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A Method to Characterize *In Vivo* Binding of Morpholinos for Drug Design

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Abstract

Antisense nucleotide oligomer analogues such as morpholinos are actively investigated as drugs to treat or manage human diseases. Their binding affinity to the complementary target sequence is a crucial parameter. The *in vitro* measures such as association constant and melting temperature are useful but do not provide the exact information about the *in vivo* binding. This paper introduces a mouse model to which a circulating vehicle molecule carrying a target morpholino (t-morpholino of interest) is first injected followed by the effecting morpholino (e-morpholino). The 3-h blood concentration level of the e-morpholino is used as an indicator for its binding to the t-morpholino. Particular to this report, an amine-derivatized t-morpholino is conjugated to an NHS-activated lipid. Mixing the conjugate with albumin as a natural vehicle molecule forms a vehicle complex and the t-morpholino can circulate on the vehicle after injection. This method can be utilized for modulation of the *in vivo* binding affinity of e-morpholino oligomers to their targets and may be applied to other nucleotide oligomers as well.

Keywords: Antisense nucleotide oligomers; DNA analogues; Morpholinos; *In vivo* applications; *In vivo* binding affinity

Abbreviations

DNA: Deoxyribonucleic Acid; NHS: N-Hydroxysuccinimide; DSPE: a lipid, 1,2-Distearoyl-Sn-Glycero-3-Phosphoethanolamine; PEG: Polyethylene Glycol; MAG_3: Mercaptoacetyltriglycine; UV: Ultraviolet; HPLC: High Performance Liquid Chromatography; HEPES: ((4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid) or 2-[4-(2-Hydroxyethyl)Piperazin-1-yl] Ethanesulfonic Acid; HSA: Human Serum Albumin; %ID: Percent of Injected Dose; %ID/g: Percent of Injected Dose Per Gram of Tissue; PB: Phosphate Buffer

Introduction

The *in vivo* use of synthetic antisense DNA analogues (antisense nucleotide oligomer analogues) as potential pharmaceuticals is the most important aspect of their applications [1-8]. In most cases, the antisense DNA analogues form duplexes *in vivo* with their targets. Thus, the binding affinity between two mutual complements is a crucial parameter for the success. *In vitro* methods measuring or calculating the binding affinity in terms of melting temperature, free energy change ΔG°, association constant, etc. have long been available [9-12]. However, these *in vitro* measurements or calculations may not exactly predict the *in vivo* binding. This is especially true for the comparatively new synthetic DNA analogues lacking *in vitro-in vivo* correlation.

The affinity of a DNA analogue to its complementary target is required to be sufficiently high to take effect. As said, although the quantitative *in vitro* metrics do provide a measure for affinity, the *in vitro* measures such as association constants or melting points are not a direct indicator of *in vivo* binding. Thus, a convenient *in vivo* approach that can characterize *in vivo* binding is needed.

In this report, we introduce an *in vivo* measure for the *in vivo* binding of morpholino oligomers. Free single strand morpholino oligomers are stable *in vivo* and are cleared very rapidly from the blood pool. If we load the complement of a free single strand onto a vehicle molecule of a long circulating half-life (Figure 1), the free single strand would bind to the complement and circulate with the vehicle molecule. At the time when the unbound single strand is essentially cleared , the retained portion would be due to the bound and can be used as a measure for the *in vivo* binding. Long circulating artificial molecules and long circulating endogenous proteins (antibodies or serum protein) may both be considered as the vehicle, but we chose the latter and employed serum albumin.

Our choice of serum albumin is partially because it circulates for weeks [13] and also because we have now a technology available to conveniently load a morpholino oligomer onto the albumin. A commercially available NH_2-derivatized morpholino can be readily conjugated to an NHS-activated lipid by mixing the two in a buffer solution. Further mixing the conjugate with the serum albumin would load the NH_2-derivatized morpholino (t-morpholino) onto the long circulating albumin. The conjugation mixture needs not to be purified because the unconjugated morpholino is non-toxic and will be cleared very rapidly. Another required technology is also available. A protocol for morpholino radiolabeling has long

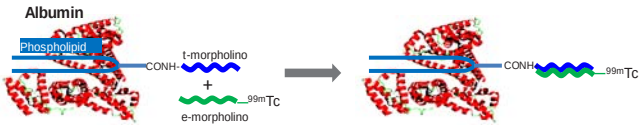

Figure 1: Binding of the labeled e-morpholino to an albumin-carried t-morpholino would allow it to circulate with the albumin vehicle.

