Synthesis and evaluation of a fluorine-18 labeled antisense oligonucleotide as a potential PET tracer for iNOS mRNA expression

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Abstract

Inducible NO synthase (iNOS) is overexpressed in inflammatory bowel diseases. An antisense oligonucleotide with good hybridization properties for iNOS mRNA was selected using RT-PCR. The oligonucleotide was reliably labeled with fluorine-18 using N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide. Cellular uptake and efflux of oligonucleotide complexed with FuGENE-6 were rapid, unlike naked oligonucleotide, which hardly accumulated. However, neither uptake nor efflux showed any selectivity for iNOS expressing cells. The oligonucleotide showed a high level of non-specific binding, which may have obscured its specific hybridization to iNOS mRNA. © 2004 Elsevier Inc. All rights reserved.

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1. Introduction

Every year, several million people worldwide are affected by idiopathic inflammatory bowel diseases (IBD), like colitis ulcerosa and Crohn's disease. These disorders are generally accompanied by symptoms that seriously reduce the patient's quality of life, such as severe diarrhea, abdominal pain, and rectal bleeding. Mucosal biopsies of the inflamed gut of IBD patients are characterized by enhanced expression of the inducible form of the enzyme NO synthase (iNOS) and increased levels of the radical nitric oxide (NO). Although the role of NO in the inflamed gut is still subject of considerable controversy, several studies have shown that overproduction of NO by iNOS has a deleterious effect on chronic bowel inflammation [1]. Many pathophysiological effects that are observed in IBD, such as mucosal vasodilatation, enhanced epithelial permeability and impairment of gut motility, are likely caused by the high concentrations of NO radicals produced by iNOS. Inhibition of iNOS may provide an opportunity for pharmacological therapy of IBD. Although iNOS inhibitors, like N^{G}-nitro-L-arginine methyl ester and aminoguanidine, have indeed

shown to reduce inflammation, the selectivity of the currently available inhibitors is still insufficient, as they also inhibit the constitutively expressed endothelial NO synthase (eNOS), resulting in adverse side-effects [2].

In theory, antisense techniques can be very selective and therefore have been investigated as a potential therapeutic approach for IBD. When the DNA sequence of a gene is known, in principle an antisense oligonucleotide (ODN) can be designed that specifically hybridizes to its target mRNA sequence. In this manner, the expression of a single target gene in the entire human genome can be inhibited. This approach has been successfully applied to inhibit iNOS expression in animal models. In a mouse model for Crohn's disease, clinical and histological symptoms of colitis completely diminished after local administration of an antisense phosphorothioate ODN that targeted the mRNA of nuclear factor NF-κB, an important transcription factor for iNOS [3]. However, NF-κB not only regulates iNOS, but also a variety of genes that are involved in survival responses of epithelial cells and consequently inhibition of NF-κB could also block important anti-apoptotic and anti-microbial survival mechanisms. Therefore, the use of ODNs that directly target iNOS mRNA seems preferable. Direct inhibition of the iNOS expression with antisense ODNs against iNOS mRNA has been demonstrated in animal models for encephalomyelitis, sepsis, cerebral and renal ischaemia [4–7].

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These studies indicate that antisense ODNs have the potential to become useful therapeutics.

Moreover, ODNs might be applicable not only as therapeutic, but also as imaging tools. Radiolabeled ODNs can be monitored *in-vivo* with nuclear imaging techniques, like single photon emission computed tomography (SPECT) and positron emission tomography (PET). These techniques allow noninvasive measurement of ODN pharmacokinetics and thus facilitate the evaluation of delivery methods for ODNs. Several methods for labeling ODNs with isotopes suitable for SPECT (e.g., ^{99m}Tc, ^{111}In, ^{123}I) or PET (e.g., ^{11}C, ^{18}F, [76]Br) have been described. Hitherto, only a few *in-vivo* imaging studies with radiolabeled ODNs have been published yet. The preliminary results reported thus far appear promising, as sequence-dependent specific uptake of ODN has been observed in some animal studies [8].

We aim to develop a noninvasive method to monitor iNOS mRNA expression using a radiolabeled ODN in combination with PET. Such an imaging method might be applicable for detecting overproduction of iNOS mRNA, for repetitive monitoring of the effect of therapeutic intervention on iNOS mRNA levels and for determining ODN pharmacokinetics in relation to the administration route. This study describes the selection of an antisense ODN with good hybridization properties to iNOS mRNA, the labeling of this ODN and the preliminary *in-vitro* evaluation studies of the radiolabeled ODN.

