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Comparative Evaluation of ^{99m}Tc-MAG2-Oligodeoxynucleotides with Phosphodiester or Phosphorothioate Backbones: Preparation, Stability and Biodistribution

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SUMMARY

Antisense oligodeoxynucleotides (ODNs) conjugated to a suitable bifunctional chelating agent and labelled with a suitable radionuclide could become useful in the diagnosis and differentiation of cancers. Radiolabelled natural DNA with phosphodiester (PO) backbone is a candidate for use in imaging, while phosphorothioate (PS) ODNs already are proving their efficacy in antisense therapy. In this study, two PO and two PS ODNs have been conjugated with the N_2SO chelator MAG2 in a procedure designed to make a N_3S tetraligand which forms stable complexes with technetium-99m. Depending on the S-protecting group used and reaction conditions employed, labelling yields of more than 90 % can routinely be obtained. The ^{99m}Tc-MAG2-ODNs are stable in solution, even in the presence of a 30-fold excess of cysteine. However, at higher concentrations of cysteine and in foetal calf serum they are not stable. In vivo in mice, the PO ^{99m}Tc-MAG2-ODNs are degraded more than 50 % in 5 min, while the PS analogues remain intact. The PS and PO ^{99m}Tc-MAG2-ODNs are distributed in a

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similar fashion in normal mice, except for a higher liver retention of the PO ^{99m}Tc-MAG2-ODNs at 60 min.

Key words: Mercaptoacetyldiglycine, technetium-99m, phosphodiester, phosphorothioate, oligodeoxynucleotides, biodistribution.

INTRODUCTION

Antisense oligodeoxynucleotides (ODNs) complementary to oncogenes and labelled with a radionuclide could be useful tracer agents in diagnosis of severe pathologies such as cancers. ODNs have previously been labelled with ^{111}In and ^{99m}Tc by the use of DTPA (1), hydrazinonicotinic acid (Hynic) (2) and mercaptoacetyltriglycine (MAG3) (3,4) as bifunctional chelating agents (BCA) for use in single photon emission computerised tomography (SPECT). Recently, a normal ODN and ODNs with backbone or ribose modifications were labelled with ^{18}F and the biodistribution in baboons monitored by positron emission tomography (PET) (5). With phosphorothioate (PS) backbones, the ODNs become more stable towards enzymatic degradation and ODNs of this type are currently in several clinical studies for antisense therapy (6). Up to this moment, the issues of cell uptake of ODNs and in vivo target recognition remain to be solved before radiolabelled ODNs can prove their clinical efficacy (7). In this process, the development of new BCA-ODN conjugates for labelling with ^{99m}Tc and other imageable radionuclides is of utmost importance.

Mercaptoacetyldiglycine (MAG2) is an N_2SO tetraligand that can be converted to a N_3S mercaptotriamide tetraligand with strong ^{99m}Tc binding properties when the N-hydroxysuccinimide (NHS) ester of S-protected MAG2 is conjugated to the free amino terminal of an aminoalkyl derivatised ODN as shown in Fig.1. In this way, the conjugate between the ^{99m}Tc binding tetraligand and the ODN is one glycine residue shorter than if MAG3 is used as the BCA.

In previous studies we conjugated phosphodiester (PO) ODNs with S-benzoyl-MAG3, labelled the conjugates with ^{99m}Tc and demonstrated intact RNA hybridisation capacity of the

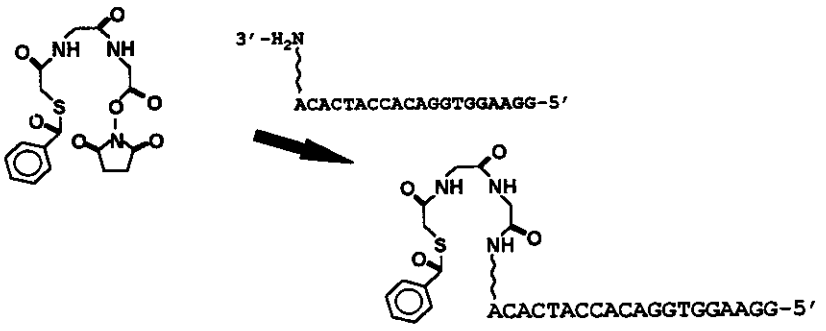

Figure 1. Conjugation of S-benzoyl-MAG2-N-hydroxysuccinimide ester to the 3'-aminohexyl modified oligodeoxynucleotide GX-1

^{99m}Tc-MAG3-ODNs (4,8). The aims of this study were to comparatively investigate the efficiency of labelling of S-benzoyl(Bz)-MAG2-ODN and S-benzyl(Bn)-MAG2-ODN conjugates of two PO ODNs and two PS ODNs and to study the stability and biodistribution of the resulting ^{99m}Tc-MAG2-ODN complexes. We also investigated the possibility of obtaining ^{99m}Tc-MAG2-ODNs without the need for a final purification step, which was found necessary in the case of the ^{99m}Tc-MAG3-ODNs reported previously. The influence of shortening the conjugation linker with one glycine residue on the hybridisation capacity of the radiolabelled ODN will be reported in a separate paper.

MATERIALS AND METHODS

Synthesis

General

Thin-layer chromatography was carried out using precoated TLC plates (Alugram® SIL G/UV254, Macherey-Nagel, Düren, Germany). For purification by column chromatography, silicagel (MN Kieselgel 60, 70-230 mesh ASTM, Macherey-Nagel) was used. ^{1}H- and ^{13}C-NMR spectra were recorded in DMSO-d_6 on a Gemini 200 MHz spectrometer (Varian, Palo Alto, CA). Chemical shifts are reported in ppm relative to TMS ($\delta = 0$).

Synthesis of S-benzoylmercaptoacetic acid and succinimidyl-S-benzoylthioglycolate was performed as described by Schneider et al (9). S-benzoylmercaptoacetyldiglycine was synthesised as described by. Brandau et al (10).

S-Benzoyl mercaptoacetyldiglycine-N-hydroxysuccinimide ester

A solution of S-benzoyl mercaptoacetyldiglycine (7.34 g; 23 mmol) and N-hydroxysuccinimide (NHS) (2.65 g; 23 mmol) in CH_3CN (100 ml) was heated to reflux and solid dicyclohexylcarbodiimide (DCC) (5.15 g; 25 mmol) was added in portions. The mixture was stirred overnight at room temperature. The precipitate was filtered off and washed twice with warm CH_3CN. The filtrate was evaporated and the residue stirred with THF (100 ml). The precipitate was filtered off, washed with hot THF (100 ml) and finally recrystallised from ethyl acetate to yield 2.65 g (28.5 %) of white solid.

^{1}H-NMR δ 2.81 (s, 4H, $COCH_2CH_2CO$), 3.81 (d, ^3J=5.8Hz, 2H, CH_2NHCO), 3.90 (s, 2H, SCH_2CO), 4.29 (d, ^3J=5.8Hz, 2H, CH_2NHCO), 7.55 (t, 2H, 3′-H_{Ar}, 5′-H_{Ar}), 7.70 (t, 1H, 4′-H_{Ar}), 7.95 (d, 2H, 2′-H_{Ar}, 6′-H_{Ar}), 8.60 (t, ^3J=5.8Hz, 2H, N*H*CO).

