Radiolabelled Oligonucleotides for Evaluation of in vivo Hybridisation Utilising PET Methodology

GÁBOR LENDVAI
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Abstract

Antisense oligonucleotides (ODN) may interfere in gene expression on the basis of hybridising to its complementary messenger RNA (mRNA) sequence in the cell thereby preventing the synthesis of the peptide. Therefore, these ODNs may be potential drugs to treat human diseases by “knocking down” the expression of responsible genes or correct the maturation process of mRNA in the field called antisense therapy. Moreover, antisense ODNs upon labelling are also potential imaging agents to monitor gene expression in vivo, i.e. to accomplish in vivo hybridisation. This would provide a non-invasive tool compared to present methods, which require tissue samples.

This goal may be reached using positron emission tomography (PET) methodology. PET is a most advanced in vivo imaging technology, which would allow exploring the fate of radionuclide-labelled antisense ODNs in the body; thereby providing information about biodistribution and quantitative accumulation in tissues to assess pharmacokinetic properties of ODNs. This kind of evaluation is important as part of the characterisation of antisense therapeutics but also as part of the development of antisense imaging agents.

The present study aimed to investigate 79Br- and 68Ga-labelled ODNs of five different modifications: phosphodiester, phosphorothioate, 2′-O-methyl phosphodiester, locked nucleic acid (LNA), and peptide nucleic acid. The study included exploration of the hybridisation abilities of these ODNs after labelling; furthermore, the biodistribution, metabolite analysis and uptake of the ODNs in rats regarding non-hybridisation and hybridisation specific uptake was conducted. Among the ODNs studied, LNA-DNA mixmer (LNA and DNA nucleotides in alternation along the sequence) displayed the most promising characteristics considering a higher retention in tissues, stability and longer plasma residence. However, biodistribution data demonstrated a non-hybridisation specific distribution in rat tissues with kidney, liver, spleen and bone marrow being the organs of high uptake. Scavenger receptors or other saturable processes unrelated to hybridisation may play a role in tissue uptake and in clearance of antisense ODNs through these organs. These processes may be sequence dependent suggesting that proof of in vivo hybridisation through imaging needs much more elaborate evaluations than just comparison of sense and antisense sequences and proving dose-dependency.

Keywords: Gene expression, Antisense oligonucleotides, Positron emission tomography, In vivo hybridisation, In vivo biodistribution, Chromogranin-A, 68Ga, RT-PCR, Scavenger receptors

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“When you think you have exhausted all possibilities, remember this — you haven’t. There is still at least one more.”

Thomas Edison

To my girls: Edit, Dóra & Eszter
Papers included in the thesis

This thesis is based on the following papers, which are referred to in the text by their roman numerals.


III  Lendvai G, Velikyan I, Estrada E, Eriksson B, Långström B and Bergström M. Biodistribution of \(^{68}\text{Ga}\)-labelled LNA-DNA mixmer antisense oligonucleotides for Rat Chromogranin-A. *Submitted to Oligonucleotides*

IV  Lendvai G*, Monazzam A*, Velikyan I, Eriksson B, Josephsson R, Långström B, Bergström M and Estrada S. Non-hybridisation saturable mechanisms play a role in the uptake of \(^{68}\text{Ga}\)-labelled LNA-DNA mixmer antisense oligonucleotides in rats. *Manuscript*

* These authors contributed equally to this work.

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The author’s contribution to the papers:

- Paper I: Planned the experiments, analysed the data, participated in paper writing.
- Paper II: Planned, performed the experiments except conjugation, labelling and HPLC-TLC analysis, which was headed by Irina Velikyan, and whole-body autoradiography, which was performed by Daniel Laryea, and wrote the paper.
- Paper III: Planned, labelled ODN, performed the experiments except conjugation, which was performed by Irina Velikyan, and wrote the paper.
- Paper IV: Planned, labelled ODN, performed the experiments except cell culturing and cell uptake studies, which was performed by Azita Monazzam, and wrote the paper.

Prof Mats Bergström, PhD acted as the main supervisor in all papers together with Sergio Estrada, PhD (paper II-IV), Prof Barbro Eriksson, MD, PhD (paper I, III, IV) and Raymond Josephsson, PhD (paper IV and PNA project).

Contribution in additional papers


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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>cDNA</td>
<td>complementary/copy DNA</td>
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<td>Chg-A</td>
<td>Chromogranin A</td>
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<td>CMC</td>
<td>carboxymethyl cellulose</td>
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<td>CT</td>
<td>computed tomography</td>
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<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>DOTA</td>
<td>1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>kD</td>
<td>kilo Dalton</td>
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<td>LNA</td>
<td>locked nucleic acid</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>ODN</td>
<td>oligonucleotide</td>
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<tr>
<td>ORN</td>
<td>oligoribonucleotide</td>
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<tr>
<td>OMe</td>
<td>2'-O-methyl</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PNA</td>
<td>peptide nucleic acid</td>
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<tr>
<td>PO</td>
<td>phosphodiester</td>
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<tr>
<td>poly-A</td>
<td>poly-adenylic acid</td>
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<tr>
<td>poly-I</td>
<td>poly-inosinic acid</td>
</tr>
<tr>
<td>PS</td>
<td>phosphorothioate</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SELEX</td>
<td>systematic evolution of ligands by exponential enrichment</td>
</tr>
<tr>
<td>siRNA</td>
<td>small/short interfering RNA</td>
</tr>
<tr>
<td>SPECT</td>
<td>single photon emission computed tomography</td>
</tr>
<tr>
<td>SUV</td>
<td>standardised uptake value</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melting temperature</td>
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Introduction

Our understanding of the molecular mechanisms underlying the normal functioning of cells and tissues has been growing enormously thanks to the revolutionary development of molecular biological methods since the 1980’s. Interest has been focused on the determination of the patterns of active genes involved in replication, migration, and signal transduction of cell communication. The progress has been exemplified most notably by the sequencing of different genomes, especially the human genome. In parallel, a belief has also been growing that the altered patterns of gene expression are an important cause behind the transition of cells to the phenotypes of disease. These alterations can evolve from interactions with the environment, hereditary defects, developmental errors, and aging processes (1-4). For example, a selective growth advantage, which results from changes in genes that directly or indirectly control cellular proliferation and cell death are generally considered the main cause of cancer. These changes involve alterations or mutations in specific oncogenes such as proto-oncogenes (e.g. K-\textit{ras}, H-\textit{ras}, Bcl-2), which promote cellular proliferation or inhibit cell death; and also, in tumour suppressor genes (e.g. APC, Rb, p53), which inhibit cell proliferation or promote cell death (5).

\textit{Molecular medicine} exploits the advances in molecular and cellular biology, especially in gene technology (DNA microarrays) to understand and explain how normal cellular processes either fail or are subverted in disease. Analysing gene expression in diseased and normal cells or tissues is obvious since, for example, cancer cells do not invent new pathways; they use the existing ones but in different ways or new combinations of these existing pathways.

Thus, the goals are to identify and validate the fundamental errors of diseases at the molecular level and to develop and validate molecular corrections for them, for example, with gene therapy (6). The knowledge of the sequence of 3 billion base pairs in the human DNA will have a great impact on finding new targets for the earlier detection of diseases, evaluating molecular markers for therapeutic responses, and using imaging for drug selection and gene expression (7).
Gene expression

In a human cell, the genetic information is stored in DNA molecules (genome) in such a way that information coding regions (~4.5%) alternate with abundant non-coding regions (95.5%) along the DNA. Basically, the genes are the regions or units of DNA sequence that contain the information to specify the synthesis of single polypeptide chains or functional RNA molecules (8). Thus, the proteins, as the end products of genes, indicate the manifestation of information providing the phenotypes of the various cell types.

![Diagram of the central dogma of molecular biology](image)

**Figure 1.** The central dogma of molecular biology describing the expression of genetic information from DNA (genotype) into proteins (phenotype). Following transcription and translation, primer RNA and peptide products undergo further maturation processes.

Expression is the process in which the encoded information of DNA is decoded into proteins. First, the information of selected regions of DNA is carried over to RNA molecules (transcription) that are then in turn used to specify the synthesis of polypeptides (translation), which subsequently form functional proteins (9). The expression of genetic information is a one-way system in all cells and thus its universality, has been described as the central dogma of molecular biology (10). The information mediator molecules specifying polypeptide are known as messenger RNA (mRNA). A human cell expresses only a fraction of its about 20,000-25,000 genes (11) but can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Therefore, the different types of cells arise because different sets of genes are expressed (12).

Monitoring of biological processes at molecular level

The indicators of an active gene can be either the presence of its mRNA or its polypeptide/protein in a cell. Protein expression may normally be tracked by using highly specific antibodies (e.g. Western blot, immunohistochemistry, protein array) while RNA transcripts can be recorded by approaches
involving either molecular hybridisation with a specific antisense nucleic acid probe (e.g. Northern blot, in situ hybridisation, microarray) or reverse transcriptase polymerase chain reaction (RT-PCR) synthesising thousands of copies of a few hundred bp long sequence of the target mRNA. However, these methods require samples of tissue, usually obtained by biopsy or after surgery. This limits the potential to follow processes with time and to observe tissues that are not readily accessible by biopsy. Therefore, a non-invasive method would be of great significance.

One of the important analytical tools in biology and medicine has undoubtedly been imaging, the technology referred to as molecular imaging evolved in the 1990s on the basis of in situ visualisation of target molecules and biological processes using optical imaging (13). The term molecular imaging was coined to separate the part of in vivo imaging that deals with the localisation of molecules, from those concerned with anatomy or the imaging of a function. It focuses on the visualisation of molecular phenotypes such as receptor density, cellular responses to drug challenge, drug concentration, and expression of specific genes (14, 15). This sensitive and informative means allows to identify, study, and diagnose the biological nature of disease early in and throughout its evolution, as well as to provide biological information for development and assessment of therapies (16). Imaging of gene expression is increasingly important for cancer diagnosis, prediction of tumour response to available therapies and monitoring response to therapy (17).

The imaging modalities include x-ray diffraction, electron microscopy, autoradiography, optical imaging, x-ray computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) (16). Among them, non-invasive in vivo molecular imaging may be attainable with nuclear (PET, SPECT), magnetic resonance, and visible infrared in vivo optical imaging systems (13, 14).