2. Materials and methods

2.1. General

Oligonucleotides were purchased from Eurogentec (Seraing, Belgium) and gel filtration columns from Pharmacia Biotech (Uppsala, Sweden). N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide was prepared as previously described [9,10]. Preparative HPLC separations were performed on a Waters system, consisting of a 600E gradient pump, a 486 multi-wavelength UV detector operated at 254 nm, and a Bicron Geiger-Müller radioactivity detector. The reversed phase column used was: Hamilton PRP-1, 250×10 mm ID, 10μ. Elution was performed with a flow of 3 mL/min using a 30 min linear gradient starting with a mobile phase consisting of 10% acetonitrile in 0.1M tetraethylammonium acetate pH 7 and ending with 70% acetonitrile in 0.1M tetraethylammonium acetate pH 7. Electrospray ionization mass spectroscopy was performed in negative ionization mode and scanned from m/z 1250 to m/z 2000. Samples were dissolved in methanol with 1% NH_4OH.

2.2. Selection of ODN sequence

RNA was isolated from cytokine stimulated DLD-1 colon carcinoma cells using Trizol reagent (Life Technologies Ltd) according to the manufacturer's instructions. Reverse transcription was carried out on 5 μg of total RNA, using random primers in a final volume of 75 μL (Reverse Transcription System, Promega, Madison, WI, USA). Polymerase chain reaction (PCR) on cDNA was performed with Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), using a single sense primer specific for human iNOS (5′-CTA-TGC-TGG-CTA-CCA-GAT-GC-3′) and 8 iNOS specific antisense primers (sequence A: 5′-CAT-GGT-GAA-CAC-GTT-CTT-GG-3′ located at bp 2186–2205, sequence B: 5′-CCA-TGA-TGG-TCA-CAT-TCT-GC-3′ located at bp 1490–1509, sequence C [11]: 5′-GGG-TTG-GGG-GTG-TGG-TGA-TGT-3′ located at bp 2704–2724, sequence D [12]: 5′-GCA-TCA-GCA-TAC-AGG-CAA-AGA-GC-3′ located at bp 1772–1794, sequence E [13]: 5′-ACG-GGG-GTG-ATG-CTC-CCA-GAC-A-3′ located at bp 1609–1630, sequence F [14]: 5′-ATG-GAA-CAT-CCC-AAA-TAC-GA-3′ located at bp 1197–1216, sequence G [12]: 5′-GTC-CAT-GAT-GGT-CAC-ATT-CTG-CTT-3′ located at bp 1488–1511 and sequence H [12]: 5′-GAT-TCT-GCC-GAG-ATT-TGA-GCC-TC-3′ located at bp 2411–2433). Antisense primers C-H were retrieved from the literature, whereas, the sense primer and antisense primers A and B were selected with the primer-3 primer design program, using accession code L09210. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sense: 5′-CCA-TCA-CCA-TCT-TCC AGG-AG-3′; antisense 5′-CCT-GCT-TCA-CCA-CCT-TCT-TG-3′), resulting in an amplified product of 576 bp, were used as control for the RT-PCR procedure. The primers and cDNA were denatured in a GeneAmp PCR system 2400 (Perkin-Elmer, Norwalk, CT, USA) at 95°C for 5 min. For each iNOS antisense primer, annealing was performed at a temperature of 56, 58, 60, 62, and 64°C. The cycling program consisted of 30 cycles for the iNOS primer sets and 22 cycles for the GAPHD primer set. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining, according to methods described previously [15]. A 100bp DNA ladder was used for determining product size. The iNOS antisense ODN sequence to be used in the subsequent labeling experiments was selected on basis of specificity (single band) and product yields in the RT-PCR assay.

2.3. Oligonucleotide conjugation with N-(4-fluorobenzyl)-2-bromoacetamide

Prior to conjugation, 90 μL 0.1N dithiothreitol was added to 10 μL of water containing 100 nmol of a oligonucleotide with the sequence 5′-GTC-CAT-GAT-GGT-CAC-ATT-CTG-CTT-3′ (sequence G), a uniformly modified phosphorothioate backbone and a hexylthiol linker at the 5′-terminus. After 30 min at room temperature, the oligonucleotide solution was transferred to a NAP-5 cartridge. The cartridge was washed with 0.4 mL of PBS and eluted with 0.5 mL 0.1M sodium phosphate buffer pH 8. To the eluate, 25 μg N-(4-fluorobenzyl)-2-bromoacetamide in

1 ml of methanol was added. The reaction mixture was allowed to react at 110°C for 2 h. After evaporation of the solvent, the product was purified by reversed phase HPLC; retention time: 12 min. The product was desalted by gel filtration over a NAP-25 column and analyzed by mass spectroscopy. MS (ESI$^-$): m/z 1339.0 (M-6H)$^{6-}$,1607.1 (M-5H)$^{5-}$. Reconstructed molecular mass (BioSpec software program): 8041 amu.