^{13}C-NMR δ 25.51 ($COCH_2CH_2CO$), 32.53 (SCH_2CO), 38.29 (CH_2NHCO), 42.24 (CH_2NHCO), 127.10 (*m*-C_{Ar}), 129.38 (*o*-C_{Ar}), 134.30 (*p*-C_{Ar}), 136.15 (*i*-C_{Ar}), 166.53 (*C*ONH), 167.50 (*C*ONH), 169.72 (*C*OS), 170.23 ($COCH_2CH_2CO$), 191 (*C*OO).

N-hydroxysuccinimidyl-S-benzylthioglycolate

S-Benzylthioglycolic acid (18.224 g; 100 mmol) and N-hydroxysuccinimide (11.5 g; 100 mmol) were dissolved in 200 ml THF. The reaction mixture was cooled to 0-5 °C and a solution of DCC (22.33 g; 110 mmol) in 100 ml THF was added dropwise over a period of 30 min. The reaction mixture was stirred at 0°C for 1 h and then for 16 h at room temperature. The precipitate formed was filtered off and the filter cake was washed with boiling THF (2x100 ml). The filtrates were combined and evaporated to yield a colourless oil. After crystallisation from hexane/diethylether a white crystalline product was obtained (19.33 g; 69.3%).

S-benzylmercaptoacetyldiglycine

N-hydroxysuccinimidyl-S-benzylthioglycolate (19.33 g; 69.2 mmol) was dissolved in 150 ml acetonitrile at 50°C and diglycine (9.142 g; 69.2 mmol) dissolved in a mixture of NaOH 1 N (70 ml) and water (70 ml) was added. The reaction mixture was stirred at room temperature overnight and then acetonitrile was removed by evaporation. The resulting aqueous solution was washed with diethylether (2x250 ml) and adjusted to pH 2 by the addition of hydrochloric acid 6 N. The resulting precipitate was filtered off, washed with water and dried under reduced pressure. Yield: 14.5 g (71%).

S-Benzylmercaptoacetyldiglycine-N-hydroxysuccinimide ester

To a solution of S-benzylmercaptoacetyldiglycine (5.92 g; 20 mmol) and N-hydroxysuccinimide (2.3 g; 20 mmol) in 100 ml acetonitrile/dimethylformamide (9:1) was added DCC (4.53g; 22 mmol). The reaction mixture was stirred overnight, the dicyclohexylurea formed was filtered off and the filtrate was evaporated under reduced pressure. Acetonitrile (50 ml) was added to the dry residue and again the precipitate was filtered off. The filtrate was evaporated and the residue was stirred with THF (50 ml) during 72 h. The white precipitate was filtered off, washed with THF (100 ml) and dried under reduced pressure at 60°C. Yield: 3.22 g (41%)

^{1}H-NMR δ 2.8 (s, 4H, COC*H*$_2$C*H*$_2$CO), 3.1 (ds, 2H, SC*H*$_2$CO), 3.8 (s, 2H, ArC*H*$_2$; d, 2H, NHC*H*$_2$CO), 4.3 (d, 2H, C*H*$_2$NHCO), 7.3 (s, 5H, Ar*H*), 8.35 (t, 1H, CO-N*H*), 8.6 (t, 1H, CON*H*).

Oligodeoxynucleotides

GX-1 is a 20-nucleotide (20-nt) antisense oligodeoxynucleotide with proven access to a site on exon 2 of the mRNA of the CAPL oncogene (11). The sequence of GX-1 is 5'-GGA AGG TGG ACA CCA TCA CA-3'. CTRL2 is a scrambled control ODN that contains the same 20 nucleotides as GX-1, but in a random order. The sequence of CTRL2 is 5'-AGT GAC CGA CTA GGC ACC AA-3'. Phosphodiester GX-1 and CTRL2 with an aminohexyl linker on the 3'-end were synthesised by Eurogentec (Ougree, Belgium). The corresponding phosphorothioate GX-1 and CTRL2 were synthesised by Oligon AS (Oslo, Norway).

In order to study the labelling process and possible in vitro/in vivo instability, a 15-nt and a 10-nt ODN with phosphodiester backbones were synthesised by Eurogentec. These shorter ODNs correspond to respectively the first 15 and 10 deoxynucleotides of GX-1 and were made with a 3'-aminohexyl linker as the other ODNs.

Conjugation of S-Bn/Bz-MAG2-N-hydroxysuccinimide ester with PO and PS ODNs

Lyophilised ODNs in a quantity range of 20-50 nmol were dissolved in 100 µl 0.05 M phosphate buffer (PB) pH 7.5. The solutions were heated in a boiling water bath for 2 min and snap cooled in ice water. A solution of 400 µg S-benzoyl (or S-benzyl-) mercaptoacetyldiglycine-N-hydroxysuccinimide ester in DMF was added under vortex mixing. The conjugation reaction was allowed to proceed at ambient temperature for at least 2 hours. In several cases, the reaction mixture was left for longer periods of time, in one experiment up to 1.5 weeks.

After conjugation, the S-benzoyl-MAG2-ODNs were purified by high performance liquid chromatography (HPLC). Isolated peaks were evaporated to dryness under reduced pressure and the residue was stored or used as such for labelling with ^{99m}Tc.

HPLC conditions: Hewlett Packard system 1050 (autoinjector, on-line degassing, gradient pump, column oven, variable wavelength detector set at 254 nm). *Column:* SynChropack RP-P C18 6.5 µm column, 4.6 mm x 250 mm (SynChrom Inc., Lafayette, In). *Mobile phases at a flow rate of 1 ml/min:* linear gradient mixtures of 0.025 M PB pH 5.85 (A), distilled water (B) and methanol (C). Gradient: 0':100% A, 15':80% A/20% C, 20':50% A/50% C, 20.1':50% B/50% C, 25':10% B/90% C, 28':10% B/90% C, 30':100% B.

The S-benzyl-MAG2-ODNs were purified on a Sep-Pak C18 Light mini-column (Waters, Milford, Massachusetts) according to the following procedure: preconditioning of the column with successively 10 ml 96% (V/V) ethanol and 5 ml 0.05 M PB pH 7.5; after application of the conjugation reaction mixture (110 µl), the column was eluted with successively 1 ml 0.05 M PB pH 7.5, 1 ml distilled water and 1 ml CH_3CN. The acetonitrile eluate was evaporated to dryness under reduced pressure to obtain a residue of the S-benzyl-MAG2-ODNs.

Labelling with technetium-99m

The S-Bz/Bn-MAG2-ODNs obtained from HPLC or Sep-Pak purification were dissolved in 100 µl 1 M carbonate buffer (CB) pH 8.5 or 9.3, and subsequently 5 mg potassium sodium tartrate, 100 µg $SnCl_2.H_2O$ dissolved in 50 µl 0.05 N HCl and 1 ml 0.1-1 GBq sodium pertechnetate eluate (IFETEC, Isopharma AS, Norway) were added. The mixture was heated in a boiling water bath for 10 min. After cooling to ambient temperature, the radiochemical purity was determined by reversed phase HPLC (as described above for purification of the S-benzoyl-MAG2-ODN conjugate). For determination of specific activity, the oligonucleotide concentration was determined using a GeneQuant spectrophotometer (Pharmacia Biotech, Cambridge, England) measuring absorbance at 260 nm. The radioactivity in the sample was determined in a VDC 404 ionization chamber (Veenstra Instruments, Joure, The Netherlands).