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Table 1: Morpholinos used in this report.

Morpholino	Sequence	MAG_3 conjugation	lipid conjugation
ATG12	5'-TGAAGTAGAAGA-NH_2		X
ATG14	5'-TGTGAAGTAGAAGA-NH_2		X
ATG16	5'-GTTGTGAAGTAGAAGA-NH_2		X
ATG18	5'-TAGTTGTGAAGTAGAAGA-NH_2	X	X
ATC18	5'-TCTTCTACTTCACAACTA-NH_2	X	
A7ATC18	5'-AAAAAAATCTTCTACTTCACAACTA-NH_2		X
ATG18T7	5'-TAGTTGTGAAGTAGAAGATTTTTTT-NH_2		X

become routine in our lab that can be used to track the binding of a morpholino (denoted as effecting morpholino or e-morpholino) to the t-morpholino on the albumin [14-16]. As the labeled morpholino is in the form of e-morpholino/t-morpholino-albumin complex (Figure 1), we can measure the *in vivo* percentage retained in blood of the labeled e-morpholino (blood concentration in %ID/g or %ID/mL) to quantify its *in vivo* binding.

Materials and Methods

Materials and mice

The morpholinos were all purchased from Gene Tools (Philomath, OR) in a modified form bearing a primary amine at the 3' equivalent terminal. The NHS-activated phospholipid (DSPE-PEG-NHS) was from Nanocs (New York, NY). The male CD-1 mice (4-5 wk old) were from Charles River laboratories (Wilmington, MA). The Albumin (Human) 25% Solution Albu Rx® 25 was from CSL Behring AG (Bern, Switzerland). The ^{99m}Mo-^{99m}Tc generator was from Perkin Elmer Life Science Inc (Boston, MA). The NHS-activated S-acetyl MAG_3 was house-made. All other chemicals were reagent grade and used without purification.

UV spectrophotometry and HPLC analysis

The concentrations of morpholinos were measured by UV spectrophotometry using the molar absorbance values provided by the vendor. Size Exclusion (SE) HPLC was used for analysis of the morpholinos and their conjugates. The HPLC system was equipped with a superpose-12 10/30 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; optimal separation range: 1×10^3 to 3×10^5 Da), a UV in-line detector, and a radioactivity in-line detector. A 0.10 M pH 7.2 phosphate buffers was used as the eluant at a flow rate of 0.60 mL/min. Radioactivity recovery for radiolabeled morpholinos was routinely measured and was always greater than 90%.

MAG_3 modification and ^{99m}Tc radiolabeling of e-morpholinos

The ^{99m}Tc-labeled morpholinos (^{99m}Tc-morpholinos) were prepared following our documented procedure [17]. The amine-derivatized morpholinos that were conjugated with MAG_3 are listed in Table 1. Each tine, prior to the use, the stock MAG_3-morpholino solutions are labeled with ^{99m}Tc (half-life 6.02 h) following our routine protocols.

Conjugation of t-morpholino to NHS-activated lipid and loading on to HSA

In a 0.2 M pH 8 HEPES buffer, mixing the NHS-activated lipid (DSPE-PEG-NHS) with the primary amine-derivatized morpholinos readily provides a morpholino-lipid conjugate. The morpholinos conjugated to lipid are also listed in Table 1. Specifically, 2-3 mg of DSPE-PEG-NHS was dissolved into a HEPES-buffered 1 mM NH_2-morpholino solution to a concentration of 6 mM. After 3 h, the conjugate was ready for use or stored in the 4 °C refrigerator for further use. The conjugation mixture was analyzed on HPLC. HSA was added , or lipid conjugates would stick to the Superose-12 column.