2.4. Radiolabeling

The oligonucleotide with a hexylthiol linker was treated with dithiothreitol and desalted over a NAP-5 column, as described above. A solution of N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide in 0.5 mL of methanol was added to a solution of 100 nmol oligonucleotide in 0.5 mL of 0.1M sodium phosphate buffer pH 8. The reaction mixture was heated at 120°C for 30 min. The solvent was evaporated at 120°C with the aid of an argon flow. The residue was dissolved in 1 mL 0.1N tetraethylammonium acetate pH 7 and injected on HPLC. The radioactive product with a retention time of 12 min was collected and transferred to a NAP-25 cartridge. The cartridge was eluted with saline, affording the radiolabeled oligonucleotide in 41±19% decay corrected yield. The oligonucleotide concentration was determined by measurement of the optical density at 260 nm (OD$_{260}$). The specific activity of the radiolabeled ODN ranged from 1 to 13 GBq/μmol. The radiolabeled oligonucleotide co-eluted with a cold reference sample.

2.5. Liposome-ODN complex

To a solution of 33 μg of radiolabeled oligonucleotide in saline, 100 μL of FuGENE 6 (Roche, Indianapolis, IN, USA) transfection reagent was added. The mixture was diluted with serum-free medium to a total volume of 7 mL. The mixture was incubated at room temperature for 15 min before use. In cellular uptake studies, 100 μL of the ODN-transfectant complex (60 pmol ODN) per well was used to ensure sufficient cell-associated radioactivity for good counting statistics at the end of the experiment.

2.6. iNOS induction

In DLD-1 colon carcinoma cells, iNOS expression was evoked with a cytokine mixture composed of human recombinant interleukin-1β (10 ng/mL), human recombinant TNF-α (10 ng/mL) and human recombinant interferon-γ (1000 U/mL) for 8 h [16,17]. Strong induction of iNOS mRNA in cytokine stimulated cells was confirmed by RT-PCR.

2.7. Cellular uptake of labeled ODN

Cellular uptake of the labeled oligonucleotide was studied in cytokine stimulated and control DLD-1 cells grown in monolayers in 24-well culture plates (approximately 5×10^5 cells/well). The culture medium was removed from the cells and replaced by 100 μL fresh serum-free culture medium containing 60 pmol of radiolabeled oligonucleotide, which was either naked or complexed with FuGENE 6. Cells were incubated at 37°C for 30, 60, or 120 min. In order to correct the uptake for extra-cellular activity, cells were incubated at 0°C for 30 min. The culture medium was removed and the cells were washed 3 times with 0.5 mL of ice-cold PBS. The cells were harvested from the culture plates by treatment with 0.1 mL of 0.5% trypsin-EDTA (Sigma Chemical Co, St. Louis, MO,USA) for 5 min and resuspended in 0.5 mL of culture medium to neutralize the trypsin. A 50 μL sample was taken to assess cell viability with trypan blue and to count the number of viable cells under the microscope. The radioactivity in the cell suspensions was measured in a gamma counter (LKB, Wallac, Turku, Finland) and normalized to the number of viable cells. Tracer accumulation was corrected for extra-cellular activity (measured at 0°C) and expressed as the percentage of the tracer dose that had accumulated per million cells (%dose/10^6 cells).

2.8. Efflux of labeled ODN

Cytokine stimulated and control DLD-1 cells were incubated with the labeled oligonucleotide-FuGENE 6 complex at 37°C and subsequently washed 3 times with PBS, as is described above. To the monolayers, 0.5 mL of fresh serum-free culture medium was added and cells were incubated at 37°C for 0, 60, or 120 min. The culture medium was removed and the cells were washed 3 times with 0.5 mL of ice-cold PBS. The cells were harvested from the culture plates by treatment with 0.1 mL of 0.5% trypsin for 5 min. The cells were re-suspended in 0.5 mL of culture medium to neutralize the trypsin. The radioactivity in the cell suspensions was measured in a gamma counter and normalized to the number of viable cells. In each experiment, the retained radioactivity per cell after 60 and 120 min of incubation in fresh medium was normalized to the radioactivity per cell at 0 min of efflux.