Stability of ^{99m}Tc-labelled MAG2-ODNs in solution

After labelling, the reaction mixtures were stored at room temperature and samples withdrawn for HPLC analysis at fixed time points up to 24 hours. After 30 min and 3 hours, each ^{99m}Tc-MAG2-ODN was analysed using Sep-Pak C18 Plus columns and the radioactivity in each elution fraction was counted using a Packard TopCount gamma counter (Packard Instrument Company, Meriden, CT). Each Sep-Pak column was conditioned with 5 ml ethanol followed by 2.5 ml 0.1 M Tris buffer pH 8; then 6 µl sample (diluted to 60 µl in distilled water) was applied and the column eluted with 5 ml distilled water followed by 2 ml CH_3CN. The activity eluting in the acetonitrile fraction was considered to be due to intact ^{99m}Tc-MAG2-ODN (see below).

Stability of ^{99m}Tc labelled MAG2-ODNs to excess cysteine

The stability of the ^{99m}Tc-MAG2-ODN complexes to cysteine challenge was studied at two cysteine concentrations. A 300- and 30-fold excess of L-cysteine over S-Bn/Bz-MAG2-ODN was added to each reaction mixture and the stability of the ^{99m}Tc labelled conjugate studied by HPLC analysis at fixed time points over the next 3 hours. A control sample without L-cysteine was analysed in conjunction with the last time point.

Stability of ^{99m}Tc-labelled MAG2-ODNs in serum

To determine the stability of the ^{99m}Tc labelled MAG2-ODNs towards transchelation, each ^{99m}Tc-MAG2-ODN (100 µl) labelling reaction mixture was mixed with 400 µl heat-inactivated foetal calf serum (FCS) and the mixture incubated for 3 hours at 23°C. At 5, 15 and 60 min, samples were analysed by HPLC after elution from a Sep-Pak C18 Plus column (5 ml ethanol, 2.5 ml 0.1 M Tris.HCl buffer pH 8, 60 µl sample, 5 ml distilled water, 2.5 ml CH_3CN). At 30 min and 3 hours, the stability was studied by Sep-Pak C18 Plus elution alone. Four elution fractions from the mini-column were analysed for radioactivity: (1) the 5 ml of water, (2) the first 0.5 ml CH_3CN, (3) the next 0.7 ml CH_3CN and (4) the final 1.3 ml of CH_3CN. The total radioactivity of each fraction and the remaining activity on the mini-column was counted using a 2-in. NaI(Tl) scintillation detector connected to a multichannel analyser (Canberra Series 20, Meriden, CT).

Stability of ^{99m}Tc-labelled MAG2-ODNs in blood

The stability of ^{99m}Tc-MAG2-GX-1 with PO or PS backbones in vivo in blood was studied in mice. An activity of 37 MBq of each radiolabelled conjugate (0.3–2.5 nmol) in a volume of 100 µl, was injected intravenously in each of three mice via a tail vein. The animals were sacrificed at 5, 15 and 60 min post injection and blood sampled in polypropylene tubes containing 50 µl heparin solution (5000 IE/ml). The blood samples were separated and analysed on Sep-Pak C18 Plus mini-columns and using HPLC. The mini-columns were first conditioned with 5 ml ethanol, followed by 5 ml 1 M CB pH 8.5 and then up to 500 µl of the blood sample was applied. Each column was subsequently eluted with 5 ml distilled water and then 2.5 ml CH_3CN, which was collected in three fractions (0.7 ml, 0.5 ml and 1.3 ml). The radioactive content in each fraction was quantified as described above.

Biodistribution in mice

After approval by the National Committee for Experiments on Animals, the biodistribution of the ^{99m}Tc-MAG2-GX-1 and ^{99m}Tc-MAG2-CTRL2 with PO as well as PS backbones was studied in normal white mice (Hsd:KRF, body mass 20-25 g). A dose of 370 kBq (3-25 pmol)

of each of the conjugates was injected via a tail vein in each of eight mice. Four animals in each group were sacrificed after 5 min and the rest at 60 min post injection. Organs were dissected and the radioactivity in each organ counted in a 3-in. NaI(Tl) well crystal sample changer (Wallac Oy, Turku, Finland). The activity in each organ was expressed as percentage of injected activity, equal to the sum of net counts in all organs. To obtain data for excretion in the urine, the floor paper in the individual cages was removed and counted along with the urinary bladder. Blood values were calculated on the assumption that blood volume is 7 % of total animal body mass. Corrections were made for background radiation and physical decay during counting.

RESULTS

Conjugation yield

S-Benzyl-MAG2 could be conjugated to all the 20-nt ODNs at pH 7.5, as judged by the ability to label the ODNs with ^{99m}Tc after conjugation in contrast to the unconjugated ODNs. However, we were not able to distinguish by HPLC the conjugate from the main ODN peak eluting at 19-20 min in HPLC either before or after purification on the mini-column. It cannot be ruled out that possibly the quantity of the conjugate was too low to be detected by the variable wavelength detector. Therefore, the conjugation yield has not been quantified.

The conjugates of S-benzoyl-MAG2 with the ODNs appeared as a separate peak eluting at 22.0-22.6 min in the HPLC. The conjugation yield could therefore be determined on the assumption that the S-benzoyl-MAG2 moiety does not contribute considerably to the absorption of the conjugate at 254 nm. The conjugation yield for all four 20-nt ODNs is presented in table 1. No differences in conjugation yield were obtained for reaction times ranging from 2 hours to 1.5 weeks (results not shown).

The 15-nt ODN conjugate to S-Bz-MAG2 eluted 1.3 min after the pure ODN peak (at 19.6 and 18.3 min, respectively). The conjugation yield was 12 %.

The 10-nt ODN conjugate to S-Bz-MAG2 eluted 1 min after the unconjugated ODN peak (at 18.5 and 17.8 min, respectively). The conjugation yield was 9 %.

Table 1. Conjugation yield of S-benzoyl-MAG2-ODN after conjugation for 24 hours as percentage (and range) of total quantity ODN present in the reaction mixture (n = 3)

ODN	PO-GX-1	PO-CTRL2	PS-GX-1	PS-CTRL2
Conjugation yield	33.9 (33.1-34.6)	5.4 (4.2-6.9)	35.6 (33.9-37.5)	25.4 (21.5-29.3)

Labelling with technetium-99m

The S-Bz/Bn-MAG2-ODN conjugates were labelled with ^{99m}Tc as described above to give ^{99m}Tc-MAG2-ODN complexes eluting at 21-22 min in the HPLC system. The final radiolabelled complex of each ODN was the same, irrespective of the nature of the S-protective group of the starting material, i.e. benzyl or benzoyl. The labelling yields obtained with S-Bn-MAG2-ODNs using purification with a mini-column, ranged from 70 to 84 % at 30 min post labelling. The only two impurities were ^{99m}Tc-MAG2 and ^{99m}Tc-tartrate, eluting at 14.8 and 3.5 min, respectively. Since the S-Bn-MAG2-ODN conjugate could not be distinguished from the pure ODN, it is important to note that attempts to label the unconjugated ODN in the absence of S-Bn/Bz-MAG2-NHS, gave only ^{99m}Tc in colloidal form, a result in good accordance with previous reports (2, 4).