**Positron emission tomography**

PET is an imaging method by which biologically interesting molecules are labelled with positron-emitting radionuclides such as $^{11}$C, $^{15}$O, $^{18}$F, $^{68}$Ga, or $^{76}$Br with half-lives of 20 minutes, 2 minutes, 2 hours, 68 minutes, and 16 hours, respectively. When the radionuclide decays, a positron will be released, which within short distance (mm) in tissue slows down until it annihilates with an electron, yielding two 511 keV photons leaving in opposite directions (18). For in vivo animal and human studies, the photons may be detected externally by animal (microPET) or human PET camera (Fig. 2). For in vitro or ex vivo studies, the detection or imaging tools may primarily be autoradiography (19), nevertheless, scintillation counting of tissue sam-
amples and cell aggregates are also powerful options. Among the developed PET-probes, there are various labelled enzyme and transporter substrates, ligands for receptor systems, hormones, antibodies, peptides, drugs, and oligonucleotides (ODN) (16).

Figure 2. The principles of detecting positron in vivo with PET camera (A) and autoradiography for in vitro detection using phosphor imaging plates (B). The plate stores the radioactive signal by the excited state of electrons of the crystals constituting the surface of the plate. The reading is performed by a laser beam when the excited electrons upon phosphorescence emits its surplus energy, which is detected and converted to a pseudocolour image where different colours correspond to signal intensity.

These tracers are usually injected into the circulation and observed by external measurements. The tracer kinetics in tissue is related to perfusion, elimination, metabolism, non-specific uptake, and selective binding to a target molecule. By proper choice of tracer and interpretation of the kinetics, parameters can be extracted that quantitatively describe the interaction between the tracer and the target molecules, receptors or enzymes. Therefore, PET is a most advanced analytical technology allowing to image and measure biochemical and physiological processes (e.g. molecular interactions) in vivo. It provides knowledge about the fate of a drug including pharmacokinetics, pharmacodynamics, and metabolism of the substance (16, 20-25). PET offers high sensitivity in terms of molecular concentrations. It is possi-
ble to measure picomolar amounts of a PET radiopharmaceutical in a tissue. With PET, it is feasible to test potent in vitro drug candidates directly in vivo in humans and thereby eliminating molecules at potential risk of failure due to poor tissue delivery (15, 26). Moreover, the fusion of PET with CT in one camera unit (PET/CT) provides a merged image where not only the physiological status but the anatomical details may also be seen on the same image opening new dimensions in diagnostics (27-29).

Monitoring of gene expression with PET

Basically, there are two main approaches to observe gene expression non-invasively using external recording of PET tracers.

- **Direct way of imaging** – by utilising labelled antisense oligonucleotide probes for recording of specific hybridisation in vivo to mRNA.

- **Indirect way of imaging** – by recording of transmitted reporter genes that generate protein products, which can be observed with selective PET tracers.

Since the presence of a protein implies that its gene has been expressed, gene expression is being imaged whenever the distribution of receptors and other cellular proteins are imaged. Molecular imaging may also involve those exogenous probes that are transported and trapped in the cells (glucose, nucleoside analogues), those directed against extracellular receptors (somatostatin, opioid, melanotropin) and cellular enzymes (aromatase, 11β-hydroxylase, aromatic amino acid decarboxylase) and those activated by extracellular proteases (14). These probes are widely used in neuroscience research (receptor density/occupancy), in clinical nuclear medicine research (cell surface-specific antigens or epitops with radio-labelled antibodies), and in oncology research (tumour cell surface receptors using radio-labelled peptides). The mere imaging of these proteins is not in the direct scope of interest of molecular imaging of gene expression. Rather the interest is directed more at cellular processes closer to transcription than translation (30). One reason is that selective protein synthesis for proteins released from the cell, such as hormones, cannot be imaged today.

Small ODN sequences, apart from antisense, may also be used for targeting specific proteins. The aptamers (“adaptable oligomers”) (31, 32) are selected by a SELEX (Systematic Evolution of Ligands by EXponential enrichment) method, which allows for the selection from a random library of ODNs those molecules that have the desired binding property for a designed protein target (15).

The second approach is indirect because the expression of a reporter gene is monitored in order to check the expression of a co-transcribed gene such as a therapy gene. These genes have been introduced into cells via a com-
mon viral vector. They share a promoter; therefore, the expression of the therapy gene leads to the expression of the reporter gene. The protein product (e.g. an enzyme or a receptor) of the reporter gene can be visualised by a radionuclide-labelled reporter probe. The concentration of the labelled product is increasing over time in cells expressing the reporter gene, while in cells without reporter gene expression the labelled probe will freely leave the cells. Common reporter gene-reporter probe systems are the HSV thymidine kinase with e.g. $^{18}$F-fluoroganciclovir (trapping by phosphorylation) or the dopamine D$_2$ receptor with $^{18}$F-labelled fluoroethylspiperone (33). However, there are reporter proteins for optical imaging modalities, as well (bacterial β-galactosidase, green fluorescent protein, and luciferase (14, 34)).

**Antisense approach**

The antisense approach aims to monitor gene expression at mRNA level *in vivo*. In principle, it is very similar to the *in vitro* methods applied to detect nucleic acid (Southern and Northern blot, *in situ* hybridization), where labelled antisense ODN probes are used in order to recognise target nucleic acid sequences. The employment of ODNs contributed significantly to the development of molecular biology and to the possibility that, in principle, any gene can be analysed *in vitro*. Nowadays, about 25 years later, the ODNs are key molecular tools in the field of molecular imaging with the ultimate purpose of allowing the detection of any expressed gene by non-invasive *in vivo* hybridisation (25).

**Antisense concept**

Antisense ODN may interfere in the gene expression process by arresting the mRNA in the cell and thereby preventing it from being translated into a protein. Upon hybridising to its complementary “sense” sequence in mRNA through Watson-Crick base-pairing, this short, synthetic nucleic acid manifests its inhibition effect. This may act at different levels: (a) either by interfering with mRNA processing through a steric blockage of the pre-mRNA splicing or of the initiation of translation, (b) or through recognising and cleaving the RNA moiety in the mRNA-ODN duplex by ribonuclease (RNase) H, which leads to degradation of the target mRNA (15, 35). In addition, antisense ODN may inhibit transcription by binding directly to the transcription factor or to DNA and thereby forming triplets (36, 37).
The therapeutic utility of antisense nucleic acids was already suggested by STEPHENSON following the first encouraging experiments to inhibit Rous sarcoma virus replication and RNA translation with synthetic ODN in cell culture (38). This then entailed the testing of several antisense ODNs in different in vitro model systems. However, the most intensive research concerning the in vivo applications has been conducted in the past decade. Successful “knock-down” of gene expression has been demonstrated in cell cultures (39-42), and in small animal experiments (43-45).

**Modified antisense oligonucleotides**

It was quickly realised that natural ODNs with their phosphodiester (PO) backbone were unstable to serum and cellular nucleases (46), which led to searching for analogues with improved stability.

*Phosphorothioate* (PS) analogue, a first generation antisense ODN, is still among the preferred modifications, irrespective of its major disadvantages, most probably because it is widely known and analysed (47). The replacement of a non-bridging oxygen atom in the phosphate group by a sulphur along the entire backbone renders a much improved resistance against metabolism (48, 49). PS ODN forms regular Watson-Crick base pairs, activates RNase H, carries negative charges for cell delivery and displays attractive pharmacokinetic properties (50). However, its major shortcomings, as non-specific binding to proteins, reduced binding affinity towards complementary RNA molecules in comparison to their corresponding PO ODN and toxic side effects (e.g. activation of the immune system), limit its applications; however, many preclinical studies are still based on this principle (51, 52). The reduced affinity results from the fact that the melting temperature of a heteroduplex is decreased by approximately 0.5°C per nucleotide. This is, in part, compensated by an enhanced specificity of hybridisation found for PS ODNs compared to natural ODNs (50).
To further improve the antisense properties, second generation ODNs were introduced with alkyl modification at the 2' position of the ribose. The most important representatives of these ODNs, which are actually RNAs, are 2'-O-methyl and 2'-O-methoxy-ethyl RNA. 2'-O-methyl RNA (OMe) has an improved binding affinity (53-55) and excellent hybridisation capacity (35, 54, 56); it is resistant to nucleases and is less toxic than PS ODNs. Nevertheless, 2'-O-alkyl RNA cannot induce the RNase H cleavage of the target RNA (57) and it proved to be less effective and less stable in vivo than PS (51, 58).

Figure 4. The structure of natural (DNA and RNA), backbone modified (phosphorothioate DNA) and sugar moiety modified (2'-O-methyl RNA) ODNs. The picture shows also the 5' aminohexyl group, which was needed for ⁷⁶Br- and ⁶⁸Ga-labelling of ODNs in the present study.

The capability of second generation antisense ODNs to induce RNase H cleavage can be restored by gapmer or chimeric ODN design meaning an ODN with a modified-natural-modified structure. Namely, a stretch of seven-eight bp long natural DNA sequence in the middle of the ODN is enough to restore RNase H activity (55), while the flanking modified ODN sequences on both sides are essential to prevent the ODN from degradation. RNase H activity seems to be very important for gene therapy approach where elimination of mRNA of a gene being involved in a disease is required. However, for antisense imaging, RNase activity is not a prerequisite when the retention of the radio label at its specific site is of interest (59). Therefore, a modification not activating RNase may be a good candidate for imaging. Nevertheless, if RNase is activated it should be considered because of target elimination.
In recent years a variety of third generation antisense ODNs has been developed to improve target affinity, nuclease resistance and pharmacokinetics. Figure 5 shows some of the important representatives of this group, namely *locked nucleic acid* (LNA), *peptide nucleic acid* (PNA) and *phosphoramidate morpholino* (PMO) ODN (60-62), among which each research group seems to have a preferred modification. They possess high specificity and affinity with minimal non-specific binding, and they are stable enough in biological systems. They do not support RNase H cleavage of mRNA.

In particular, LNA is one of the promising candidates. It is an oligoribonucleotide (ORN) containing a methylene bridge that connects the 2’-oxygen of the ribose with the 4’-carbon (63). This locked conformation leads to a highly improved binding affinity to complementary RNA and DNA sequences (64) and increases the melting temperature (T_m) by several degrees, up to 9.6°C per LNA introduced into an ODN (65). The high affinity and specificity provides remarkable hybridisation properties, especially with respect to improved mismatch discrimination. In addition, LNA possesses high stability, it displays good aqueous solubility, low toxicity and poor immunostimulation (66-68), which makes it an obvious candidate for antisense gene silencing. Examples of dose-dependent and sequence-specific inhibition have already been published (66). When designed as a gapmer, it accelerates RNase H cleavage (55) and leads to a much higher potency of chimeric LNA-DNA-LNA ODNs in suppressing gene expression in cell culture, compared to isosequential PS ODNs or OMe modified gapmers (respectively, 175- and 550-fold superior) (35, 51). Furthermore, full LNA and chimeric DNA-LNA ODNs offer a stability against nucleolytic degradation with up to 10-fold increased half-lives in human serum compared with unmodified antisense ODNs (51, 55).
In comparative studies, LNA bound more strongly than PNA oligomer (64), and LNA might also rival the small interfering RNA (siRNA) approach for gene silencing (69, 70). In addition, the exceptional properties of LNA are also exploited in other areas of life sciences, such as detection of single-nucleotide polymorphism (67).

