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Unexpected side products in the conjugation of an amine-derivatized morpholino oligomer with p-isothiocyanate benzyl DTPA and their removal

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Abstract

In connection with pretargeting, an amine-derivatized morpholino phosphorodiamidate oligomer (NH₂-cMORF) was conjugated conventionally with p-isothiocyanate benzyl-DTPA (p-SCN-Bn-DTPA). However, after ¹¹¹In radiolabeling, unexpected label instability was observed. To understand this instability, the NH₂-cMORF and, as control, the native cMORF without the amine were conjugated in the conventional manner. Surprisingly, the ¹¹¹In labeling of the native cMORF conjugate was equally effective to that of the NH₂-cMORF conjugate (>95%) despite the absence of the amine group. Furthermore, heating the radiolabeled NH₂-cMORF and native cMORF conjugates resulted in respectively a 35% loss and a complete loss of the label. Since the ¹¹¹In labeled DTPA is known stable, the instability in both cases must be due to some unstable association of DTPA to the cMORF, presumably unstable conjugation of the DTPA to some endogenous sites in cMORF. Based on this assumption, a post-conjugation, pre-purification heating step was introduced and the labeling efficiency and stability were again investigated. By introducing the heating step, the side products were dissociated and, after purification and labeling, the NH₂-cMORF conjugate provided a stable label and high labeling efficiency with no need for postlabeling purification. The biodistribution of this radiolabeled conjugate in normal mice showed significantly lower backgrounds compared to the labeled unstable native cMORF conjugate. In conclusion, the conventional conjugation procedure to attach the p-SCN-Bn-DTPA to NH₂-cMORF resulted in side product(s) that were responsible for the ¹¹¹In label instability. Adding a post-conjugation, pre-purification heating step dissociated the side products, improved the label stability, and lowered tissue backgrounds in mice.

Keywords

Chelator; Conjugation; Radiolabeling; DNA analogues

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INTRODUCTION

Several methods have been reported to radiolabel DNAs, RNAs, and their analogs for inhibition of gene expression [1-6], for antisense targeting [7-15], and for numerous other applications including pretargeting [16-19]. One amine-derivatized morpholino phosphorodiamidate oligomer (cMORF) has been labeled with ^{99m}Tc [20-22] and ^{111}In [23,24] via MAG_3 and DTPA respectively. The cMORF has also been radiolabeled with ^{188}Re via MAG_3 and used for radiation therapy in a mouse tumor model [21,25]. The MORF/cMORF pretargeting is currently under consideration for pancreatic beta cell imaging [23,24] and therefore we are interested in labeled cMORF oligomers with minimal background radioactivity, especially in the lower abdomen.

A bifunctional chelator, p-SCN-Bn-DTPA, can be conjugated to amine-derivatized biologicals for radiolabeling with radionuclides such as ^{111}In . As an alternative to DTPA used in several previous studies [23,24], the p-SCN-Bn-DTPA is used herein for the reported increased chelation stability resulting from the extra chelation arm provided by this chelator [3,26-28]. Interestingly, although electrophilic groups are expected to attach to the terminal amine on the cMORF (we earlier confirmed the exclusive attachment in one occasion [24]), the p-SCN-Bn-DTPA also reacts with other endogenous sites. Fortunately, the side products can be dissociated by heating. We now report on the conjugation of p-SCN-Bn-DTPA to an amine-derivatized cMORF, the identification of ^{111}In instability resulting from the side conjugation products, and the development of a modified conjugation method to dissociate the DTPA side products.

EXPERIMENTAL PROCEDURES

Materials and methods

The MORF (5'-TCTTCTACTTCACAAGTA) and cMORF (5'-TAGTTGTGAAGTAGAAGA) were obtained from Gene-Tools (Philomath, OR) with and without a primary amine attached to the 3' equivalent terminal via a 3-carbon linker. The p-SCN-Bn-DTPA was from Macrocyclics (Dallas, TX). The P-4 resin (Bio-Gel P-4 Gel, medium) was from Bio-Rad Laboratories (Hercules, CA). The $^{111}\text{InCl}_3$ was from Perkin Elmer Life Science Inc (Boston, MA). All other chemicals were reagent grade and were used without purification.