Measurement of 3-h e-morpholino blood level in mice receiving t-morpholino-lipid/albumin

Variation of the chain length of either the t-morpholino on the albumin or the e-morpholino labeled with ^{99m}Tc would alter their binding affinity. We chose to vary the t-morpholinos because of the easier lipid conjugation than the MAG_3 conjugation, the instant lipid binding to HSA, and the fixed pharmacokinetics of the e-morpholino. Specifically, the lipid conjugation mixture of a t-morpholino (ATG12, 14, 16, or 18) prepared following the above procedure was dissolved into a 1.25% HSA solution to a concentration of 26 nmol morpholino/mL. 4 mice were used and each received 100 µL of the injectate by the tail vein. As a control, another 4 mice received only the HSA. After an hour, a tracer amount of the ^{99m}Tc-labeled e-morpholino (0.4 µg, 20 µCi of ATC18) was injected to both study and control mice and they were euthanized 3 hours thereafter for biodistribution. The 3-h time point was decided based on our previous observation that the blood clearance of free labeled morpholinos essentially completes at 3 h [18,19].

Replacement and release of the labeled e-morpholino from the HSA vehicle

Any contribution due to binding to an unknown *in vivo* target would reduce the validity of the current method. We designed an experiment to examine any possible interaction contributing to the 3-h blood radioactivity levels other than the t-morpholino binding. As the ATC18/ATG18 interaction among the four tested provided the highest blood level (will be shown), this pair was interrogated. Because the ATC18 showed higher kidney accumulation in the above study (will be shown), the ATG18 was labeled instead and the ATC18 was conjugated to the lipid correspondingly. Furthermore, the ATC18 was elongated at the 5'-terminal with an extension of 7 adenines (A7ATC18). The extended A7ATC18 binds the ^{99m}Tc-ATG18 and, with a stronger affinity, its elongation of ATG18 (ATG18T7). Challenging the ^{99m}Tc-ATG18/ATCA7-lipid/HSA by the ATG18 elongation would replace and release the ^{99m}Tc-ATG18, as shown in (Figure 2). To ensure a sufficient time for the challenge, the ATG18T7 was also loaded onto the HSA. Thus, a group of 4 mice was injected 1-h apart with the ATCA7-lipid/HSA (2.4 nmol t-morpholino-lipid conjugate in 1.25% HSA), the ^{99m}Tc-ATG18 (0.4 µg, 50 µCi), and the challenging ATG18T7-lipid-HSA. At 3 h post the last injection, the mice were euthanized for biodistribution. A control group underwent an identical procedure except for not receiving the challenge. Historical 3-h biodistribution data of free ^{99m}Tc-ATG18 was used as another control [20] (Figure 3).

Results

Morpholino-lipid conjugation

Figure 3A illustrates the HPLC trace of the ^{99m}Tc-labeled ATC18 alone and Figure 3B shows the traces of the labeled ATC18 in 1.25%

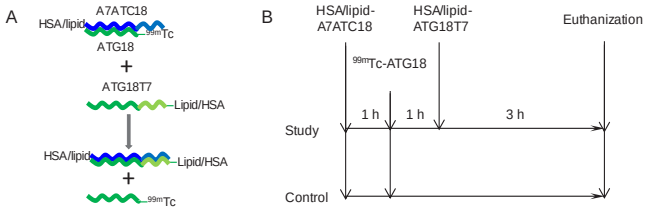

Figure 2: The replacement of the labeled ATG18 by the ATG18T7 (A) and the experimental procedure (B).

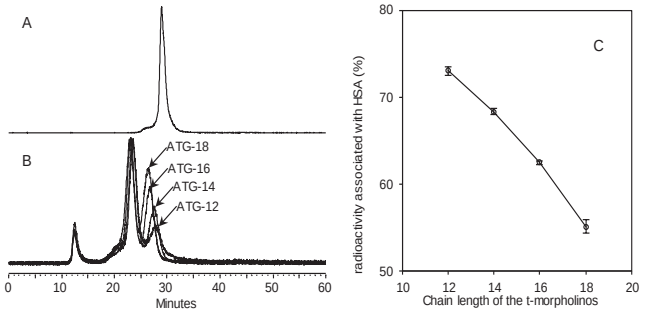

Figure 3: Radio chromatographic traces of ^{99m}Tc-ATC18 in pH 7.2 PB buffer (A) and in 1.25% HSA solution that containing the DSPE/t-morpholino as 6 conjugation mixtures at a t-morpholino concentration of 2.58×10^{-5} M (B). The conjugation efficiencies to the NHS-activated lipid are shown in panel (C) for different t-morpholinos.