In PNAs, the entire sugar-phosphate backbone is replaced by N-(2-aminoethyl) glycine polyamide linkages (71, 72). This renders high resistance to nuclease and protease digestion and favourable hybridisation properties. They can form very stable duplexes or triplexes with nucleic acids, with both single or double-stranded DNA and RNA due to the lack of charge repulsion between PNA and DNA/RNA strands (in contrast, the decreased binding affinity of PS is caused by the opposing electrical charges). PNA suffers from little to no cellular uptake and have poor solubility because of being a charge-neutral molecule (35, 57). Consequently, employment of cell penetrating peptides, such as penetratin or nuclear localisation signal (NLS), is necessary (73). The inhibitory effect of gene expression by PNA is similar to that of siRNA in potency and duration (74) and may be accomplished by forming a triplet with the gene sequence in the nucleus preventing the transcription or by binding to the premature mRNA preventing the right splicing. Because of its beneficial properties, PNA is mainly utilised in genetic diagnostics for detecting nucleic acids since it significantly reduces the hybridisation time (62).

PMO is unique in the sense that the sugar moiety is replaced with a 6-membered morpholino ring, and the charged phosphodiester linkage is replaced with phosphorodiamidate linkages rendering a non-ionic chemical structure that is still highly water soluble. It is resistant to nuclease and free from toxic degradation products; however, it is sensitive to degradation when exposed longer to low pH. PMO-RNA duplexes are more stable than corresponding DNA-RNA duplexes and are much more stable than PS-RNA duplexes (62). PMO is virtually free of non-specific interactions (75). Studying of gene expression is the main application field of PMO either in antisense therapy/imaging (76, 77) or in developmental biology (62).

Criteria for an effective antisense oligonucleotide

To fulfil the effective imaging of mRNA, the requirements that an antisense ODN has to meet are very high and complex. First of all, the antisense ODN should reside in circulation sufficiently long and in such a way as to be delivered to the cells of interest. Then, it should enter cells and, once inside the cell, it should be accessible to the target recognising it with high binding affinity and specificity. Moreover, it has to be stable against nuclease in vivo. The antisense ODN-mRNA duplex should have a melting temperature high enough to prevent dissociation of the base pairs at 37°C (i.e. high enough GC content in the antisense sequence). For an acceptable specific-to-
non-specific ratio, the antisense ODN should possess minimal non-specific interactions and the excess non-hybridised ODN should also be able to leave the cells sufficiently rapidly. Prerequisites are raised from the side of the target mRNA, as well. The concentration of mRNA may need to exceed a certain level for antisense imaging and the sequence or target region cannot be involved in a hairpin structure or bound to a protein (4, 34, 59, 78, 79).

Uptake into cells

ODNs, regardless of having anionic charge or being charge-neutral possess very little or no ability to diffuse across cell membranes. In addition, they are large molecules. Therefore, they are mainly taken up through energy-dependent mechanisms, such as adsorptive endocytosis and fluid-phase pinocytosis but, also receptor-mediated endocytosis transport ODNs across cell membranes (80-83), particularly ODNs <30 bases in length. The relative proportion of internalised material depends on time, temperature and concentration of ODN. Adsorptive endocytosis is predominant at low ODN concentration, and fluid-phase endocytosis is predominant at higher concentration (57, 84). An ~80 kDa cell surface protein was isolated from several cell types seeming to be responsible for ODN binding and internalisation, which was saturable (80, 81, 85). Other ODN-binding proteins have also been identified (83). Furthermore, a 45 kDa protein localised in the plasma membrane of rat kidney cells was demonstrated to act as a nucleic acid channel allowing the passage of both PS and PO ODNs (86). A similar channel was observed in rat brain tissue, through which relatively small ODNs can enter the cells (87). Because of discrepancies and inconsistencies in results, as well as the still unknown mechanism of escape from endosomes, multiple mechanisms of uptake were suggested (88).

![Figure 6. The two major categories of endocytosis, phagocytosis or cell eating (uptake of large particles) and pinocytosis or cell drinking (uptake of fluid and solutes). The figure shows only pinocytosis for larger volumes of extracellular milieu (89).](image-url)
uptake of PS ODNs in liver, spleen, bone marrow and kidney was reported to be receptor-mediated and saturable, whereas uptake in muscle, skin and intestine was dose-independent (90-92).

In PS uptake, a plateau was achieved after approximately 1 to 24 h of incubation (93, 94). In a comparative study, early cellular uptake (1-3 h) was similar for an OMe ODN; however, by 9-24 hours, cellular accumulation of PS exceeded that of OMe by 3-to 5-fold. Interestingly, the uptake was much higher in transformed cells, suggesting that transformation enhances cellular uptake (95). In addition, several studies have provided evidence that ODNs enter cells in organs. It was calculated that up to $10^8$ molecules of antisense DNA accumulate in each cell over 5-10 h of incubation (96). Autoradiographic, fluorescent, and immunohistochemical approaches have shown that these ODNs are localised in convoluted tubular cells, various bone marrow cells, skin cells, and liver cells (97). It has also been demonstrated that dead cells accumulate large amounts of ODNs (98).

A major remaining challenge is to achieve efficient and non-toxic delivery of ODNs into the cells in a broad range of target tissues. At present, intensive research is ongoing to find potent delivery agent candidates (99, 100), such as liposomes, charged lipids, polymers (polyethylene derivatives), nanoparticles (nanocapsules) and peptides (83). The main role of these compounds is to enhance uptake by binding to negatively charged cell membrane or to receptors and facilitate endocytosis. For example, a transferrin receptor antibody was utilised to cross the blood-brain barrier (101, 102). These transfection reagents may also help to disrupt the endosomal membrane to release the ODNs from the endosomal compartment.

**Target accessibility**

Once the ODNs are internalised in the cell, they must be released from the endocytotic vesicles in order to reach the cytosolic compartment and their target RNA (78). However, as a result of endocytosis, ODNs become trapped in endosomes and/or lysosomes (57, 87) and they usually become degraded there. Cationic lipids are able to accomplish destabilisation of the endosomal membrane and help to translocate ODNs into the cytosol (103). Nevertheless, several groups have shown that exogenously administered ODNs appear to localise readily in the nucleus, cytoplasm or mitochondria (66, 88) and there are suggestions that certain ODN could escape the endosome.

**Elimination from cells**

It is necessary for unhybridised ODNs to leave the cells, especially for antisense imaging, in order to achieve high specific versus non-specific ratio. It seems that membrane passage is difficult even from inside the cell; however studies demonstrated that both PO and PS were effluxed by exocytosis from cells, where they previously showed to be localised in intracellular vesicles.
PO seemed to be effluxed more rapidly from cells than PS with a half-life of 5-15 min (78). The efflux of PS ODNs was biphasic, with a rapid efflux in the first phase (half-life 10-30 min), followed by a much slower phase. Another study demonstrated that efflux was sequence dependent and delivery agents, such as liposome, facilitated the elimination process (104).

Affinity and specificity
Affinity reflects the stability of the hybrid between the ODN and the target, while specificity displays the ability of ODN to recognise the target sequence and not to form a mismatched binding.

The affinity of a single-stranded DNA to its complement (characterised as Tm, the melting temperature at which half of the duplex has dissociated into single strands) depends on the sequence and chain length. The binding affinity of antisense drugs for mRNA is usually in the nanomolar range (105). A single mismatch can drop the affinity by as much as 300-fold (34). Compared to a PO ODN as reference, Tm is lower for a PS and higher for an OMe with the same sequence (53). Dewanjee et al. reported the kinetics of hybridisation of an 111In-labelled ODN targeted to c-myc mRNA and maximal association was observed at one hour (106).

While the nanomolar affinity of an ODN appears ideal for a radiopharmaceutical, the kinetics of binding is a more critical consideration for ODNs in vivo. For antisense, the binding is a multi-step process induced by a time-consuming recognition of a nucleation site followed by a quick hybridisation step, similarly to a zipper mechanism. Therefore, the hybridisation rates of antisense ODNs are low (107) and depend on the secondary structure of the target (39).

Specificity is an essential parameter for in vivo antisense applications and they should ideally discriminate point mutations on mRNA. Mathematical analysis demonstrated that only 11 to 15 bases need to be targeted in order to hybridise uniquely to mRNA in the human genome and an antisense probe of 15-20 nucleotides long sequence may be enough to target a given mRNA (39, 41, 45, 108). Lengthening improves the affinity, but at possible expense of reducing cell membrane transport; furthermore, it might lower the specificity as small regions find unintended matches (59).

A study on the possibility to distinguish between the oncogenic form of H-ras, found in a single-point mutation on codon 12, and wild type ras showed that the best antisense achieved a five-fold discrimination between the two forms. If maintained in vivo, this difference would be compatible with selective imaging (39). Both LNA and PNA have a high mismatched discrimination property compared to DNA based on the significant decrease in Tm for the mismatched duplexes (67, 109).
Stability

In blood, tissues and cells, the stability of antisense ODNs is endangered by nucleases such as exonucleases (cleaving off nucleotides from the 3’- or 5’ prime side of an ODN) and endonucleases (cutting the ODNs at an insider nucleotide). Natural PO has a half-life of a couple of minutes \textit{in vivo} (110), while its stability \textit{in vitro} ranges from 15 to 60 min varying as a function of length and sequence and also depends on the type of sera evaluated (111-115).

The half-life of many PS ODNs in biological fluids is reported to be longer than 24 h (93). It has a half-life in human serum of approximately 9-10 h compared to 1 h for natural ODNs (116, 117). It is degraded slowly by cells in tissue culture with a half-life of 12–24 h and are slowly metabolised in animals (112, 118, 119). The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases. However, several evidences suggest that, in many cells and tissues, endonucleases are important in the metabolism of ODNs (120). \textit{In vivo}, PS was found intact two hours after intravenous (i.v.) injection (58, 110, 121).