The cMORF concentrations were determined by UV spectrophotometry. Size exclusion (SE) HPLC was used for the cMORF analysis. The HPLC system was equipped with a SuperdexTM 75 column (optimal separation range: 1×10^2 to 7×10^3 Da; Amersham Pharmacia Biotech, Piscataway, NJ) and with both UV and radioactivity in-line detectors. The eluant was 0.10 M pH 7.2 phosphate buffer at a flow rate of 0.60 mL/min. Radioactivity recovery was routinely measured and was always greater than 90%.

Conventional and modified conjugation procedures

Conventional conjugation was performed in a NaHCO_3 buffer following published procedures [3,10,29]. Specifically, the amine-derivatized or the native cMORF (1-2 mg) was dissolved in a 0.5 M Na_2CO_3 - NaHCO_3 buffer (pH 9.80) containing p-SCN-Bn-DTPA (10 mg/mL) at a chelator:cMORF molar ratio of about 10:1. The mixture was allowed to react at room temperature over night. A 1×50 cm P-4 column with 0.25 M pH 5.2 NH_4AcO as eluent was used for purification as described previously [24]. Radiolabeling was achieved by adding 1 μL of $^{111}\text{InCl}_3$ in 0.05 M HCl to a 20 μL aliquot of the pooled peak fraction off the P-4 column.

As will be described below, both the NH₂-cMORF and native cMORF after the above conventional conjugation could be radiolabeled with ¹¹¹In with equal effectiveness, but heating resulted in partial loss of ¹¹¹In from the labeled NH₂-cMORF and complete loss from the labeled native cMORF. Since the radiolabeling efficiency before conjugation was less than 1 % for both NH₂-cMORF and native cMORF, the high labeling efficiencies after conjugation confirmed that DTPA groups were attached. In the case of the native cMORF that lacks a terminal primary amine group, the DTPA group must have attached to some endogenous sites within the cMORF sequence. Since the ¹¹¹In-DTPA chelate itself was stable to heating, a reasonable assumption was that the label instability was due to dissociation of the DTPA at the endogenous sites along with its radiolabel. Since the same endogenous sites were present in the conjugated NH₂-cMORF, it was not a surprise that the NH₂-cMORF after conjugation and radiolabeling also showed some instability to heating. To test this assumption and to prepare a conjugate that would provide a stable label, we considered a modified procedure that included a post-conjugation, pre-purification heating step to dissociate the DTPA at the endogenous sites.

Thus 1 mL of 0.25 M NH₄AcO (pH 5.2) was added to the conjugation mixture and the solution was heated at 100 °C for 1 h before purification on a 1×50 cm P-4 column. The NH₂-cMORF conjugated by this modified procedure was again labeled at room temperature and the stability of the label was evaluated by heating at 100 °C as before. The results were compared to those for the NH₂-cMORF and the native cMORF conjugates prepared by the conventional procedure.

Serum stability and biodistribution

The serum stability evaluation and animal studies were performed to compare the label stability and biodistributions of ¹¹¹In when attached to cMORF exclusively at a stable DTPA site and when attached to cMORF exclusively at unstable DTPA site(s). Both preparations were incubated in mouse serum at 37 °C for up to 24 h with periodic measurements by SE HPLC. For biodistribution, 1 µg (10 µCi) labeled cMORF for each preparation was administered to normal CD-1 mice by tail vein with sacrifice at 10 min, 30 min, 1 h, 3 h, or 6 h. The animal procedure was approved by the IACUC of University of Massachusetts Medical School and described previously [30].