With S-Bz-MAG2-ODNs, labelling yields above 90 % were repeatedly reached at 30 min post labelling (table 2). The main impurity was ^{99m}Tc-MAG2 in all preparations.

Labelling of the S-Bz-MAG2 conjugate of the 15-nt ODN gave 86.2 % of a ^{99m}Tc-MAG2-ODN peak eluting at 20.6 min. The main impurity was ^{99m}Tc-MAG2. Labelling of the conjugate of the 10-nt ODN gave a labelling yield of only 29.1 % ^{99m}Tc-MAG2-ODN eluting at 20.3 min, while the ^{99m}Tc-MAG2 peak amounted to 58 %. The low labelling yield is possibly due to contamination with S-Bz-MAG2 in the fraction collected from the HPLC. The S-Bz-MAG2 peak in the purification of the reaction mixtures of these conjugation experiments shows pronounced tailing and probably contaminates the peak collected at 18.5 min.

***Table 2.** Labelling yields for the ^{99m}Tc-MAG2-ODNs obtained from S-benzoyl-MAG2-ODNs (average of 3 experiments)*

Substance	Labelling yield, % (range)
^{99m}Tc-MAG2-GX-1 (PO)	93.2 (90.4-96.9)
^{99m}Tc-MAG2-CTRL2 (PO)	94.3 (92.4-95.1)
^{99m}Tc-MAG2-GX-1 (PS)	91.4 (89.5-94.7)
^{99m}Tc-MAG2-CTRL2 (PS)	95.7 (94.2-97.2)

Stability

The stability of the ^{99m}Tc-labelled 20-nt ODNs with PO and PS backbones was studied in the reaction mixture, i.e. a predominantly aqueous solution. A tendency for an increase in radiochemical purity was seen with three out of four substances (fig 2). The relative amounts of the fourth substance (PO ^{99m}Tc-MAG2-CTRL2) remained unchanged during the time course of the study. The increase is probably due to transchelation from the main impurity, ^{99m}Tc-MAG2, as the proportion of this substance decreased in accordance with the ^{99m}Tc-MAG2-ODN increase.

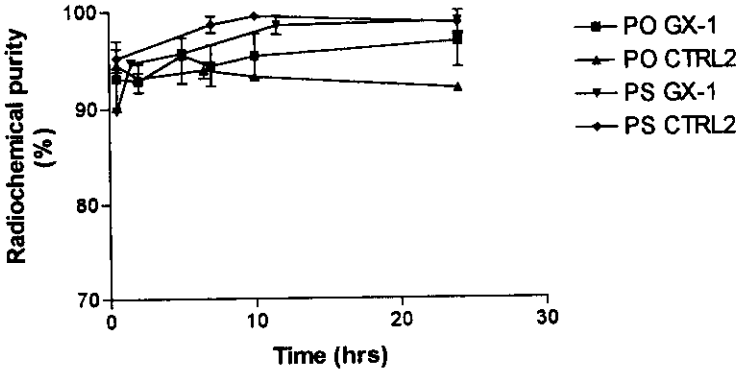

Figure 2. Stability of four ^{99m}Tc-MAG2-ODNs in aqueous solution. All values are means of 2 or more results, except for PO CTRL2 at 24 hours. Ranges of observations are indicated.

Cysteine challenge has frequently been applied to technetium-99m labelled compounds in order to study the chelation strength of the molecule and to get an indication of the likely stability of the complex in vivo (12, 13). The stability of the 20-nt ^{99m}Tc-MAG2-ODNs to challenge from a 30- and 300-fold excess of L-cysteine was studied over a period of 3 hours after addition of the challenger. All complexes were stable in the presence of a 30-fold excess of cysteine (table 3). In fact, cysteine at this concentration did not interfere with the increase in radiochemical purity observed in aqueous solution. However, all ^{99m}Tc-MAG2-ODNs showed instability to a 300-fold excess of cysteine. Particularly the phosphorothioates expressed pronounced instability.

Table 3. Radiochemical purity (%) of ^{99m}Tc-MAG2-ODNs after 3 hours with and without challenge of a 30- and 300-fold excess of L-cysteine

ODN	Without cysteine	30:1 Cysteine:ODN	300:1 Cysteine:ODN
GX-1 (PO)	95.3	> 99	9.5
CTRL2 (PO)	94.0	92.0	68.9
GX-1 (PS)	98.3	98.3	< 1
CTRL2 (PS)	> 99	> 99	< 1

After incubation in foetal calf serum, the stability of the ^{99m}Tc-MAG2-ODNs was first assessed by elution through a mini-column at 30 min and 3 hours after mixing with the serum. In the first 5 ml of water, contaminants like $^{99m}TcO_4^-$ and ^{99m}Tc-MAG2 were eluted along with the serum proteins. The next 2.5 ml CH_3CN contained the ^{99m}Tc-labelled ODNs regardless of PO or PS backbone, as determined in a separate experiment (data not shown). Fig. 3 shows that the percentage of the activity eluting with the CH_3CN-fractions was relatively constant, starting at more than 99 % and staying well above 80 % for all ^{99m}Tc-MAG2-ODNs even after 3 hours. Both of the PO ^{99m}Tc-MAG2-ODNs are less stable than the corresponding PS ^{99m}Tc-MAG2-ODNs.

For one PO-ODN (^{99m}Tc-MAG2-CTRL2) and one PS-ODN (^{99m}Tc-MAG2-GX-1), samples were removed from incubation in FCS at 5, 15 and 60 min, separated on a mini-column and the fractions subsequently analysed on HPLC. The analyses revealed the radioactive species eluting with each fraction off the mini-column, and the overall content of intact ^{99m}Tc-MAG2-ODN could be calculated (fig. 4). The ^{99m}Tc-MAG2 contaminant eluted with the aqueous eluate along with some of the ^{99m}Tc-MAG2-ODN. In the 0.5 ml and 1.3 ml CH_3CN fractions,

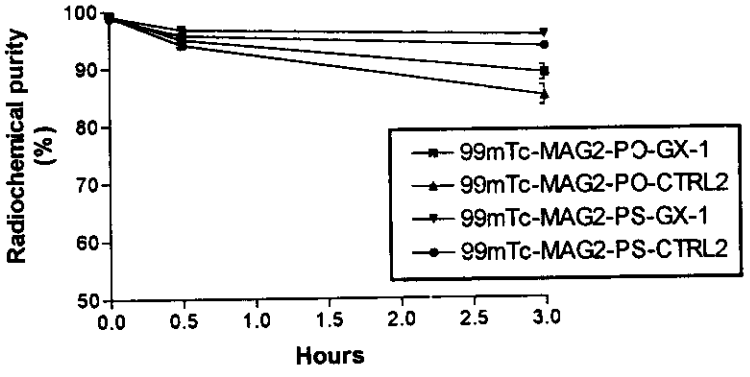

Figure 3. Radiochemical purity of four ^{99m}Tc-MAG2-ODNs after incubation in foetal calf serum (n = 2) as measured by separation on a mini-column. Ranges of observations are indicated.

pure ^{99m}Tc-MAG2-ODNs were eluted, while in the middle 0.7 ml fraction (and only there), an unknown impurity appeared. On HPLC, this impurity eluted as a very broad peak 1-4 min prior to the ^{99m}Tc-MAG2-ODN peaks. In the HPLC UV trace, no peak was accompanying the unknown radioactive impurity. In all experiments, the radioactivity remaining on the column accounted for less than 0.3 %.