The half-life of 5 h has been reported for an OMe in cell culture medium (51), while \textit{in vivo}, OMe proved to be stable for two hours (110).

In the only \textit{in vivo} metabolite analysis performed so far with LNA, most of the injected 16-mer full LNA ODN was still intact after 30 min of incubation with only minor peaks for 13-15-mers (68). In serum, the same LNA remained stable for more than 2 h. Also a 16-mer LNA gapmer with thiolated backbone showed great stability in human serum within 24 h (122). A 15-mer LNA-DNA mixmer (LNA and DNA nucleotides in alternation) incubated in rat serum was also stable even after 20 h (123). In human serum, in case of an 18-mer LNA-DNA-LNA gapmer ODN, the introduction of three or four LNA at each end increased the half-life in human serum to ~15 h (55). This indicates that LNA ODN is remarkably stable. It imposes significant protection against nucleolytic degradation, especially if more than one LNA nucleotide is incorporated into an ODN (67, 70). The terminal positioning of LNA nucleotides is important as the 3’ terminal LNA residue slows the 3’→5’ exonucleolysis, while an LNA group in the second position from the 3’ terminal gives complete resistance to degradation (124).

PNA was reported to be highly stable against nuclease and protease attack both \textit{in vitro} and \textit{in vivo}. In one \textit{in vivo} analysis, metabolites of PNA ODN were not detected in mice plasma and urine samples for up to 6 h (125). Incubation of PNA in human serum and with nucleases and proteases did not result in significant degradation (109).

Non-specific binding

Binding of ODNs to undesired RNA sequences may pose little problems, because dissociation constants of ODNs are in the nanomolar range and de-
pend strictly on the complementarity of the two strands of the duplex. In contrast, non-specific binding to proteins is a major issue, especially for the PS derivatives (126, 127) possibly due to increased lipophilicity resulting from the presence of sulphur atoms in the backbone (59). Binding to serum proteins, including albumin, prevents rapid clearance by glomerular filtration, thus providing prolonged plasma half-time and the opportunity to distribute to peripheral tissues (120). The binding to albumin was reported to be saturable for PS, PO and OMe ODNs and the extent of binding decreased in that order (58). In contrast, third generation ODNs possess minimal non-specific binding or off-target effects (those interactions that are not due to Watson-Crick base pairing) (75).

Target mRNA concentration

The number of mRNA copies per cell may be critical to the success of antisense imaging. Typically, mRNA is present at levels of a few to a few thousand molecules per cell (128) or in the range of 1 to 1000 pmol/L if expressed in concentration. In disease conditions such as cancer and viral infections, some mRNAs may be expressed 100-10,000 times higher (4). In an assumption, there may be around 0.4 pg total mRNA per cell representing around 500,000 fully mature mRNA molecules. Assuming about 2000 different mRNA species at any moment, there are on average 250 molecules or about 100 pM of a given mRNA present per cell. This calculation is relevant for imaging if the tissue in question expresses homogeneously the mRNA in the cells contained in the volume of interest (129). Yet, information on absolute cellular concentrations of mRNA is still insufficient. As an example, about 1800 molecules of tyrosine hydroxylase mRNA per catecholaminergic cells in the rat adrenal medulla was estimated in a study (130). In principle, PET is able to detect 100-1000 targets per cell, which corresponds to about 40-400 pM.

It was estimated that mRNA concentrations as low as 1 pmol/L tissue can be imaged with PET, by use of radionuclide-labelled antisense ODN probes with specific radioactivity of 3.7x10^4-3.7x10^5 MBq/μmol, which is within the range of present positron labelling (34). According to another assumption, imaging of target tissue by antisense approaches should be feasible if about 7.4 kBq would accumulate in each gram of target tissue assuming 10^8 cells per gram and saturation with a 22-base oligomer labelled with a positron emitter at a specific radioactivity of 74 MBq/μg (96). This calculation is based on mRNAs that are over-expressed, possibly with 100 copy numbers per cell. Assuming only one target mRNA per cell, the radioactivity accumulation would be only about 0.074 kBq (34), a value which is currently below the limit of detection by a PET scanner.

The lifespan and production rate of mRNAs are also important factors. In the cytoplasm, the mRNAs have widely variable half-lives, ranging from one or a few minutes for mRNA coding for cytokines, cell-cycle regulated genes,
or proto-oncogenes such as c-myc, to hours or days for housekeeping genes, and appear to be highly regulated (59, 129, 131). For successful antisense targeting, target mRNA turnover rate seems to be more important than the steady-state mRNA level, as it would increase the accumulation of antisense ODNs in target cells compared to the accumulation level in non-target tissues that would remain unchanged (132). Another study also suggested this because the specific uptake per cell at 24 h was much higher than the estimated steady-state target mRNA level indicating that feedback mechanisms could stimulate the production of additional mRNA that could explain the high specific uptake.

Target mRNA structure
The mRNA molecules are typically several hundred to thousands base pairs long, but not all of these bases are accessible to a probe because of the secondary and tertiary structure of the mRNA molecule and/or proteins bound to the RNA. Intracellular single-strand mRNAs form extensive secondary structures and are invariably protein bound (133). The secondary structures are composed of double stranded regions, stem-loops, hairpins, pseudo-knots, etc. These may be required for stability and possibly for recognition by those proteins regulating translation. However, this leaves only short stretches of single strand available for base pairing (15). The affinity of antisense DNA for duplex, compared to singlet regions of mRNA is $10^5$-10$^6$-fold lower (134). Therefore, antisense strategies usually seek to target only single-strand regions. Also therefore, it is important to identify the optimal target sites and test a number of ODNs for their antisense efficiency with careful studies (35, 59). It was demonstrated that, at best, no more than 6%–12% of ODNs targeting an RNA sequence are efficient at forming the duplex necessary for the antisense effect (135).

In order to identify favourable local target sequences and to predict the secondary structure of RNA, different computer-based methods have been established (52, 136, 137). These methods demonstrated that 17 of the 34 antisense ODNs tested showed significant inhibition (>50%) of ICAM-1 expression in mammalian cells. This may help significantly in ODN design but, these methods are unable to provide an antisense ODN targeting with 100% success.

Considerations about toxicity
Since ODNs are modified and used as drugs it is essential to investigate the potential toxic effects. Toxicities of the PS ODNs have been attributed to its negative charge and protein-binding affinity. In monkeys, the principal dosage-limiting toxicities are hypotension and bradycardia, probably associated with complement activation and prolonged clotting time, probably associated with thrombin inactivation (138). In rodents, the most prominent toxicity is
immune stimulation (120). Nevertheless, appreciable toxicity in animals was only observed after prolonged administration (59). In patients, administration of antisense ODNs entailed no significant toxicity (139). PS ODNs have also been found to be non-mutagenic (59).

In contrast, LNA ODN is well tolerated in rats and mice. No toxic effects or any histologically detectable toxicity could be observed with LNA or DNA-LNA mixmer, not even after 14 days of continuous administration at a dose of 1 mg/kg/day. However, treatment with 5 mg resulted in elevated liver enzyme values without histological changes in liver (69). Furthermore, 4 days after a single administration of LNA ODN significant hepatotoxicity in mice was observed in one study (140). It was concluded that intact LNA with thioated backbone was responsible for the observed toxicity. In contrast, no signs of liver toxicity were observed following a 4-day, 25 mg/kg/day treatment of an LNA-DNA mixmer (141). PNA was reported to have unspecific toxic reactions at concentrations above 5-10 µM (37).

Another possible mode of toxicity could result from unintended hybridisation of antisense ODNs to non-target mRNAs with low affinity (59). This binding could interfere with the expression of normal genes and, consequently, with proper cellular function. Nevertheless, the probability is extremely low that an ODN analogue could be integrated into the genome and produce mutagenic events. Viral DNA integration is itself a rare event and, of course, viruses have evolved specialised enzyme-mediated mechanisms to achieve integration. Another genotoxicity concern is that ODNs might be degraded to toxic or carcinogenic metabolites. However, metabolism of PS ODNs would release normal bases, and the hydrolysed PS bonds would be rapidly oxidized to natural (non-toxic) nucleoside phosphates (120).

In general, antisense ODNs are much less toxic than conventional chemotherapeutic drugs and ODNs will be administered at low dosages for antisense imaging (59).

In vivo imaging of hybridisation

Being aware of the many hurdles and barriers, which an ODN has to overcome, accomplishing in vivo hybridisation seems to be more complicated than it was originally thought. Methods are still under development and although slowly, it is steadily progressing. It seems that many years of research is still necessary for success. For example, in vivo imaging of endogenous gene expression in animals and humans has still not been demonstrated in a conclusive manner since antisense imaging agents have displayed limited delivery, low localisation specificity, high background noise, and several biological barriers to pass through (128). Not to mention further necessities such as improved target/non-target radioactivity ratios and proofs that real antisense mechanism is behind the successful tumour targeting ex-
experiments (59, 128, 142). At present, imaging of the bio-distribution of ODNs is a reality but imaging of hybridisation using ODNs is still a major challenge (25).

A small number of imaging attempts has been published so far. DEWAN-JEE et al. were the first to demonstrate the targeting of endogenous gene expression with antisense probes using gamma camera imaging on a tumour-bearing mice model (143). This study demonstrated rapid targeting to tumour expressing c-myc oncogene compared to a control tumour during a 2 h long imaging follow-up after i.v. injection. The uptake of the antisense in the tumour was 10%/g at 30 min, compared to less than 1%/g with control ODNs. No subsequent investigations were able to report verification of these preliminary, but encouraging results.

In an ex vivo study of rat glioma with a 25-mer 11C-labelled PS ODN, targeted to glial fibrillary acidic protein mRNA, KOBORI et al. reported prominent radioactivity uptake in the glioma compared with control sense sequences. This suggests that accumulation in the glioma was correlated with recognition by the antisense probe of the target mRNA over-expressed in tumoural tissue (144).

WU et al. reported the pharmacodistribution of 76Br-labelled PS ODNs of different lengths (6, 12, 20 and 30 bp) targeting rat Chromogranin A (Chg-A) mRNA. The distribution was clearly dependent on length, with highest uptake in the kidney cortex for the shortest and in the liver and spleen for the longest ODNs. Significant uptake in the adrenals, the organ targeted by the Chg-A antisense, was observed only with the 20- and 30-mer ODNs, and remained at modest values (145).

A proof of hybridisation of two complementary sequences was brought by a study of MARDIROSSIAN et al. in which a 99mTc-labelled 15 bp PNA was shown to target its complementary PNA (cPNA) sequence coupled to polystyrene beads implanted intra-muscularly in the thigh of a mouse (146). A 10-fold difference in the binding to the cPNA-coupled versus uncoupled beads was reached during the first hour, and was maintained for at least 24 h.

