able to separate the influence on the biodistribution of the oligomer from that of the streptavidin. Because of the dominance of streptavidin in the case of the MORF nanoparticles, the clearance from circulation, and from normal organs of the radiolabeled MORF, was markedly slowed. The increased accumulations in liver and kidneys from 3 to 21 hours may have been due to the positive charge of the carriers and/or the properties of the streptavidin.^{27–29} We speculate that the delivery nanoparticles by using streptavidin as a bridge and MORF oligomers as antisense agents may have applications in antisense chemotherapy, if proper carrier molecules are used. In this case, the slow blood clearance may not have been a big concern. Future studies will compare results without the carrier and with avidin and other avidin-like proteins replacing streptavidin.

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Disclosure Statement

No competing financial interests exist.

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