The in vivo stability of one PO-ODN (^{99m}Tc-MAG2-GX-1) and one PS-ODN (^{99m}Tc-MAG2-GX-1) in blood was studied in normal white mice. The elution profile of a 0.5-ml blood sample

separated on a mini-column is described in table 4. The activity in each fraction was too low to allow qualitative radio-HPLC analysis of all fractions. Only the 0.5-ml and 0.7-ml CH_3CN-fractions of the 5-min experiments could be analysed by radio-HPLC. The chromatograms of each of the 0.5-ml CH_3CN-fractions from PO and PS ^{99m}Tc-MAG2-GX-1 are shown in fig. 5. The PS ^{99m}Tc-MAG2-ODN did not show major signs of degradation, as only the original ^{99m}Tc-MAG2-ODN peak eluting at 21.6 min could be detected. The PO ^{99m}Tc-MAG2-GX-1, however, appears only partially intact at 5 min post injection, as a new radioactive peak at 20.3 min appears (43 % and 57 %, respectively).

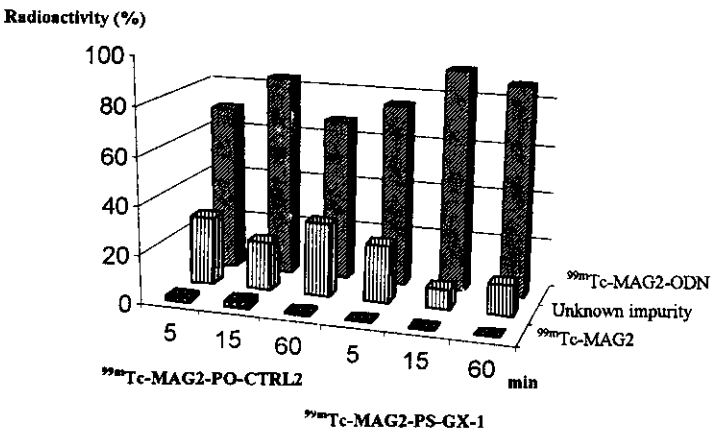

Figure 4. Content of radioactive components in mini-column elution fractions 5, 15 and 60 min after incubation of two ^{99m}Tc-MAG2-oligodeoxynucleotides (ODNs) in foetal calf serum at 23°C and quantitative analysis by radio-HPLC.

The elution profiles after the metabolite experiments are quite similar to the elution profile observed for the FCS experiments. Also in the FCS-experiments, 10-20 % of the radioactivity could be found in the 5-ml water fraction. Radio-HPLC of those fractions revealed that, in all cases, leakage of the intact ^{99m}Tc-MAG2-ODNs into the water fraction in the presence of serum proteins accounted for more than 95 % of this activity.

Table 4. *Percentage radioactivity in mini-column elution fractions after separation of whole blood from mice injected with PO and PS ^{99m}Tc-MAG2-GX-1*

Substance and incubation time	5 ml water (%)	2.5 ml acetonitrile (%)	Column (%)
^{99m}Tc-MAG2-CTRL2 (PO), 5 min	15.2	81.9	2.9
^{99m}Tc-MAG2-CTRL2 (PO), 15 min	15.2	61.1	23.7
^{99m}Tc-MAG2-CTRL2 (PO), 60 min	26.8	60.5	12.7
^{99m}Tc-MAG2-GX-1 (PS), 5 min	7.5	90.9	1.6
^{99m}Tc-MAG2-GX-1 (PS), 15 min	9.0	87.6	3.4
^{99m}Tc-MAG2-GX-1 (PS), 60 min	27.4	62.2	10.4

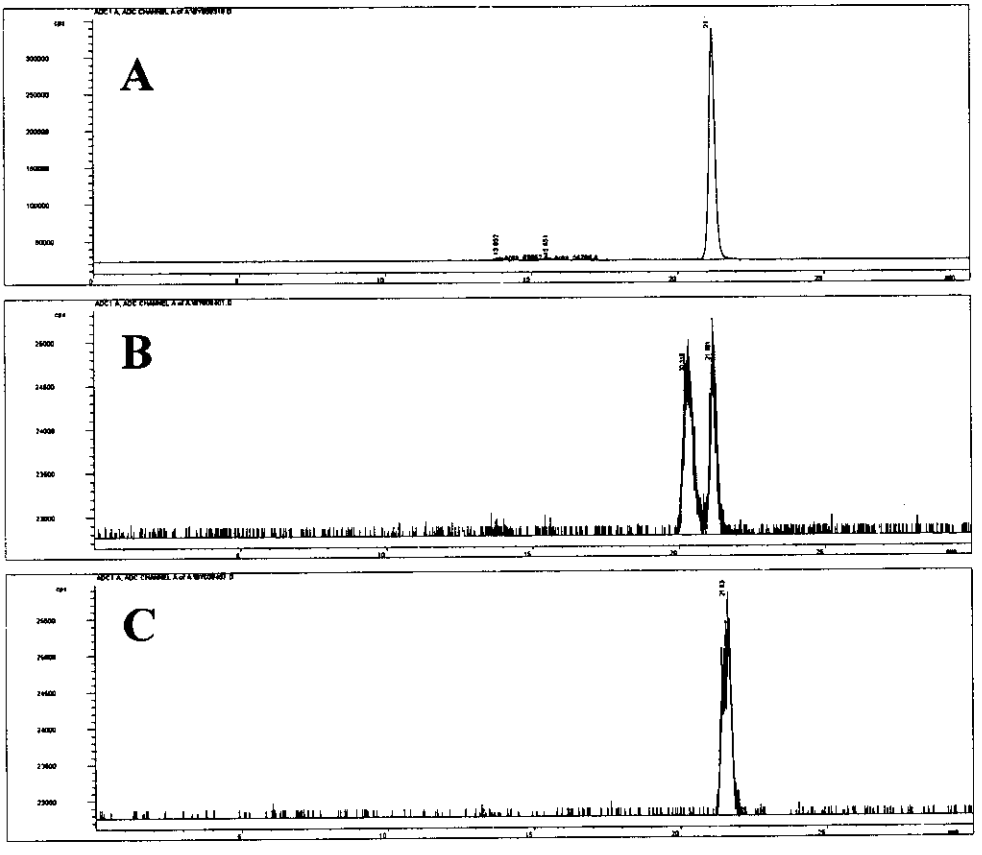

Figure 5: Radiochromatograms of ^{99m}Tc-MAG2-GX-1 with phosphodiester (PO) backbone 30 min after labelling (A), the content of 0.5 ml CH_3CN fractions from mini-column separation of blood samples withdrawn 5 min after injection into mice of ^{99m}Tc-MAG2-GX-1 with phosphodiester (B) and phosphorothioate (C) backbones.