3. Results

3.1. Selection of iNOS antisense ODN sequence

Strong and selective hybridization to the target sequence is the primary prerequisite for a radiolabeled antisense ODN to become a suitable PET tracer. For the selection of an antisense ODN sequence, the hybridization properties of 8 antisense phosphodiester ODN probes against iNOS mRNA were tested in a standard RT-PCR assay using a single sense primer (Fig. 1). Each probe was tested at several annealing temperatures between 56 and 65°C. Four antisense probes (sequences A, B, C, and H) gave multiple products in the PCR assay, indicating that these probes also had affinity for
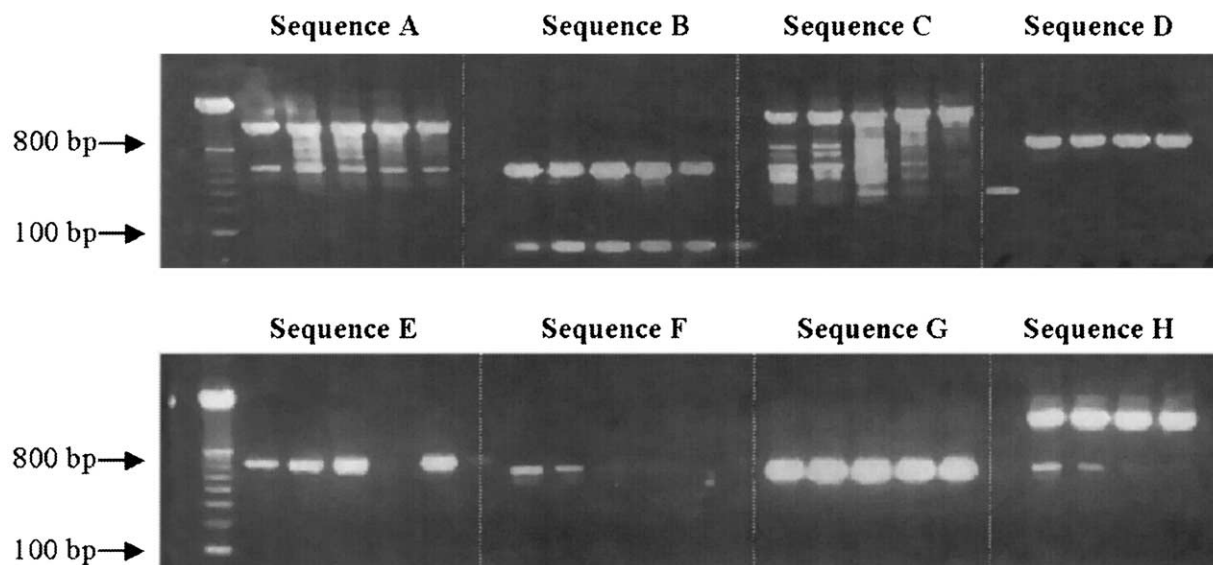
Fig. 1. Gel electrophoresis of the products of the RT-PCR assay for eight antisense sequences (A-H) at annealing temperatures ranging from 56 to 65°C. The first lane is a 100 bp DNA ladder. For each probe, five lanes with the PCR results at an annealing temperature of 56, 58, 60, 62, and 64°C are represented (from left to right). The results of the gel electrophoresis were in agreement with calculated molecular weights of the PCR products, except for sequence F (Sequence A: 1202 bp, Sequence B: 506 bp, Sequence C: 1721 bp, Sequence D: 791 bp, Sequence E: 627 bp, Sequence F: 213 bp, Sequence G: 508 bp and Sequence H 1430 bp). See Sec. 2 for the antisense sequences.

mismatched sequences. Because of this lack of selectivity, these sequences were disregarded as potential probes for imaging. Among the remaining sequences, antisense sequence G (5′-GTC-CAT-GAT-GGT-CAC-ATT-CTG-CTT-3′), which is directed against exon 12 of iNOS mRNA [12], gave highest PCR product yields at all annealing temperatures, suggesting that hybridization to the target sequence was strongest for this probe. Consequently, sequence G was selected for radiolabeling.