RESULTS

Conventional and modified conjugation procedures

While the labeling efficiency by HPLC of greater than 95% was expected for the NH₂-cMORF conjugate by the conventional procedure, it was a surprise that the same labeling efficacy was obtained with the native cMORF conjugate. Also surprising were the identical HPLC profiles of both labeled cMORFs as shown in Fig 1, top traces, left and middle panels. The figure also presents at bottom the traces for the same conjugates but after 30 min of heating at 100 °C. The radiolabeled NH₂-cMORF conjugate lost roughly a third of its label and the loss for the native cMORF conjugate was complete. The chemical forms of ¹¹¹In released by the heating were unidentified but, to judge by retention times, most likely they were some structures similar to the radiolabeled hydrolyzed SCN-Bn-DTPA (not a single peak). After radiolabeling, the HPLC analysis of the overnight-hydrolyzed SCN-Bn-DTPA in the conjugation medium showed multiple peaks covering the range of retention time although with different spectrum of intensity (data not presented). The figure also shows the identical HPLC results for the NH₂-cMORF conjugate by the modified procedure (right panel, top trace), but as shown in the bottom trace, the modified procedure provided a label entirely stable to heating in boiling water bath. To confirm that the labeled cMORF was not denatured by the heating step, an excess of its complement (i.e. MORF) was added.

The HPLC profile showed essentially a complete shift to higher molecular weight, consistent with effective hybridization (data not presented). The small peak in all three top traces at 20 min was due to the cMORF itself. The intensity of this peak varied with temperature and disappeared entirely after heating.

Serum stability and biodistributions

When the NH₂-cMORF was conjugated by the modified procedure and radiolabeled, no change in HPLC profile was observed during 24 h incubation in mouse serum at 37 °C (data not presented). In contrast, as shown in Fig 2, incubation in serum of the labeled native cMORF conjugate from the conventional procedure resulted in a steady partial shift to higher molecular weight, most likely due to binding to serum proteins. The high molecular weight peaks appearing at 13 and 16 min correspond to two mouse serum peaks in the UV 280 nm channel. If the partial protein binding is occurring in vivo, the unstable radiolabeled cMORF conjugate may be expected to slightly elevate the blood and tissue backgrounds.

Table 1 lists the biodistributions over 6 h of the NH₂-MORF conjugated by the modified procedure and the native cMORF conjugated by the conventional procedure, both after radiolabeling. The results confirm that the former labeled NH₂-cMORF preparation provides lower radioactivity levels virtually in all tissues after 1 h. The results for blood, liver, lung, and spleen from the table have been plotted in Fig 3.

DISCUSSION

We expected and have confirmed that NH₂-cMORF can be readily conjugated with pSCN-Bn-DTPA and the resulting DTPA-cMORF can be labeled with ¹¹¹In at a high labeling efficiency. However, in what was unexpected, we observed a surprisingly high ¹¹¹In labeling efficiency for the control native cMORF after conjugation and labeling in an identical manner. Furthermore, the label instability towards heating was evident for both cMORF conjugates and especially for the native cMORF conjugate. Clearly, under the conditions of this investigation, one or more endogenous sites in the cMORF sequence were being conjugated with DTPA. Since the phosphorodiamidate moiety and the morpholino ring in the backbone of cMORF were not reactive nucleophiles, the endogenous sites for attachment were most likely to be the base residues of the cMORF oligomer. To our best knowledge, reactions between an isothiocyanate group and the nitrogenous bases have not been reported. However there are reports on similar reactions of isothiocyanates with aromatic amine and heterocyclic nitrogen [31-36]

The biodistributions in normal mice were measured to evaluate whether the NH₂-cMORF conjugated by the modified procedure would provide a more stable label in vivo. The improved in vivo stability would be reflected by lower tissue backgrounds because the instability was related to serum binding and therefore to higher blood level and normal tissue backgrounds. Rather than comparing with the NH₂-cMORF conjugate by the conventional procedure that provided a mixture of stable and unstable labels, the comparison was against the native cMORF conjugated by the conventional procedure that provided only the unstable labels. The NH₂-cMORF conjugate by the modified procedure was confirmed with a more favorable biodistribution as shown in Table 1 and Fig 3, showing less accumulation in blood and normal tissues ($p < 0.05$ at all time points for liver and spleen and after 2 h for blood and lung).