The group of HNATOWICH imaged 99mTc-labelled PO and PS 22-mer ODNs in normal mice (147). Both compounds showed low molecular weight metabolites, demonstrating that PS is not totally stable in vivo and showed high levels of protein binding.

TAVITIAN et al. reported the bio-distribution of antisense probes (PO, PS, and OMe) in baboons in vivo with PET (110). This study demonstrated that each of the three types of 18F-labelled ODNs studied behaved very differently in vivo, that the labelling did not affect the bio-distribution of each probe, and that PET could be used to quantify the bio-distribution of ODNs. This study focused on the in vivo pharmacokinetics of the antisense ODNs and did not attempt to target any particular mRNA. The specific radioactivity of the antisense ODN used in this study was 7.4x10^4 MBq/μmol (4).
ROIIVAINEN et al. used $^{68}$Ga-labelled 17-mer antisense ODNs against point mutationally activated K-ras mRNA. The intravenously injected tracer revealed high-quality PET images that allowed quantification of the bio-kinetics in major organs and in tumours containing K-ras point mutation versus tumours with wild type K-ras oncogene (58).

Finally, SUN et al. imaged MCF-7 tumour cells in mice with $^{64}$Cu-labelled PNA-permeation peptide conjugate using microPET camera (148). All the four PNA sequences were able to image the tumour xenograft, but the image contrast varied with the sequence. One of them showed tumour/muscle ratio of $6.6 \pm 1.1$ at 24 h post-injection, which is among the highest reported for radio-labelled ODNs.

**Existing criteria for proving antisense mechanism**

Besides the pending major challenges mentioned for the purpose of achieving efficient and non-toxic delivery of ODNs into the cells in a broad range of target tissues, another major challenge is how to prove that a real antisense mechanism is behind the successful downregulation or tumour targeting experiments. This seems to be a difficult task, especially, in *in vivo* imaging applications, where the concentration of the probe is sub-therapeutic and hence may not affect the magnitude of mRNA or protein production. So far, to the best of our knowledge, only HNATOWICH and his research group have reported indirect evidences of antisense mechanisms in cell culture experiments and in xenograft models (132, 149-151).

For a real antisense mechanism, one has to prove that the localisation of ODN in cells was due to its binding to target mRNA and not to an aptameric mechanism. According to Hnatowich’s research group, the existing criteria for proving specific hybridisation are the demonstration of (1) higher accumulation of antisense ODN in comparison to a control (sense, random or scrambled); (2) higher accumulation of antisense demonstrated in a variety of cell types; (3) higher accumulation of antisense demonstrated for different mRNA targets; and (4) higher accumulation of antisense shown to decrease with increasing unlabelled antisense ODN concentrations, i.e. concentration dependency.

**Antisense therapy**

Antisense ODNs are tested in clinical trials in order to find molecular corrections for diseases at gene expression level, which, actually, goes back longer in time than application for imaging. Nevertheless, antisense imaging could contribute to these clinical applications. For example, PET could help in validating new antisense sequences for therapy by monitoring them in the body. Furthermore, antisense ODNs for either imaging or therapy share
The chances for successful achievement in therapeutics compared to images were predicted to be higher but there have been a lot of ups and downs in the development of antisense ODNs for therapeutics in human diseases (152). The breakthroughs in the area of nucleic acid-mediated gene silencing have not come yet (153). Nevertheless, third generation ODNs are promising candidates in therapy, as well. There are already early indications from phase 1 and phase 2 clinical trials suggesting gapmers to be promising clinical candidates (154) together with LNA, PMO and even siRNA. These trials include a wide field of diseases, mainly cancer. The only antisense drug available on the market, Vitravene, is for cytomegalovirus retinitis for AIDS patients (155). However, only relatively few people have been treated with Vitravene and it is administered by intraocular injection, which is a specialised case. Nevertheless, there is no such a class of synthetic compounds that are more dissimilar from traditional drugs regarding the hurdles such as the definition, for example, of the mechanism of action of ODNs and systemic administration at a reasonable cost (154).

Additional background for the present study

The intention with this section is to present background information that is relevant to the performed experimental studies.

Radionuclide-labelling of antisense oligonucleotides

An ODN can be recorded with PET methodology with respect to biodistribution study if it is labelled with a radionuclide of sufficiently long half-life matching the pharmacokinetics, with the expectation that the labelling method does not perturb the property of the molecules with respect to metabolism, selective hybridisation or non-specific interactions.

The labelling methods available include both gamma emitters, such as $^{90}$Y, $^{99m}$Tc, $^{111}$In, $^{125}$I (114, 143, 156, 157), and positron emitters, such as $^{11}$C, $^{18}$F, $^{68}$Ga, and $^{76}$Br (110, 144, 158-160). The reported methods modify the ODNs by addition of labelled groups or radio label chelating groups. Compared to radio-halogenetion, however, radio-metal ion complexation reactions have simpler chemistry and allow tracer production kits. Generator available radio-metals are preferable to costly cyclotron produced ones. The modification of ODNs for labelling increases the stability of the ODN to
degradation by exonucleases and allows labelling (160). For choosing the isotope and labelling method, it should be considered that PET has high sensitivity, acceptable resolution and good quantification accuracy compared with other imaging modalities such as SPECT.

**Targeted genes**

The Chromogranin-A (Chg-A) gene, which is significantly expressed by neuroendocrine cells and neurons (161), was chosen as target because of its specific localisation and high expression in neuroendocrine tumours. The prominent release of Chg-A protein in these tumours is used for diagnosis and treatment monitoring (162). As an alternative target, the K-ras oncogene was chosen as a model because of its significance in cancer biology and oncology.

Chg-A is a glycoprotein of 439 amino acids (163), which is released in the extracellular environment and after secretion, Chg-A can reach the blood stream via the capillaries or the lymphatic vessels (164). Although the extracellular function of Chg-A is not yet clearly understood, it is believed that this protein is a multivalent precursor of several polypeptides that may exert autocrine, paracrine, and endocrine effects (165). Chg-A is abnormally expressed by various tumours, including pheochromocytoma, carcinoid tumours, medullary thyroid carcinoma, pancreatic islet cell tumours, small cell lung cancer, prostate cancer, and many others (166). Chg-A, released in high amounts in the blood of patients, has proven to be a sensitive and specific serum marker for the diagnosis of various types of neuroendocrine tumours. Moreover, serum Chg-A is an independent marker of prognosis in patients with carcinoid tumours (162). It is suggested that Chg-A expression by tumour cells can affect tumour development and architecture. It has been shown that the expression of Chg-A decreases with increasing malignancy in neuroendocrine tumours, being higher in well-differentiated carcinomas (low grade) and lower in poorly differentiated (high grade) carcinomas (167).

Members of the ras gene family, H-ras, K-ras and N-ras, are thought to be involved in normal cell growth and maturation (168). However, a point mutation induced by carcinogens or environmental factors causing an amino acid alteration at one of the three critical positions in the protein results in conversion to a form that is involved in the formation of tumours (169, 170). Approximately 10-20 % of all human tumours have a mutation in one of the ras oncogenes; however, over 90 % of pancreatic adenocarcinomas, about 50 % of adenocarcinomas of colon, lung adenocarcinomas, thyroid carcinomas, and a large fraction of haematological malignancies have been found to be associated with point mutationally activated ras oncogenes (169). The ras oncogene mutations are found in a variety of human tumours but not in normal tissue, which makes ras mRNAs suitable targets for demonstration of gene expression by non-invasive imaging.
### Table 1. Examples of bio-distribution studies reported in the literature

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Target gene</th>
<th>Type of animal</th>
<th>Time point (h)</th>
<th>Length of ODN (bp)</th>
<th>Type of ODN</th>
<th>Organs of high uptake</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C</td>
<td>E2 (HPV)</td>
<td>Rat</td>
<td>1</td>
<td>20</td>
<td>PS</td>
<td>Liver, kidney, spleen, bone marrow</td>
<td>(118)</td>
</tr>
<tr>
<td>$^{3}$H</td>
<td>–</td>
<td>Mouse</td>
<td>1</td>
<td>20</td>
<td>PO</td>
<td>Spleen, kidney, liver, heart</td>
<td>(46)</td>
</tr>
<tr>
<td>$^{99m}$Tc, $^{111}$In</td>
<td>–</td>
<td>Mouse</td>
<td>2.5</td>
<td>22</td>
<td>PO</td>
<td>Kidney, liver, stomach, intestines</td>
<td>(156)</td>
</tr>
<tr>
<td>$^{3}$H</td>
<td>c-raf-1</td>
<td>Rat</td>
<td>6</td>
<td>20</td>
<td>PS</td>
<td>Kidney, liver, spleen</td>
<td>(121)</td>
</tr>
<tr>
<td>$^{3}$H</td>
<td>–</td>
<td>Mouse</td>
<td>24</td>
<td>20</td>
<td>PS</td>
<td>Kidney cortex, liver, spleen</td>
<td>(110)</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>–</td>
<td>Baboon</td>
<td>1</td>
<td>18</td>
<td>PO</td>
<td>Urinary bladder</td>
<td>(110)</td>
</tr>
<tr>
<td>$^{76}$Br</td>
<td>chr A</td>
<td>Rat</td>
<td>20</td>
<td>20</td>
<td>PS</td>
<td>Kidney, liver, spleen, adrenal gland</td>
<td>(145)</td>
</tr>
<tr>
<td>$^{35}$S</td>
<td>human intracell. adh. mol-1</td>
<td>Rat</td>
<td>20</td>
<td>20</td>
<td>PS</td>
<td>Kidney, liver, spleen, adrenal gland</td>
<td>(119)</td>
</tr>
<tr>
<td>$^{3}$H</td>
<td>EGFP-654</td>
<td>Mice</td>
<td>24</td>
<td>16</td>
<td>Mixmer</td>
<td>Kidney, liver, colon, small intestine</td>
<td>(141)</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>human Tenascin-C</td>
<td>Mice</td>
<td>1</td>
<td>39</td>
<td>Gapmer*</td>
<td>Kidney, liver, spleen</td>
<td>(171)</td>
</tr>
<tr>
<td>$^{3}$H</td>
<td>RNA polymerase II</td>
<td>Mice</td>
<td>0.5</td>
<td>16</td>
<td>LNA</td>
<td>Kidney, liver, skin, bone</td>
<td>(68)</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>–</td>
<td>Mice</td>
<td>2.5, 24</td>
<td>15</td>
<td>PNA</td>
<td>Kidney</td>
<td>(146)</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>unr</td>
<td>Mice</td>
<td>0.3, 1, 4</td>
<td>19</td>
<td>PNA†</td>
<td>Kidney</td>
<td>(148)</td>
</tr>
</tbody>
</table>

Mixmer = LNA-DNA in alternation; Gapmer = LNA-DNA-LNA; *aptamer (targeting protein); †PNA conjugated to a permeation peptide.
Pharmacokinetics

Pharmacokinetics deals with the study on how drug molecules behave in the body after administration, which includes the evaluation of absorption, distribution, metabolism and elimination (ADME). For the usual PET pharmacokinetic studies however, the PET tracer is administered i.v., escaping therefore the absorption step, and providing a rapid distribution all over the body with the blood stream (22). Under these conditions, the other advantage is that PET methodology allows recording the kinetics of the tracer in an organ while plasma kinetics is also recorded by blood sampling preferably associated with radio-metabolite evaluation with respect to time. Since the present study embraced only the preclinical aspects (in vitro, ex vivo) of radio-labelled ODNs, distribution and metabolism have been relevant.