3.2. Radiolabeling

Antisense oligonucleotides with a phosphodiester backbone are rapidly degraded by nucleases *in vitro* and *in vivo*. To increase the stability of the radiolabeled oligonucleotide, ODN sequence G with a uniform phosphorothioate backbone was used as the labeling precursor. At the 5′-terminus, the precursor was modified with a hexylthiol group as a linker to facilitate the labeling with N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide (Fig. 2). Prior to labeling, the ODN precursor was treated with dithiothreitol to reduce any disulfide bridges that might have led to dimerization—and thus inactivation—of the precursor. Conjugation of the radiolabeled bromoacetamide to the ODN precursor was carried out in an acetonitrile / phosphate buffer mixture at 120°C for 30 min. The product was separated from unreacted precursor and impurities by gradient ion-exchange HPLC. After desalting, the radiolabeled antisense ODN was obtained in $41\pm19\%$ radiochemical yield (correct for decay). The specific activity of the labeled oligonucleotide ranged from 1 to 13 GBq/μmol.

3.3. Cellular uptake of radiolabeled ODN

In patients with inflammatory bowel diseases, iNOS is predominantly expressed in affected gut epithelium [18].
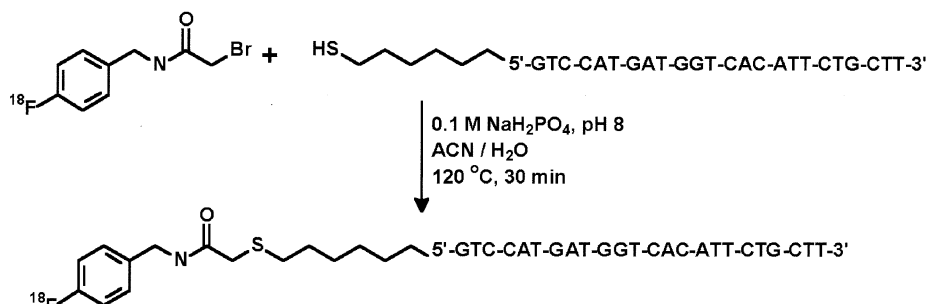

Fig. 2. Synthesis of the fluorine-18 labeled phosphorothioate antisense ODN for iNOS mRNA, using N-(4-[^{18}F]fluorobenzyl)-2-bromoacetamide as the labeling synthon.
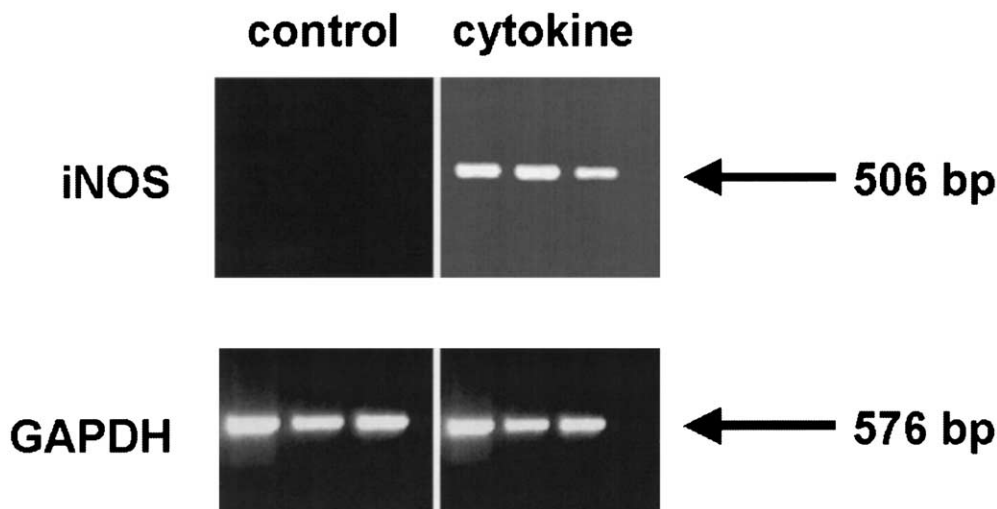
Fig. 3. Cytokine stimulated and control DLD-1 cells were analyzed for iNOS mRNA with RT-PCR, using antisense primer B. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. A 100 bp DNA ladder was used for determining product size. In cytokine simulated cell, a 506 bp PCR product was obtained, which was in agreement with the calculated size. GAPDH primers were used as controls for the RNA isolation and PCR procedure.