Biodistribution

Table 5 gives an overview of the biodistribution of the ^{99m}Tc-MAG2-ODNs and ^{99m}Tc-MAG2 in normal mice at 5 min and 60 min post injection. In general, the four ^{99m}Tc-labelled ODNs were evenly and similarly distributed throughout the test animals. However, there were some differences in blood, liver and intestinal uptake. The main route of excretion was urinary. After 5 min, 28-38 % of the injected activity had been excreted to the urine, increasing to 67-76 % after 60 min, irrespective of ODN backbone. Some hepatobiliary excretion was also observed,

Table 5. *Biodistribution in mice of two PO and two PS ^{99m}Tc-MAG2-ODNs 5 min and 60 min after injection (n = 4). Values are expressed as mean % uptake of injected dose (ID) per gram tissue ± standard deviation (SD), except for values (*) expressed as total radioactivity excreted in urine and bladder*

5 min

Organ	^{99m}Tc-MAG2-PO-GX-1	^{99m}Tc-MAG2-PO-CTRL2	^{99m}Tc-MAG2-PS-GX-1	^{99m}Tc-MAG2-PS-CTRL2	^{99m}Tc-MAG2
Urine	38.3 (±5.3)*	32.7 (±9.3)*	28.7 (±18.9)*	36.9 (±9.1)*	14.8 (±2.1)*
Kidneys	10.4 (±8.8)	9.7 (±2.1)	9.6 (±2.7)	9.7 (±3.4)	14.9 (±1.2)
Liver	25.9 (±5.3)	29.3 (±4.9)	26.2 (±4.6)	20.4 (±5.4)	6.5 (±0.3)
Spleen	0.4 (±0.1)	0.8 (±0.5)	0.3 (±0.1)	0.3 (±0.1)	2.3 (±0.2)
Intestines	3.1 (±0.4)	3.3 (±0.4)	5.2 (±2.4)	3.4 (±0.9)	3.1 (±0.1)
Stomach	0.3 (±0.1)	3.9 (±7.0)	0.4 (±0.3)	0.3 (±0.1)	1.3 (±0.4)
Lungs	1.1 (±0.3)	1.1 (±0.2)	1.6 (±0.5)	0.9 (±0.1)	6.5 (±0.4)
Heart	0.7 (±0.2)	0.9 (±0.1)	0.9 (±0.1)	0.7 (±0.2)	5.3 (±0.8)
Blood	2.1 (±0.2)	2.4 (±0.3)	3.1 (±0.6)	2.5 (±0.4)	17.2 (±0.5)

60 min

Organ	^{99m}Tc-MAG2-PO-GX-1	^{99m}Tc-MAG2-PO-CTRL2	^{99m}Tc-MAG2-PS-GX-1	^{99m}Tc-MAG2-PS-CTRL2	^{99m}Tc-MAG2
Urine	74.0 (±3.9)*	67.7 (±1.1)*	76.4 (±4.4)*	72.5 (±5.8)*	48.2 (±7.1)*
Kidneys	0.7 (±0.2)	3.9 (±5.1)	0.7 (±0.5)	0.5 (±0.2)	5.1 (±1.0)
Liver	5.5 (±1.1)	8.0 (±3.8)	1.5 (±0.5)	1.2 (±0.7)	1.7 (±0.3)
Spleen	0.1 (±0.1)	0.4 (±0.2)	0.1 (±0.1)	0.1 (±0.1)	0.8 (±0.3)
Intestines	5.3 (±0.5)	7.2 (±2.6)	5.5 (±0.9)	6.9 (±1.9)	6.9 (±0.5)
Stomach	0.1 (±0.1)	1.3 (±1.7)	0.3 (±0.2)	0.4 (±0.4)	0.4 (±0.3)
Lungs	0.1 (±0.1)	0.4 (±0.2)	0.1 (±0.1)	0.1 (±0.1)	1.7 (±0.1)
Heart	0.1 (±0.1)	0.3 (±0.2)	0.1 (±0.1)	0.1 (±0.1)	1.0 (±0.2)
Blood	0.3 (±0.1)	0.7 (±0.3)	0.3 (±0.1)	0.2 (±0.1)	4.2 (±1.0)

** percent of injected dose in total urine*

with an approximately equal initial uptake of about 31 % for the PO and PS ODNs at 5 min. At 60 min, the liver retention of the ^{99m}Tc-labelled PO ODNs was significantly higher than that of the PS ODNs (p=0.002, 2-sided student's t-test).

DISCUSSION

When S-benzoyl-MAG2 was conjugated to 20-nt ODNs, up to 35 % of the ODN reacted to give an ODN-BCA conjugate. The conjugation yield was apparently independent of PO or PS backbone, as GX-1 (PO), GX-1 (PS) and CTRL2 (PS) all gave comparable conjugation yields. CTRL2 (PO) continuously gave lower yields, without any obvious reason. However, all four S-benzoyl-ODN conjugates appeared as distinct peaks in the HPLC chromatograms and were easily collectable. This was not the case when S-benzyl-MAG2 was conjugated to the ODNs. No new HPLC-peaks could be detected at 254 nm, except for the appearance of S-Bn-MAG2-NHS active ester and the free acid S-Bn-MAG2. This could be due to a very low conjugation yield or to co-elution of the conjugate with the unconjugated ODN. However, with the mini-column separation applied in this study,

the mixture of ODN and ODN-MAG2 conjugate was collected and labelled to give ^{99m}Tc-MAG2-ODN complexes identified as the same as those obtained when the S-Bz-MAG2-ODN conjugates were labelled. Unconjugated ODN can not be labelled directly.

A benzyl thioether is by far more stable than a benzoyl thioester. While thioesters are efficiently hydrolysed at slightly alkaline pH, benzyl thioethers require quite drastic conditions for their decomposition, such as treatment with sodium in liquid ammonia (14). In the conditions of exchange labelling normally used to label S-benzyl protected mercaptoacetyltripeptides with ^{99m}Tc, only trace amounts of free thiols are generated, but this is sufficient to allow a reasonable binding of ^{99m}Tc (15). However, this high stability of the thioether could be the reason why labelling yields above 84 % could not be obtained with the S-Bn-MAG2-conjugate, regardless of prolonged heating of the labelling mixture (results not shown). When

benzoyl was the protective group, all four ODN-conjugates could easily be labelled to a radiochemical yield better than 90 % at 100°C for 10 min.

The ^{99m}Tc-MAG2-ODNs were stable in solution once formed. In general, no sign of degradation could be observed 24 hours after labelling. On the contrary, an increase in radiochemical purity over a 24-hour period was observed when S-Bz-MAG2-ODNs were labelled, eventually resulting in more than 99 % pure ^{99m}Tc-MAG2-ODNs. A simultaneous decrease in the ^{99m}Tc-MAG2 impurity was observed, leading to the conclusion that ^{99m}Tc was transchelated from the N_2SO-chelator MAG2 to the stronger N_3S-chelator in the MAG2-ODN conjugate. It can be assumed that, as a function of time, an increasing fraction of the thiols of the conjugate are present in deprotected form when the S-Bz-MAG2-ODN conjugate is used for labelling. Therefore, transchelation is likely to occur. The same observation was not seen for S-Bn-MAG2-ODNs, even though these ^{99m}Tc-MAG2-ODNs were also very stable once formed. This may be due to the more stable benzyl protecting group which is not split off at ambient temperature in the conditions present in the labelling reaction mixture.