Pharmacokinetics is the resulting combination of the elementary properties of ODNs: stability, solubility, cellular penetration, uptake in the target tissue, blood clearance, urinary excretion, etc. (107). Obviously, any chemical modification of the ODN could be expected to have effect on the specific hybridisation, sensitivity to nucleases, elimination, membrane passage, protein binding etc., and may thereby cause major alterations in the pharmacokinetics, affecting its biological activity. Therefore, it is essential to learn the basic pharmacokinetics of a modified ODN, especially for ODNs with possible therapeutic potential (110) but also for imaging purposes when ODN is administered to humans.

Bio-distribution

The bio-distribution seems to depend mainly on the length and modification of ODNs, type of labelling and species used. Table 1 provides a summary on the reported findings relevant to the present study. It is clear from the results that kidney and liver are the two organs showing the highest uptake. In addition, the organ uptake pattern of the same ODN modification displays similarities across species (mouse, rat, monkey), which provides a level of confidence that preclinical animal models can be predictive towards exposure in the clinic.

Metabolite analysis

A drug introduced into the body is endangered by the attack of enzymes already in the plasma and upon that it loses its biological activity. Evaluating the process of metabolism is therefore important from two perspectives: (1) to know how fast the drug becomes degraded and, (2) to know which metabolites are created, because the drug may stay intact while the radio-labelled group becomes removed thereby causing a false detection.

The metabolism of antisense ODNs is summarised under stability.
**Scavenger receptors**

Scavenger receptors in liver, bone marrow and kidney were suggested to be contributing to the elimination of PS and PO ODNs from blood (172, 173). It was demonstrated that, respectively, 89% and 54% of the membrane-bound $^{32}$P-PO ODN binding to liver endothelial cells and Kupffer cells was internalised within 10 min (172). It was also observed that PO ODN uptake in scavenger receptor-transfected cells increased (174).

In general, scavenger receptors, as their designation indicates, have a scavenging function facilitating the clearance of microbial pathogens, senescent cells or altered plasma proteins by phagocytic cells. Nevertheless these receptors are also involved in fatty acid transport. Class A type I and II scavenger receptors (SR-AI/II), which are expressed on macrophages, endothelial liver cells and to a lesser extent on Kupffer cells, are the best characterised from this receptor family of six classes. Due to their broad poly-anionic ligand specificity, polynucleotides also bind to them and become internalised (175, 176). Poly-inosinic acid (poly-I) polyribonucleotide effectively inhibits the binding of a ligand to SR-AI/II, whereas poly-adenylic acid (poly-A), with a different tertiary structure, is a poor inhibitor. Nevertheless, these agents might not be fully specific.

Several blocking studies of scavenger receptors identically report that poly-I and dextran sulphate resulted in a 40-60% reduced hepatic uptake of ODNs compared to poly-A implying the participation of scavenger receptors in liver uptake (92, 175, 177). In contrast, uptake in kidney was significantly reduced by poly-A, but not by poly-I indicating an uptake by scavenger receptors other than the liver receptors. Spleen, muscles, and skin were not significantly affected by either polyribonucleotide.
The present study

Aims

The overall aim of the present study was to evaluate and characterise radio-labelled ODNs for \textit{in vivo} imaging of hybridisation using PET methodology.

- Initially, the focus was on the exploration of differently modified ODNs in order to select the most potent ODN for imaging purposes, which was based on investigation of the
  - effect of radio-labelling on the hybridisation abilities of ODNs, and
  - bio-distribution and metabolite analysis of ODNs.

- After having realised that antisense ODNs may reach the targeted cells in a multi-factorial process, the aim focused on the understanding of uptake specificity of radio-labelled ODNs by different tissues.
Materials and Methods

Oligonucleotides

The ODNs used in the present study are listed in Table 2. Antisense ODNs possessed different type of modifications and an aminohexyl linker in the 5’-position (Fig. 4) for conjugation and ⁶⁸Ga-labelling. The backbone modifications included phosphodiester (PS), phosphorothioate (PS) and, the sugar modifications involved 2’-O-methyl (OMe) and locked nucleic acid (LNA) (both with phosphodiester backbone; however OMe contained uracil instead of thymidine). Mixmer ODNs consisted of equal number of alternating LNA and DNA nucleotides along the sequence. In contrast, sense ODNs were made up of natural phosphodiester backbone and deoxyribose sugar. Their sequence matched with their corresponding antisense ODN.

Table 2. Antisense and Sense sequences used

<table>
<thead>
<tr>
<th>Designation</th>
<th>Modification</th>
<th>Target</th>
<th>Sequence bp</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO&lt;sub&gt;Chg&lt;/sub&gt;, PS&lt;sub&gt;Chg&lt;/sub&gt;</td>
<td>PO-PS</td>
<td>ChgA</td>
<td>5’-caccctagggtgtccccctttgtcatagggct-3’</td>
<td>30</td>
</tr>
<tr>
<td>PO, PS, OMe</td>
<td>PO-PS-OMe</td>
<td>K-ras</td>
<td>5’-ctacgccactagctca-3’</td>
<td>17</td>
</tr>
<tr>
<td>LNA1</td>
<td>Mixmer</td>
<td>ChgA</td>
<td>5’-GtGtCcCcTrTtGtCaTaGg-3’</td>
<td>20</td>
</tr>
<tr>
<td>LNA2</td>
<td>Mixmer</td>
<td>ChgA</td>
<td>5’-gTgTcCcCtTrTgTcAtAgG-3’</td>
<td>20</td>
</tr>
<tr>
<td>Mismatched</td>
<td>Mixmer</td>
<td>ChgA</td>
<td>5’-gTgTcCcCtAcTgTcAtAgG-3’</td>
<td>20</td>
</tr>
<tr>
<td>Sense</td>
<td>Mixmer</td>
<td>ChgA</td>
<td>5’-cCtAtGaCaAaAgGgGcCaC-3’</td>
<td>20</td>
</tr>
<tr>
<td>sense&lt;sub&gt;K&lt;/sub&gt;</td>
<td>PO</td>
<td>K-ras</td>
<td>5’-tggagctagtggcctag-3’</td>
<td>17</td>
</tr>
<tr>
<td>sense&lt;sub&gt;Chg&lt;/sub&gt;</td>
<td>PO</td>
<td>ChgA</td>
<td>5’-cctatgaaaaaggggtac-3’</td>
<td>20</td>
</tr>
</tbody>
</table>

PO = phosphodiester; PS = Phosphorothioate; OMe = 2’-O-methyl phosphodiester; Mixmer = LNA-DNA in alternation; bp = base pair; UPPER CASE LETTERS = LNA; lower case letters = DNA; underlined letters = mismatched nucleotides; Chg = Chromogranin; K = K-ras.

In paper I, III and IV, the antisense sequences specific for rat Chg-A mRNA were used (NCBI accession number: NM_021655). In paper II, the antisense sequences complementary to human K-ras oncogene targeting codon 12 point mutation were applied (based on the sequence of human K-ras oncogene exon 1 (40). The BLAST search (Blast Sequence Similarity Searching; NCBI) revealed no homology of the antisense sequences to other known rat genes.
Radio-labelling of oligonucleotides

Labelling with $^{76}$Br (paper I)
The $^{76}$Br was produced by the $^{76}$Se(p,n)$^{76}$Br nuclear reaction using a cyclotron. Through a 5' aminohexyl linker (Fig. 4), PO Chg or PS Chg ODN was labelled by reacting with $N$-succinimidyl 4-[${}^{76}$Br]bromobenzoate, which resulted in $[{}^{76}$Br]bromobenzoate-ODN (159). The specific radioactivity for 4-$[{}^{76}$Br]bromobenzoate was 20-200 MBq/nmol (178).

Labelling with $^{68}$Ga (paper II-IV)
The ODN tracer synthesis was performed by a two-step procedure where ODN bearing an aminohexyl linker (Fig. 4) was initially conjugated to a bifunctional chelator, DOTA, and thereafter labelled with $^{68}$Ga via a complexation reaction of $^{68}$Ga with the chelator (160). The purified DOTA-conjugated ODN was stored in a refrigerator until use and was found to be stable for at least six months.

Table 3. Specific radioactivity of $^{68}$Ga-ODN (mean ± S.E.) after labelling

<table>
<thead>
<tr>
<th>ODN type</th>
<th>MBq/nmol</th>
<th>MBq/mg</th>
<th>SRA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO</td>
<td>3.17 ± 1.57</td>
<td>604.7 ± 345.6</td>
<td>–</td>
</tr>
<tr>
<td>PS</td>
<td>0.93 ± 0.24</td>
<td>167.7 ± 42.6</td>
<td>–</td>
</tr>
<tr>
<td>OMe</td>
<td>1.40 ± 0.27</td>
<td>244.9 ± 47.3</td>
<td>–</td>
</tr>
<tr>
<td>LNA1, LNA2</td>
<td>3.59 ± 0.51</td>
<td>545.1 ± 77.8</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Mismatched</td>
<td>4.27 ± 0.64</td>
<td>650.4 ± 98.0</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td>Sense</td>
<td>2.56 ± 0.46</td>
<td>384.8 ± 69.6</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

SRA* is the average of the normalised specific radioactivity calculated as follows: spec. radioactivity (MBq/nmol) divided by the corresponding $^{68}$Ga radioactivity from the generator multiplied by the amount of ODN used for the labelling. It reflects the incorporation efficiency of the labelling. The low variation between the normalised values indicates high reproducibility of the labelling.