Therefore, we selected an epithelial cell line (DLD-1) to investigate the cellular uptake of the radiolabeled antisense ODN against iNOS. Strong expression of iNOS mRNA was induced by incubating the cells with a mixture of cytokines for 8 h, as was shown by PCR analysis (Fig. 3). In untreated control DLD-1 cells, no iNOS mRNA could be detected. Cytokine stimulated and control DLD-1 cells were incubated with the radiolabeled phosphorothioate antisense ODN with sequence G, either as the naked probe or complexed with the transfection reagent FuGENE-6. Cellular uptake was corrected for extracellular binding of the probe on the outer surface of the cell membrane, as was determined at 0°C. Extracellular activity was 1.4 ± 0.3 %ID/10^6 cells when the probe was administered as naked DNA and 2.0 ± 0.4 %ID/10^6 cells for the ODN-FuGENE-6 complex. This difference was statistically significant (t-test, $p = 0.03$). In both cases, however, no significant differences in extracellular activity were observed between control cells and cytokine stimulated cells. As shown in Fig. 4, the naked ODN hardly accumulated in either control or stimulated cells. Complexation of the radiolabeled ODN with FuGENE-6 resulted in a 2- to 3.5-fold increase in tracer uptake (two-sided, unpaired student's t-test; $P < 0.05$). However, tracer accumulation was not selective for the iNOS expressing cells, as similar uptake (8.2–8.6 pmol/10^6 cells) was observed in cytokine stimulated and control DLD-1 cells at all time points ($P \geq 0.1$), irrespective as to whether the radiolabeled ODN was naked or complexed with FuGNE-6.

3.4. Efflux of labeled ODN

To study the efflux of the labeled ODN, cytokine stimulated and control DLD-1 cells were loaded with tracer complexed with FuGENE-6, washed with ice-cold PBS and incubated in fresh medium. Efflux of the tracer was rapid, as more than 70% of the radioactivity was already cleared from the cells within 60 min (Fig. 5). Remarkably, the cell associated radioactivity remained constant between 60 and 120 min, suggesting that approximately 30% of the radioactivity was irreversibly bound in the cell. No statistically significant differences in efflux were found between cytokine stimulated and control cells ($P > 0.1$), showing that iNOS expression did not significantly influence the efflux of the radiolabeled ODN.
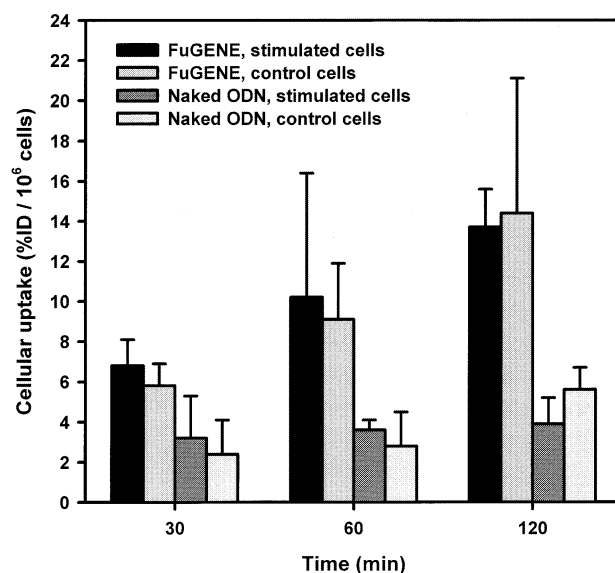

Fig. 4. Cellular uptake in cytokine stimulated and in control DLD-1 cells of naked or FuGENE-6 complexed fluorine-18 labeled antisense ODN for iNOS mRNA.
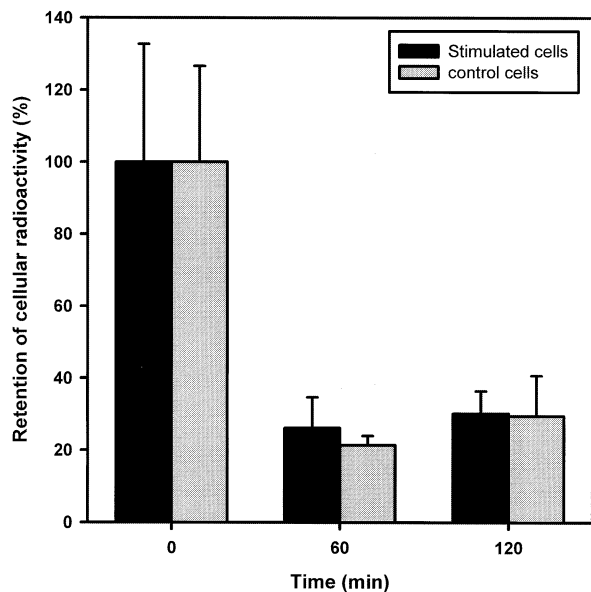
Fig. 5. Efflux of radioactivity from cytokine stimulated and control DLD-1 cells that were loaded with fluorine-18 labeled antisense ODN for iNOS mRNA as a complex with FuGENE-6.