For potential use as a tracer agent in diagnostic imaging, the radiolabelled ODNs must be relatively stable in vivo towards degradative enzymes as well as against competitive ^{99m}Tc-binding ligands such as free thiols from glutathione or proteins (16, 17). Three different assays were utilised to study the stability of the ^{99m}Tc-MAG2-ODNs. Cysteine challenge was studied first as a test for complex strength of the radiolabelled ODNs. Then, heat-inactivated foetal calf serum was used in order to further study the transchelation process and possibly protein association with only minor interference from degradative enzymes (18). The last test was to study the resistance of the ^{99m}Tc-MAG2-ODNs to enzymes, transchelation and protein binding in circulating blood in vivo. Unmodified PO ODNs have a survival time ranging from seconds to minutes in blood due to the presence of exonucleases, in particular those attacking from the 3'-end (19, 20, 21). With a PS modification, ODNs can survive for hours in vivo (22, 23, 24). The PO ODNs of this study were protected on the 3'-end by the aminohexyl linker and the ^{99m}Tc-MAG2 chelate as a protective cap (25). With such an end capping, more than 40 % of

the PO ^{99m}Tc-MAG2-ODN was found to be intact after 5 min in circulating blood. The PO ^{99m}Tc-MAG2-ODN was most likely degraded to smaller chain ^{99m}Tc-MAG2-ODNs, as a new radioactive peak eluting in the same area as 10-nt and 15-nt ^{99m}Tc-MAG2-ODNs was observed. The PS ^{99m}Tc-MAG2-ODN remained completely intact 5 min after injection. At 15 and 60 min, the radioactivity had been distributed to such an extent throughout the test animals that the radioactivity remaining in the blood was too low to be detected in quantitative radio-HPLC analysis. However, the elution profile through a mini-column provided the information that a further breakdown, transchelation or protein binding to more polar components did not occur. In FCS, up to 30 % of the radioactivity of the PO ^{99m}Tc-MAG2-ODNs and 24 % of the PS ^{99m}Tc-MAG2-ODNs was found to be transformed to an unknown impurity eluting as a broad and poorly shaped peak a few minutes before the intact ^{99m}Tc-MAG2-ODNs in radio-HPLC. This transformation occurs without any recognisable pattern at the time points studied, but is most likely due to a transchelation reaction. In the cysteine experiments, transchelation from the ^{99m}Tc-MAG2-ODNs was observed when the concentration of cysteine was in a 300-fold excess. Instability in the presence of cysteine is known for 15-nt ^{99m}Tc-MAG3-ODNs at a 650-fold excess of L-cysteine (3). Transchelation at a 300-fold excess of cysteine does not appear to have any clinical importance for PO and PS ^{99m}Tc-MAG2-ODNs, as transchelation was not a predominant event in the in vivo blood metabolic studies.

The biodistribution of the PO and PS ^{99m}Tc-MAG2-ODNs was studied in normal mice. The level of liver uptake of 1-8 % at 60 min is in the same range as in previous reports with ^{3}H-labelled 20-nt (26) and ^{111}In-DTPA-coupled 15-nt ODNs (1). However, the ^{3}H-ODNs, without any 3'- or 5'-end modifications were distributed in the reverse order with respect to the backbone at 60 min, i.e. the phosphorothioate backbone ODNs showed the highest liver retention. The ^{111}In-DTPA-aminohexyl- PO and PS ODNs distributed in equal amounts to the liver. With a PO ^{99m}Tc-Hynic-ODN (22-nt), a 1.9 % ID/g uptake in liver was observed after 4 hours (27), comparable to the observations in this study. However, a very high uptake of 47 %

ID/g was observed for the ^{99m}Tc-Hynic-ODN PS analogue, possibly due to an increased affinity for protein of this particular compound.

As a further demonstration of the hepatobiliary excretion, the intestines were the only organ in addition to the urine that displayed increasing uptake of radioactivity during the time interval studied. In the intestines, there was a tendency for higher uptake of the PS over PO ODNs at 5 min as well as at 60 min p.i., but the difference was not significant.

The urinary excretion of the PS ^{99m}Tc-MAG2-ODNs was higher than that observed with 20-nt PS ODNs internally labelled with ^{35}S (25). Probably, the negatively charged and rather polar ^{99m}Tc-MAG2 moiety contributes in making the PS ODNs more prone to be excreted through the kidneys.

The biodistribution of all ^{99m}Tc-MAG2-ODNs was distinctly different from the biodistribution of ^{99m}Tc-MAG2 alone, supporting the conclusion that the bifunctional chelating agent has formed a stable conjugate to the ODNs and the radiolabelled chelating agent was not cleaved in vivo.

The radiolabelled chelating agent MAG2 displayed significantly higher blood retention and was excreted much slower through the kidneys as well as the hepatobiliary system.

The blood metabolites study demonstrated that the PO ^{99m}Tc-MAG2-ODNs were degraded more than 50 % in 5 min. Some of the differences observed between the PO and PS ^{99m}Tc-MAG2-ODNs may be attributable to the faster formation of shorter chain oligomers for PO than for PS ODNs. Probably, the ^{99m}Tc-MAG2-aminohexyl portion stays intact, while the 20-nt ODN portion may be degraded to some extent. A possible explanation why the differences in biodistribution for the PO and PS ^{99m}Tc-MAG2-ODNs were not greater than those observed, could be a partial, but not complete destruction of the ODN chain. This could also explain why an accumulation of radioactivity is not seen in the spleen, as has been observed with internally labelled PO ODNs (26), with the possible explanation that oligomeric degradation products are retained in this organ. In the case of the PO ^{99m}Tc-MAG2-ODNs, the

spleen would retain the smaller unlabelled PO oligomers and not the residual oligomers with the intact aminohexyl-MAG2 portion actually carrying the radionuclide.

In a previously published biodistribution study (4) on two ^{99m}Tc-MAG3-ODNs with PO backbones, a similar excretion pattern to that of the ^{99m}Tc-MAG2-ODNs was observed, with comparable values for urinary excretion and intestinal uptake. However, clear differences can be seen in initial blood retention and in liver uptake. On average, 9 % of the ^{99m}Tc-MAG3-ODNs were found in the blood 5 min p.i., i.e. 2.5 times higher than with the PO ^{99m}Tc-MAG2-ODNs. Liver uptake of the ^{99m}Tc-MAG3-ODNs at 5 min p.i. was less than half of that of the ^{99m}Tc-MAG2-ODNs. At 60 min, the ^{99m}Tc-MAG3-ODN liver activity was still less than half of the PO ^{99m}Tc-MAG2-ODN, but now slightly higher than the PS ^{99m}Tc-MAG2-ODN activity.