The labelling of the conjugates was performed using either non-concentrated (paper II) (160) or pre-concentrated (paper II-IV) (179-181) $^{68}$Ga-eluate obtained from a $^{68}$Ge/$^{68}$Ga generator ($T_{1/2} = 270.8$ days, about 1.5 years life span). When it was needed, the amount of initially available $^{68}$Ga was increased by pre-concentration of $^{68}$Ga-eluates from two generators. About 10-40 nanomoles of DOTA-ODN were used per labelling and complexation was performed in a microwave oven. Following purification and product analysis the 50-80% EtOH solvent was either not, or partly, or completely evaporated. In the later case, ODN was re-dissolved in an appropriate buffer solution for subsequent application. Table 3 summarises the specific radioactivities of the ODNs.
Hybridisation in solution

Hybridisation in solution was performed to examine the hybridisation abilities of the ODNs after labelling (paper II-III). Following the evaporation of the solvent, the $^{68}$Ga-labelled antisense PO, PS, OMe, LNA1 or LNA2 ODN was dissolved in buffer. The hybridisation mixture was prepared as follows: a gradually increasing amount of complementary sense ODN was added to a constant concentration of $^{68}$Ga-antisense-ODN. As reference solutions, $^{68}$Ga-antisense-ODN and unlabelled sense ODN were used. All mixtures were kept on ice until commencement of hybridisation at 40°C for 10 min.

The subsequent gel electrophoresis was performed with 20% non-denaturing polyacrylamide gel (PAGE). After electrophoresis, the gel was stained using ethidium bromide to visualise the DNA and photographed under UV light. Subsequently, the gel was exposed to a phosphor imaging plate overnight, scanned and analysed.

RT-PCR

Reverse transcription polymerase chain reaction was performed to examine the binding abilities of antisense ODN after labelling (paper I), to learn the Chg-A mRNA expression pattern in rat tissues (paper III – semi-quantitative RT-PCR), and to investigate the presence of target Chg-A mRNA in human and rat cell lines (paper IV). The objective of semi-quantitative RT-PCR was to amplify Chg-A mRNA in rat tissues and compare it quantitatively to the amplification of 18S-ribosomal RNA (18S-RNA) endogenous gene as an internal standard. Since it lacks the typical 3' poly-A tail, an 18S-RNA sequence specific primer combined with oligo-(dT) were applied in the initial reverse transcription (RT) reaction in order to create copy DNA (cDNA) from the RNA pool in the samples. The RT-PCR was performed according to ZHU and ALTMANN (182). Table 4 summarises the primers used in the study.

Table 4. Primer sequences for RT-PCR

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>Location</th>
<th>Amplicon</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChgA-F</td>
<td>5'-ATCACCGCCACTGCACCACCA-3'</td>
<td>23-44</td>
<td>I, III, IV</td>
<td></td>
</tr>
<tr>
<td>ChgA-R</td>
<td>5'-CACCTTAGTGTCCTTTTGCTTTTATAGGGCT-3'</td>
<td>218-247</td>
<td>225 bp</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>18S-RNA-RT</td>
<td>5'-GAGCTGGAATTACCCGGGCT-3'</td>
<td>621-640</td>
<td>134 bp</td>
<td>III</td>
</tr>
<tr>
<td>18S-RNA-F</td>
<td>5'-GGGAGTGATGGACGAAATAAATAAAT-3'</td>
<td>507-532</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>18S-RNA-R</td>
<td>5'-TTGCCCTCAATGGATCTCT-3'</td>
<td>589-607</td>
<td>101 bp</td>
<td>III</td>
</tr>
</tbody>
</table>

Upper case stands for DNA; RT = reverse transcription; F = forward; R = reverse. Bold italic letters indicate the Chg-A antisense sequence of LNA-DNA mixmers.
RNA isolation

Total RNA was isolated either by the guanidine isothiocyanate method (TRIzol- Reagent) (paper I) or using RNeasy Midi kit (paper III-IV) from heart, lung, liver, intestine, pancreas, spleen, kidney, adrenal gland, testis, urinary bladder, muscle, bone marrow, and parotid gland or from BT474, U87MG and AR42J cells (described under cell lines). The quantity of total RNA sample was determined by spectroscopic measurements.

Reverse transcription (RT)

As described in paper III and IV, any remains of contaminating genomic DNA were removed by treating with DNase-I before performing RT. Reverse transcription was carried out using 1st Strand cDNA Synthesis Kit with oligo-dT15 primer. For semi-quantitative RT-PCR (paper III), 18S-RNA-RT primer were also included in the reaction mixture. All the cDNA samples were stored at –20°C.

PCR

Each cDNA template was amplified by a second PCR with ChgA-F and ChgA-R primers. In paper I, different reverse (antisense) primers were used; one of the following ODNs of the same sequence but with different modifications: (1) ChgA-R, (2) unlabelled PSChg ODN, (3) 76Br-labelled PSChg ODN, (4) unlabelled POCChg ODN, and (5) 76Br-labelled POCChg ODN. For semi-quantitative RT-PCR (paper III), 18S-RNA-F, 18S-RNA-R, ChgA-F and ChgA-R primers were applied. PCR profile consisted of 30-35 cycles at three temperatures, while semi-quantitative RT-PCR was run with 40 cycles at two temperatures. Finally, the samples were kept at 4°C.

The PCR products were separated in 2-2.5% agarose gel. The gels were stained with ethidium bromide and photographed on Polaroid film under UV light. Subsequently, the gels were exposed to phosphor imaging plate overnight, scanned and analysed. For semi-quantitative RT-PCR (paper III), the bands were evaluated with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/), and mean of pixel intensity was calculated for each band.

DNA sequencing

In order to check that the right product was amplified by PCR (paper I), sequencing of the product was performed. PCR amplification products were run on 1% of low melting agarose gel and purified with QIAquick Gel Extraction Kit. Purified PCR products were sequenced using the PRISM BigDye terminator with ChgA-F and ChgA-R as sequencing primers. The samples were run on ABI 377 automated DNA sequencer. Sequencing data were collected and analysed with Sequence Navigator software.
**Animals**

Male Sprague-Dawley rats (8-12 weeks, average weight about 360 g) were used. Permission for the animal use was granted by the local Research Animal Ethics Committee, diary numbers C 241/98, C 266/01, C2/05, C 234/05 and C60/07. The animals were kept at a constant temperature (20\(^\circ\)C) and humidity (50\%) in a 12 h light-dark cycle, and given free access to laboratory animal food and water. The animals were not anaesthetised.

Table 5. Injected tracer amount vs body weight of rats (mean \(\pm\) S.E.)

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Organ distribution</th>
<th>Whole-body autoradiography</th>
<th>Metabolite analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/kg</td>
<td>nmol/kg</td>
<td>µg/kg</td>
</tr>
<tr>
<td>PO</td>
<td>35 ± 10.1</td>
<td>6.7 ± 1.9</td>
<td>41 ± 1.3</td>
</tr>
<tr>
<td>PS</td>
<td>34 ± 4.9</td>
<td>2.4 ± 0.4</td>
<td>106 ± 8.8</td>
</tr>
<tr>
<td>OMe</td>
<td>52 ± 6.3</td>
<td>9.0 ± 1.1</td>
<td>85 ± 2.4</td>
</tr>
<tr>
<td>LNA1, LNA2</td>
<td>38 ± 3.7</td>
<td>5.8 ± 0.6</td>
<td>27 ± 9.5</td>
</tr>
<tr>
<td>Mismatched</td>
<td>8.3 ± 0.4</td>
<td>1.3 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td>Sense</td>
<td>15 ± 1.0</td>
<td>2.2 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>(^{68})GaCl(_3)</td>
<td>4.4 x10(^{-6})</td>
<td>2.5x10(^{-5})</td>
<td>3.8x10(^{-6})</td>
</tr>
<tr>
<td>(^{68})Ga-DOTA</td>
<td>0.6 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

**Organ distribution**

Bio-distribution studies in rats were performed to evaluate the quantitative accumulation of radio-labelled ODNs in various tissues, besides the observation of potential sites of elimination and the plasma levels of radioactivity. Of the number of rats used, one group received \(^{76}\)Br-labelled PO\(_{Chg}\); and for the rest each received one of the following \(^{68}\)Ga-labelled tracers: PO, PS, OMe, LNA1, LNA2, Mismatched or Sense ODN via i.v. administration. Each rat received a bolus dose of 0.5-1.0 MBq \((^{76}\)Br) or 1.5-2 MBq \((^{68}\)Ga) with saline as the vehicle. In paper II, separate rats that served as controls received 2 MBq \(^{68}\)GaCl\(_3\) or \(^{68}\)Ga-DOTA. In paper IV, rats were pre-treated with poly-I (10 mg/kg), or poly-A (10 mg/kg), or phosphate buffered saline only (1 ml/kg) 1 min prior to the injection of \(^{68}\)Ga-LNA2. Table 5 shows the injected tracer amount versus the body weight of the rats. After 2 h (paper I), 20, 60 or 120 min (paper II-III), and 60 min (paper IV), the rats were sedated with a CO\(_2\)-O\(_2\) mixture and samples of blood, plasma (200 µl), heart, left kidney, pancreas, adrenal gland, urinary bladder, testis, parotid gland, brain, and also pieces of lung, liver, spleen, intestine, muscle, bone, bone marrow, and skin were removed, weighed, and radioactivity measured in a calibrated well-counter for 60s \((^{76}\)Br) or 30s \((^{68}\)Ga). The radioactivity concentration was expressed as SUV (Standardised Uptake Value).
organ radioactivity (MBq) / organ weight (g)

SUV = \frac{\text{total given radioactivity (MBq)}}{\text{rat body weight (g)}}

This notation allows readily the calculation of tissue concentration by multiplying the respective SUV value by the amount ODN injected/kg. For example, with 5.8 nmol/kg administered, an organ with an SUV of 2 would have an equivalent ODN concentration of 2*5.8 nmol/kg, assuming that all organ radioactivity is representative of the labelled ODN.