4. Discussion

Antisense ODNs can bind via Watson-Crick base pairing with extremely high specificity and affinity to their target mRNA sequence. By varying the nucleotide sequence, an antisense ODN can be designed and synthesized that in principle can be directed towards any target gene of interest. These properties make radiolabeled antisense ODNs potentially attractive radiopharmaceuticals for nuclear imaging. Such an imaging technique would provide an opportunity to assess mRNA expression of the gene of interest in-vivo and thus would allow monitoring the effect of therapeutic intervention on gene expression. Imaging of radiolabeled ODNs can also be applied to determine the pharmacokinetics of the corresponding therapeutic antisense ODN, which provides information that is essential for improving the efficacy of these drugs. The aim of the present work is to investigate the potential of using a radiolabeled ODN as a radiopharmaceutical for imaging of iNOS mRNA, which is overexpressed during inflammation. In order to become a suitable radiopharmaceutical the ODN must fulfill a number of requirements, including (1) a high affinity and selectivity for the target mRNA, (2) in-vivo stability, (3) the availability of a labeling method, (4) rapid uptake into the target tissue, (5) rapid efflux of ODN that is not hybridized, and (6) negligible nonspecific interactions.

4.1. Affinity and selectivity

In the literature, various antisense ODN sequences for iNOS mRNA have been described. We have tested a selection of these antisense phosphodiester ODNs in a standard RT-PCR assay, using a single sense primer. This RT-PCR assay gives a good impression of hybridization properties of the antisense ODN with the target mRNA and thus allows a quick and simple qualitative comparison of the affinity and selectivity of the ODNs. Four of the antisense ODN sequences tested in this study gave multiple products in the PCR assay, suggesting that these primers had hybridized to multiple mRNA sites. Statistically, an 13-mer ODN should be sufficiently long to bind to a specific RNA sequence [19]. Since the ODNs used in this study were all 20–24 nucleotides long, apparently some of the ODNs had hybridized to mismatched sequences as well. The product yield in the PRC assay strongly depends on the ability of the antisense ODN to bind to its target sequence and thus can be used as a measure for its binding affinity. Thus, ODN sequence G (5′-GTC-CAT-GAT-GGT-CAC-ATT-CTG-CTT-3′), which targets exon 12 of iNOS mRNA [12], was selected for the labeling studies, because it gave the highest yield of a single PCR product at all annealing temperatures, reflecting its high selectivity and affinity.

4.2. Stability

In biological fluids, phosphodiester ODNs are rapidly degraded by endo- and exonucleases [20]. In contrast, ODNs with a phosphorothioate backbone are quite resistant towards nuclease activity. To improve its stability, the antisense ODN selected in the PCR assay was modified with a uniform phosphorothioate backbone. As a trade-off, this modification decreases the melting temperature of the ODN with approximately 0.5°C per nucleotide [19]. Still, the binding affinity of the modified ODN should be more than sufficient [21], as the phosphodiester ODN gave high product yields in the RT-PCR assay, even at an annealing temperature as high as 65°C.

4.3. Labeling

To facilitate labeling, a hexylthiol spacer was attached to the 5′-terminus of the phosphorothioate ODN. At the hexylthiol moiety, the ODN was labeled with fluorine-18 via alkylation with the synthon N-(4-[18F]fluorobenzyl)-2-bromoacetamide. The labeling procedure was reliable and proceeded in good yield. Tavitian and coworkers have shown that the attachment of this synthon to an ODN does not significantly influence its binding affinity [22].

4.4. Cellular uptake

In this study, the cellular uptake experiments with the naked ODN showed a rapid initial association of activity to the cells that hardly increased over time. No significant difference in uptake between iNOS expressing and control cells was observed. Phosphorothioate ODNs have been shown to specifically bind to cell surface proteins [23], which might explain the initial association of activity to the

cells. Internalization of naked ODNs generally takes place via a combination of pinocytosis, adsorptive and receptor-mediated endocytosis, which are slow and inefficient processes [24]. The low levels of internalized naked ODN, in combination with high levels of cell surface bound ODN, could have caused the lack of selectivity of the ODN for iNOS expressing cells. An alternative explanation could be that the target sequence is inaccessible, due to duplex formation of iNOS mRNA at exon 12.