CONCLUSION

The conventional procedure for the conjugation of NH₂-cMORF with p-SCN-Bn-DTPA resulted in side conjugation products(s) responsible for the ¹¹¹In label instability. Adding a

post-conjugation, pre-purification heating step improved the ^{111}In label stability and lowered tissue background in mice.

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Size exclusion HPLC radiochromatograms of NH₂-cMORF (left panel) and native cMORF (middle panel) after conventional conjugation and of NH₂-cMORF after modified conjugation (right panel), each after radiolabeling. The figure includes radiochromatograms both after incubation at room temperature (top traces) and at 100 °C for 30 min (bottom traces).

Fig 2.
HPLC radiochromatograms over time in 37 °C mouse serum of the labeled native cMORF after conventional conjugation.

Fig 3. Accumulations in selected organs over time of $\text{NH}_2\text{-MORF}$ conjugated by the modified procedure (solid circles) and native cMORF by the conventional procedure (triangles), both after labeling with ^{111}In . Data reproduced from Table 1. Error bars represent one standard deviation.

Table 1

A comparison of biodistribution in normal mice over time of NH₂-MORF conjugated by the modified procedure and native cMORF conjugated by the conventional procedure, both after radiolabeling with ¹¹¹In. Mean ± sd, N=4.

%ID/g	NH ₂ -cMORF				native MORF					
	10 min	30 min	1 h	3 h	6 h	10 min	30 min	1 h	3 h	6 h
Liver	0.66±0.11	0.33±0.04	0.21±0.04	0.18±0.06	0.16±0.03	1.08±0.10	0.77±0.08	0.56±0.09	0.58±0.07	0.68±0.08
Heart	1.11±0.23	0.38±0.11	0.15±0.03	0.09±0.02	0.08±0.01	1.15±0.15	0.42±0.07	0.19±0.04	0.13±0.01	0.12±0.01
Kidney	11.5±1.4	9.01±1.23	8.22±0.51	9.70±3.83	5.98±1.32	13.3±1.0	10.58±1.5 3	10.82±1.5 7	8.27±0.92	9.31±1.13
Lung	1.68±0.24	0.71±0.19	0.29±0.06	0.13±0.02	0.11±0.01	1.93±0.14	0.86±0.36	0.33±0.14	0.32±0.01	0.39±0.21
Spleen	0.44±0.03	0.23±0.01	0.12±0.02	0.11±0.02	0.10±0.02	0.72±0.06	0.54±0.20	0.40±0.13	0.28±0.09	0.42±0.10
Muscle	0.93±0.06	0.33±0.07	0.2±0.09	0.10±0.06	0.07±0.01	1.03±0.11	0.43±0.10	0.16±0.03	0.10±0.02	0.10±0.01
Pancreas	1.11±0.07	0.47±0.13	0.47±0.39	0.11±0.02	0.09±0.02	1.22±0.13	0.54±0.06	0.27±0.02	0.15±0.02	0.15±0.01
Salivary	1.17±0.13	0.44±0.11	0.28±0.13	0.15±0.02	0.15±0.01	1.27±0.13	0.51±0.03	0.27±0.02	0.19±0.02	0.21±0.03
Blood	3.71±0.50	1.32±0.33	0.39±0.10	0.18±0.04	0.11±0.02	3.75±0.35	1.22±0.08	0.59±0.15	0.31±0.06	0.25±0.04
%ID/organ										
Stomach	0.31±0.02	0.14±0.04	0.05±0.02	0.06±0.03	0.02±0.00	0.33±0.03	0.16±0.03	0.08±0.02	0.06±0.02	0.07±0.03
Sm. Int	0.93±0.09	0.37±0.08	0.22±0.09	0.13±0.03	0.08±0.02	1.22±0.10	0.86±0.02	0.58±0.13	0.21±0.05	0.19±0.03
Lg. Int.	0.51±0.03	0.17±0.04	0.14±0.12	0.10±0.01	0.08±0.03	0.47±0.02	0.16±0.02	0.13±0.05	0.63±0.02	0.57±0.22