HSA containing the different t-morpholino DSPE conjugates (DSPE/t-morpholino = 6). The labeled ATC18 bound to the t-morpholino-lipid/HSA elutes at 23 min and that bound to the free t-morpholinos failed to attach to the HSA elutes at 26-28 min. The peak at 13 min represents a complex of ^{99m}Tc-ATC18/t-morpholino-lipid/aggregated HSA. The relative peak heights reflect the conjugation efficiencies. We measured the % of the t-morpholinos conjugated to the lipid (conjugation efficiency) by analyzing each conjugate three times, and Figure 3C shows the data. They reduce with the increased chain length, suggesting a synergistic contribution of spatial hindrance with the competitive hydrolysis of the NHS-ester.

Biodistributions of ^{99m}Tc-labeled ATC18 in mice receiving t-morpholinos loaded on HSA

Figure 4 shows the biodistributions of ^{99m}Tc-labeled ATC18 in mice receiving the t-morpholino-lipid/HSA vehicles along with its biodistribution in non-treated control mice. Without the vehicle, the concentrations in blood and normal tissues (except for kidney) are minimal. This agrees with our documented observation that small morpholinos are essentially not retained in animal tissues except for the kidneys [18,19]. The 6 cytosines in the ATC18 sequence explain the high kidney accumulation [18]. When circulating with the morpholinos loaded on the HSA, the blood and normal tissue concentrations increase, to a higher extent if the matched chain length is longer. Interestingly, when the blood level is higher, the kidney accumulation becomes lower. This indicates that, when bound to the HSA vehicle, the ^{99m}Tc-labeled ATC18 does not accumulate in kidney and the accumulation may just represent the radioactivity in the "concentrated" blood.

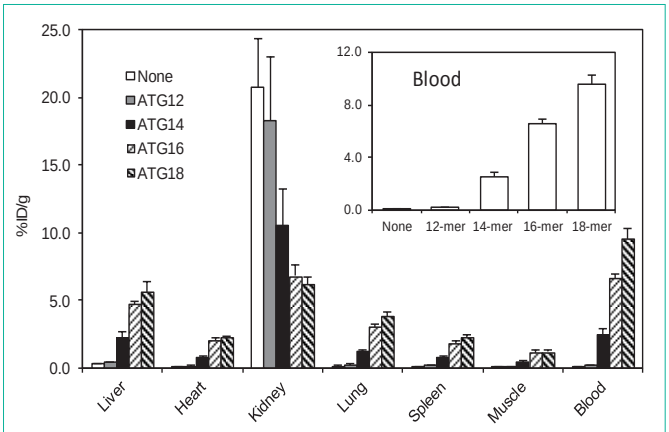

Figure 4: Biodistributions at 3 h of the ^{99m}Tc-ATC18 in normal mice and the t-morpholino-lipid/HSA treated mice.

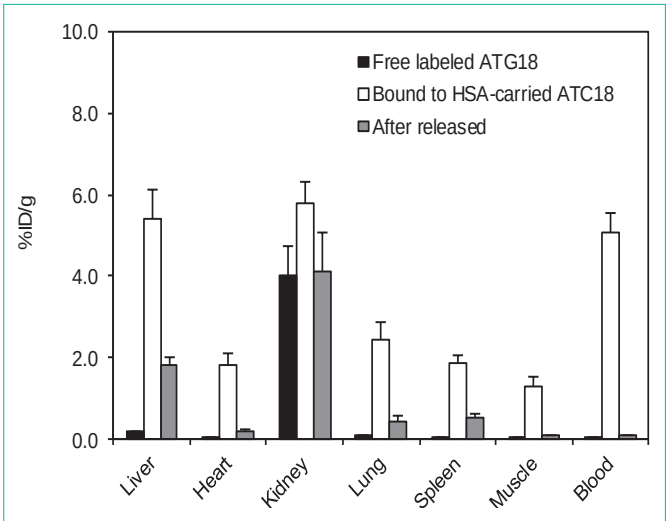

Figure 5: The 3-h bio distributions of ^{99m}Tc-ATG18 in normal mice, in the A7ATC18-lipid/HSA treated mice, and in the treated mice after release by ATG18T7-lipid/HSA.