To enhance the intracellular delivery, the ODN probe was complexed with FuGENE-6. FuGENE-6 is a multi-component lipid-based transfection reagent that demonstrates virtually no cytotoxicity. Complexation of the radiolabeled ODN with FuGENE-6 resulted in a 3.5-fold increase in the cell-associated activity after 2 h of incubation. In contrast to naked ODN, cellular uptake of the ODN-FuGENE-6 complex increased over time, suggesting that the ODN complex is internalized. In the uptake experiments, however, we could not demonstrate any specific hybridization of the radiolabeled ODN to iNOS mRNA, as cellular uptake in iNOS expressing cells was not significantly higher than in control cells. After internalization, FuGENE-6 complexed phosphorothioate ODNs have been shown to be widely distributed in the cytoplasm and the nucleus [25]. Apparently, only a small fraction of the total cell-associated activity was specifically bound to iNOS mRNA.

4.5. Efflux

A high signal to background ratio is a prerequisite for successful imaging. Thus, it is necessary that any unbound tracer is rapidly cleared from tissue and plasma. The efflux experiments showed that the majority of the cell-associated activity is rapidly cleared from the cells. However, 25–30% of the activity could not be washed from the cells during the cause of the experiment. The amount of non-removable activity was similar for iNOS expressing and control cells and thus was due to nonspecific binding.

4.6. Non-specific binding

The efflux experiments revealed that a relatively large fraction of the radiolabeled ODN was nonspecifically bound in the cell. We have calculated from the specific activity of the ODN and the non-removable cell-associated activity that the amount of nonspecific binding in our experiments corresponded to approximately 300.000 ODN copies per cell. We estimated that the cytokine stimulated DLD-1 cells contained about 1.000–10.000 copies of iNOS mRNA on basis of the Northern blot using GAPDH as a reference, of which only a few hundred copies per cell are present [26]. This estimation indicates that the amount of non-specifically bound ODN exceeds the number of target mRNA molecules by 1–2 orders of magnitude and thus precludes monitoring of specific hybridization of this radiolabeled ODN to iNOS mRNA. Other *in-vitro* studies using [^{99m}Tc]-labeled anti-

sense ODNs, also could not demonstrate specific uptake of the oligonucleotide [27,28]. In the study by Hjelstuen et al. [27], cellular uptake (5–9 pmol/10^6 cells) at 3 h was in the same range as was found in this study and probably also exceeded the number of target mRNA molecules. Since phosphorothioate ODNs are polyanions, they are able bind to a variety of proteins in a nonsequence specific manner [29]. Perhaps, the problem of the non-specific binding could be overcome by choosing another modification of the backbone of the ODN (e.g., PNA chimera). In contrast with the aforementioned results, Zang and co-workers [30] were able to show sequence specific uptake and efflux of a [^{99m}Tc]-labeled phosphorothioate antisense ODN in *in-vitro* experiments comparable to those in this study. Remarkably, the amount of specific uptake in that study was calculated to be approximately 10^5 antisense ODN molecules per cell at 24 h, which was several orders of magnitude higher than the estimated steady-state target mRNA level. The authors hypothesize that hybridization of the radiolabeled ODN to its target mRNA results in translation arrest and consequently in a decrease in the concentration of the target protein. Subsequently, feedback mechanisms could have stimulated the production of additional target mRNA, which could explain the high specific uptake observed in that experiment. If this hypothesis is correct, the short half-life of fluorine-18 might be a limiting factor for application of fluorine-18 labeled ODNs as radiopharmaceuticals for PET imaging.

5. Conclusion

An antisense oligonucleotide with good *in-vitro* hybridization properties against exon 12 of iNOS mRNA was reliably labeled with fluorine-18. Cellular uptake and efflux of the labeled oligonucleotide was sufficiently rapid for imaging. However, no selectivity for iNOS expressing cells was observed due to the large fraction of the tracer that was non-specifically bound in the cell. This nonspecific binding was estimated to exceed the number of target iNOS mRNA molecules by more than one order of magnitude. Therefore, modifications of the labeled oligonucleotide that reduce the amount of nonspecific binding are needed before imaging of iNOS expression will be feasible.

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