The differences observed in biodistribution pattern between oligodeoxynucleotides carrying the radioactive label by various chelating agents like MAG2, MAG3, Hynic, DTPA or via internal labelling, strongly indicate that the chelating agent and the radiolabel are important determinants for the biological distribution of the ODNs. This is important knowledge in the proceeding effort to make radiolabelled ODNs for scintigraphic procedures that, on the one hand can enter cells for mRNA target association, and on the other hand possess properties for a rapid background clearance.

CONCLUSIONS

S-Benzoyl-MAG2-NHS can be conjugated to 20-nt oligodeoxynucleotides to give a 25-35 % conjugation yield for three of the four ODNs included in this study. The fourth ODN (PO CTRL2) had a mean conjugation yield of 5%. All conjugates appeared as separate peaks in reversed phase HPLC, and could easily be collected and labelled with ^{99m}Tc with radiochemical yields over 90 %. When S-benzyl-MAG2-NHS was used for conjugation and the conjugate was separated from the free chelator on a mini-column, maximum labelling yields of 84 % were reached.

The ^{99m}Tc-MAG2-ODNs were stable in solution. In fact, an increase in radiochemical purity over 24 hours was observed for three out of four complexes. The increase in radiochemical purity is assumed to be a consequence of a transchelation of ^{99m}Tc from ^{99m}Tc-MAG2 to ^{99m}Tc-MAG2-ODN.

The ^{99m}Tc-MAG2-ODNs were stable towards transchelation to a 30-fold excess of cysteine, but when the cysteine concentration was raised to a 300-fold excess, the ^{99m}Tc-MAG2-ODNs expressed great instability, particularly the PS ODNs. In foetal calf serum, up to 30 % of the radioactivity could be recovered as an unknown impurity after 60 min. In blood in vivo, the PO ^{99m}Tc-MAG2-ODNs are degraded more than 50 % in 5 min, while the PS analogues remain intact. During the 60 min duration of the study, up to 28 % of the radioactivity was associated with the protein fraction eluting off a C18 mini-column.

In biodistribution studies in normal mice, the PO and PS analogues of the ^{99m}Tc-MAG2-ODNs were distributed in a similar way at the time points studied. A significant difference was observed in the liver retention at 60 min, as the PO ^{99m}Tc-MAG2-ODNs were retained to a higher extent than the PS analogues. This could be due to a higher retention of shorter chain PO oligomer breakdown products with the aminohexyl-MAG2-^{99m}Tc label intact.

The biodistribution of the ^{99m}Tc-MAG2-ODNs was found to be similar to the biodistribution of ^{99m}Tc-MAG3-ODNs, but very different from the biodistribution of ^{99m}Tc-Hynic-ODNs. The additional information gained from this study leads to the conclusion that the choice of bifunctional chelating agent for ^{99m}Tc is of great importance for the in vivo distribution of technetium-99m labelled oligodeoxynucleotides.

REFERENCES

1. Dewanjee M. K., Ghafouripour A. K., Kapadvanjawala M., Dewanjee S., Serafini A. N., Lopez D. M. and Sfakianakis G. N. - J. Nucl. Med. 35: 1054-1063 (1994)

2. Hnatowich D. J., Winnard Jr. P., Virzi F., Fogarasi M., Sano T., Smith, C. L., Cantor C. R. and Rusckowski M. - J. Nucl. Med. 36: 2306-2314 (1995)

3. Winnard, Jr. P., Chang F., Ruskowski M., Mardirossian G. and Hnatowich D. J. - Nucl. Med. Biol. 24: 425-432 (1997)

4. Hjelstuen O.K., Tønnesen H.H., Bremer P.O. and Verbruggen A.M. - Nucl. Med. Biol. 25: 651-657 (1998)

5. Tavitian B., Terrazzino S., Kühnast B., Marzabal S., Stettler O., Dollé F., Deverre J. R., Jobert A., Hinnen F., Bendriem B., Crouzel C. and Di Giamberardino L. - Nature Medicine. 4: 467-471 (1998)

6. Matteucci M.D. and Wagner R.D. - Nature 384 Supp.: 20-22. (1996)

7. Wagner R. W. and Flanagan W. M. - Molecular Medicine Today. 1:31-38 (1997)

8. Hjelstuen O.K., Mælandsmo G.M., Tønnesen, H.H., Bremer P.O. and Verbruggen A.M. – Nucl Med Comm 19: 803-812 (1998)

9. Schneider R.F., Subramanian G., Feld T.A., McAfee J.G., Zapf-Longo C., Palladino E. and Thomas D. - J Nucl Med 25: 223-229 (1984)

10. Brandau W., Bubeck B., Eisenhut M. and Taylor D.M. - Appl. Radiat. Isot. 39: 121-129 (1988)

11. Mælandsmo G.M., Hovig E., Skrede M., Engebraaten O., Florenes V.A., Myklebost O. Grigorian M., Lukanidin E., Scanlon K. J. and Fodstad Ø. - Cancer Research. 56: 5490-5498 (1996)

12. Verbeke K., Hjelstuen O., Debrock E., Cleynhens B., De Roo M. and Verbruggen A. - Nucl. Med. Comm. 16: 942-957 (1995)

13. Hnatowich D. J. Virzi F. Fogarasi M. Rusckowski M. and Winnard Jr. P. - Nucl. Med. Biol. 21: 1035-1044 (1994)

14. Greene T.W. and Wuts P.G.M. - Protective Groups in Organic Synthesis, John Wiley & Sons, New York, 1991

15. Bormans G., Cleynhens J., Adriaens P., Vanbilloen H., De Roo M. and Verbruggen A. - Nucl. Med. Biol. 22:339-349 (1995)

16. Stein C. A. and Cheng Y. C. - Science. 261: 1004-1012 (1993)

17. Piwnica-Worms D. - J. Nucl. Med. 35: 1064-1066 (1994)

18. Morvan F., Porumb H., Degols G., Lefebvre I., Pompon A., Sproat B. S., Rayner B., Malvy C., Lebleu B. and Imbach J. L. - J. Med. Chem. 36: 280-287 (1993)

19. Agrawal S. and Iyer R. P. - Pharmacol. Ther. 76: 151-160 (1997)

20. Srinivasan S. K. and Iversen P. - J. Clin. Lab. Analysis. 9: 129-137 (1995)

21. Uhlmann E. and Peyman A. - Chem. Rev. 90: 544-584 (1990)

22. Agrawal S., Temsamani J. and Tang J. Y. - Proc.Natl.Acad.Sci.USA. 88: 7595-7599 (1991)

23. Rojanasakul Y. - Advanced Drug Delivery Reviews 18: 115-131 (1996)

24. Milligan J. F., Jones R. J., Froehler B. C. and Matteucci M. D. - New York Academy of Sciences. Annals. 716: 228-241 (1994)

25. Temsamani J., Tang J. Y., Padmapriya A., Kubert M. and Agrawal S. - Antisense Research and Development. 3: 277-284 (1993)

26. Sands H., Gorey-Feret L. J., Cocuzza A. J., Hobbs F. W., Chidester D. and Trainor G. L. - Molecular Pharmacology 45: 932-943 (1994)

27. Hnatowich D. J., Mardirossian, G., Fogarasi, M., Sano, T., Smith, C. L., Cantor, C. R., Ruskowski, M. and Winnard Jr. P. - J. Pharmacol. Exp. Ther. 276: 326-334 (1996)