Validation of the 3-h blood level as a measure for *in vivo* affinity

The labeled ATC18 shows minimal blood concentration level alone but the level is high after binding to the t-morpholino -HSAs. This indicates the 3-h level is due to the radio-label on the HSA. The elevation of blood level with the increase of the matched length should have confirmed the retention mechanism by e-morpholino/t-morpholino binding. However, the replacement and release results below serve as solider evidences that the binding is solely due to the e-morpholino/t-morpholino interaction. Figure 5 demonstrates the 3-h blood concentrations of the free ^{99m}Tc-ATG18, the ^{99m}Tc-ATG18 bound to the HSA vehicle, and the ^{99m}Tc-ATG18 bound to the HSA but subsequently released by the ATG18T7. The release reduces the blood concentration level from 5.07±0.48% ID/g to 0.07±0.00% ID/g, a level comparable to the 0.02±0.00% ID/g of the free ^{99m}Tc-ATG18. This complete release (over 99%) confirms the ATG18 is completely bound to A7ATC18 in the blood.

Parenthetically, Figure 5 also indicates that in some normal tissues

there is some binding to other targets. The accumulations in liver, heart, lungs, and spleen after releasing are still significantly higher than that for the free ^{99m}Tc-ATG18, indicating some of the ^{99m}Tc-ATG18 has entered certain compartments inaccessible for the A7ATC18-HSA before releasing. In kidney, the labeled ATG18 accumulates due to binding to other targets but the accumulation while bound to the HSA may be just due to the high blood concentration. The accumulation mechanism in kidney after releasing should be similar to that for the free labeled ATG18 but it may have happened after being shed off the vehicle.

Discussion

It is our judgment that the 3-h blood concentration of the an e-morpholino in the mice treated with the t-morpholino-HSA is an indicator of the *in vivo* binding of the e-morpholino to the t-morpholino. This *in vivo* quantitation parameter may not be linearly related to the association constant. It provides information about whether a sequence would bind *in vivo* with a sufficient affinity. In the current investigation, we tested four sequences in a series up to 18 matched bases. We expect further elongation may provide higher blood level but this increase would plateau at a certain point. Beside the 3-h level, the levels at longer time points may also be worth examination in the future, as the impact of the dissociation on blood level would increase and therefore, when coupled with the 3-h level, may be a more sensitive indicator of *in vivo* dissociation. Correlation of these blood levels with dissociation constants or melting points may be made, but for a study focusing on several sequences, this correlation may not be necessary, as it would reduce neither the work nor the use of animals.

In the current investigation, we use lipid as a mediator to load the t-morpholino onto albumin. The upside of this approach is the convenience. Beside the one-step conjugation, purification is not necessary because the unreacted morpholinos are not toxic and are cleared into the urine very rapidly. The downside is the accumulation in some normal tissues (Figure 5), probably due to the lipid metabolic pathway. A direct chemical conjugation to albumin can be another option. Nevertheless, as the binding in blood is solely a result of e-morpholino/t-morpholino interaction, the lipid approach should not impede its use to construct the vehicle to measure the *in vivo* binding.

The current focus of our own group is on morpholino tumor pretargeting. In this technology, any sequence can be used, but the affinity has to be sufficient to ensure a firm bond to the t-morpholino prelocolized in the tumor. In optimizing the e-morpholinos, we previously emphasized on the pharmacokinetics of the e-morpholino alone, not fully extended to the binding to the t-morpholinos [19,21]. The current method would allow a convenient testing on other sequences in the future. Directly conjugating a t-morpholino to an antibody also serve the purpose, but the conjugation is more complicated as compared to the lipid conjugation and furthermore the vehicle residence life is shorter and varies with different antibodies.

The current method may also be useful to other applications, such as antisense targeting. It may be used for *in vivo* binding modulation. For example, nucleotide oligomers are used as aptamers to target protein for disease management. In certain settings, there is a danger of overdosing and it is proposed to be addressed by complementary oligonucleotide antidotes [3,22]. Its binding affinity to the aptamer may need to be fine-tuned. A pre-screening tool for the candidate antidotes might accelerate the drug discovery process.

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