**Whole-body autoradiography**

To obtain anatomically detailed information about tracer accumulation in tissues, whole-body autoradiography was performed (papers II-III). The animals received an i.v. injection of 10 MBq of $^{68}$Ga-labelled PO, PS, OMe, LNA1 or LNA2 ODNs, or in a separate experiment, $^{68}$GaCl$_3$ or $^{68}$Ga-DOTA (paper II), with saline as the vehicle. Table 5 shows the injected tracer amount versus the body weight of the rats. After 20 or 60 min, the animal was sacrificed with CO$_2$-O$_2$ mixture and embedded in carboxymethyl cellulose (CMC) gel that formed a mould around the animal after freezing in liquid hexane kept around -60°C by dry ice. The resulting CMC block containing the frozen animal was each mounted in a cryomicrotome where several 50 μm thick sections at various sagittal levels of each animal were cut and adhered to tape. The sections were heat-dried for a few minutes and then exposed to phosphor imaging plates overnight, scanned and analysed.

**Metabolite analysis**

To enable examination of *in vivo* and *in vitro* stability of differently modified $^{68}$Ga-labelled ODNs (paper II-III), metabolite analysis was carried out. The $^{68}$Ga-labelled PO, PS, OMe, LNA1 or LNA2 was i.v. injected into the rats as a bolus with saline as the vehicle. Table 5 shows the injected tracer amount versus the body weight of the rats (21.6 nmol of 23 MBq/rat (paper II) and 22 nmol of 90.7 MBq/rat (paper III)). After 20 or 60 min, the rats were sedated with CO$_2$-O$_2$ mixture and blood was collected from cardiac puncture. The plasma was extracted by phenol-chloroform (paper II) or by phenol-chloroform-1xTE (Tris-EDTA) (paper III). Aqueous phases were analysed using 20% non-denaturing PAGE, UV-radio-HPLC and thin layer chromatography (TLC) (paper II), or aqueous fractions were concentrated using a vacuum centrifuge and analysed with PAGE (paper III).

For *in vitro* studies (paper III), 0.5 MBq of $^{68}$Ga-labelled LNA1 or LNA2 was added directly to 200 μl of rat plasma, and incubated for 20, 60, and 120 min at 37°C or 4°C. Then the samples were extracted by phenol-chloroform-1xTE and analysed with 20% non-denaturing PAGE.
The gel was exposed to phosphor imaging plate overnight, scanned and analysed. The intensity was measured by drawing lines over the lanes from the top to the bottom of the gel.

**Cell cultures and multicellular tumour spheroids**

BT474 human breast carcinoma, U87MG human glioblastoma astrocytoma, and AR42J rat exocrine pancreatic tumour cell lines were used (paper IV). The latter served as positive control known to express Chg-A (184).

Multicellular tumour spheroids (MTS) of BT474 and U87MG were prepared by seeding the desired cell suspension in 1% agarose-coated 24-well plates. MTSs of AR42J were prepared by a hanging drop method (185) where MTSs were formed in a drop of cell suspension hanging from a tray within ten days. The spheroids were kept at 37°C and grown for six days. Then, the MTSs were transferred to 1% agarose-coated 24-well plates and grown for six days.

Digital images of MTSs were evaluated with respect to volumes of total spheroid, rim of viable cells and central necrosis using a semi-automated size determination software (SASDM), as previously described (186).

**Incubation of $^{68}$Ga-LNA2 in cell cultures**

In one type of experiment, the MTSs were incubated for 10, 20, 30, 60, 120 and 180 min at 37°C in medium with or without serum containing 0.7 MBq of $^{68}$Ga-LNA2 per well. In another type of experiment, the MTSs were incubated for 120 min at 37°C in medium with or without serum containing different concentrations (2, 6.7, 13.3, 22 and 66.7 μM) of $^{68}$Ga-LNA2 (approximately 0.2-5.6 MBq) per well. In a third type of experiment, the MTSs were first incubated with poly-I or poly-A, or in phosphate buffered saline only, for 60 min prior to the addition of the tracer. Then, $^{68}$Ga-LNA2 was added (0.6 MBq) and incubated for 120 min at 37°C. After incubations, the MTSs were washed with medium and transferred to tubes with 20 μl washing medium for $^{68}$Ga-LNA2 uptake measurement (30-45 s in a calibrated well-counter). A 20 μl sample of the incubation medium was measured as reference. The $^{68}$Ga-LNA2 uptake in aggregates was defined as:

\[
\text{Relative uptake} = \frac{\text{Measured radioactivity of aggregate (Bq)}}{\text{Viable volume of aggregate (ml)}} / \frac{\text{Volume of reference (ml)}}{
\text{Measured radioactivity of reference (Bq)}}
\]

or expressed in another way as radioactivity concentration in aggregates divided by radioactivity concentration in medium.
Results

Preparation of $^{68}$Ga-ODN tracers
When the labelling of the conjugates was performed using pre-concentrated generator $^{68}$Ga-eluate, higher radioactivity incorporation was achieved. HEPES buffering system resulted in higher yields. The radiochemical purity of the tracers in the study exceeded 95%. The position of the $^{68}$Ga-label was assessed by performing the labelling reaction with both conjugated and unconjugated ODNs. No radioactive product was detected in the reaction with unconjugated ODNs, indicating that the label was attached to the chelator. In addition, $^{68}$Ga-labelled ODNs were found to be stable in buffer for at least 4 h (58, 160).

Hybridisation abilities
The objective of these experiments was to investigate the hybridisation abilities of ODNs after labelling to mRNA or complementary DNA templates with RT-PCR (paper I) or hybridisation in solution (paper II-III) methods.

Binding ability of ODNs to Chg-A mRNA
In a PCR, 30-mer antisense ODNs having the same sequence but differing in backbone, either with or without 5'-end modification, $^{76}$Br-labelled or unlabelled, served as reverse primers. In the reactions, rat adrenal gland was used as a template known to express Chg-A mRNA. An expected 225 bp PCR fragment was successfully amplified using ChgA-R primer (PO without 5' aminohexyl linker), $^{76}$Br-labelled and unlabelled POCchg ODN (having 5' aminohexyl linker) as reverse primers. In contrast, no amplification was obtained using PSChg ODN (having 5' aminohexyl linker) regardless of being labelled or unlabelled (Fig. 7A).

Determination of binding specificity
Also by PCR, reverse transcribed mRNA samples of rat adrenal gland, heart, liver and kidney served as templates. Irrespective of the use of POCchg or $^{76}$Br-labelled POCchg ODN as reverse primer, positive PCR products were only detected in the sample prepared from the adrenal gland. The autoradiogram and the Polaroid picture of the gel were confirmatory to one another. The position of the radioactive spots corresponded to the amplified products, whereas $^{76}$Br-labelled POCchg ODN was seen at a position different from the product in the cases of the heart, liver and kidney samples, where no amplification occurred (Fig. 7B).
Figure 7. A) PCR with modified ODNs as a reverse primer run in 2% agarose gel. Lanes: (1–3) PO^{Chg}, (4–6) PS^{Chg}, (7–9) ChgA-R, (10) Molecular weight marker. In lane 9, the adrenal cDNA template was substituted for H₂O. The PCR product appeared at 225 bp position. B) Photographed gel (left) and autoradiogram of the exposed gel (right) after PCR with PO^{Chg} (Lanes 1-4) and ^{76}Br-PO^{Chg} (Lanes 5-9) as a reverse primer using cDNA templates generated from the mRNA of heart (Lanes 1 and 5), liver (Lanes 2 and 6), kidney (Lanes 3 and 7) and adrenal gland (Lanes 4, 8 and 9). Lane 10: Molecular weight marker. The upper radioactivity spots correspond to the PCR product, whereas the lower ones correspond to ^{76}Br-PO^{Chg} being non-used reverse primers in PCR.

Sequencing result
The PCR products amplified from the adrenal gland template using ChgA-R, PO^{Chg} or ^{76}Br-labelled PO^{Chg} ODN as reverse primers, respectively, were bi-directionally sequenced to confirm the specificity of PCR amplification. The sequence of the PCR products, with three different pair primers, completely matched the expected rat Chg-A mRNA sequence (accession number: X06832) obtained from EMBL database (http://www.ebi.ac.uk/embl/).

Hybridisation in solution to sense ODN
The stained PAGE gels and the corresponding autoradiograms showed that ^{68}Ga-labelled PO, PS, OMe, LNA1 and LNA2 antisense ODNs retained their potency and were able to hybridise. The duplex formation between the labelled antisense and complementary sense ODNs, as well as, the increase of radioactive signal of ^{68}Ga-labelled antisense ODNs appearing in the hybrid band were proportional to the increase of concentration of sense ODNs (Fig. 8). Both the antisense and sense bands appearing at concentrations when they were in excess and the hot spots of antisense ODNs correlated with the references.

The hybrid of PO, PS and OMe appeared at 17 bp heights on the gel corresponding to the similarly double-stranded molecular weight marker, whereas, the hybrid of LNA1 and LNA2 appeared to be heavier than 20 bp on the gel most probably due to LNA modification. However, the single-stranded antisense and sense used for control did not emerge at the same heights because of their folded structure. When non-conjugated ODN was
run on PAGE as reference, it appeared at different height from DOTA-conjugated ODN.

Figure 8. Photographed polyacrylamide gels (left) and autoradiograms of the exposed gels (right) after hybridisation in solution with $^{68}$Ga-LNA2 antisense ODN to their complementary sense Chg ODN. The respective applied antisense:sense concentration ratios in the lanes are: (1) 1:1/45, (2) 1:1/15, (3) 1:1/5, (4) 1:1/2, (5) 1:1, (6) 1:2, (7) 1:4, (8) sense Chg ODN (unlabelled), (9) $^{68}$Ga-LNA2 antisense ODN, (10) Molecular weight marker (10 bp ladder).

**Presence of Chg-A mRNA in rat tissues and cell lines**

Semi-quantitative RT-PCR was applied to be able to discern which rat tissues express Chg-A mRNA and to compare the expression quantitatively to the expression of a standard endogenous gene such as 18S-RNA (paper III). Chg-A mRNA expression was found in the intestine, pancreas, adrenal gland, testis and AR42J rat exocrine pancreatic tumour cell line as can be seen on Figure 9A.

The employment of 18S-RNA as an internal standard made it possible to relate the expression of Chg-A mRNA observed in those rat tissue samples. The graph on Figure 9B shows this comparison. With intensity decreasing in magnitude, Chg-A expression was the highest in the AR42J rat cell line and adrenal gland (63 and 61% of 18S-RNA expression), followed by the intestine (49%), testis (47%) and pancreas showing the lowest percentage (27%). 18S-RNA resulted in a consistent expression in the studied rat tissues with a pixel intensity of $135 \pm 11$ (expressed as mean ± S.D.).

The RT-PCR was performed to investigate the presence of target rat mRNA in the cell lines used in the study. The results revealed that PCR failed to amplify Chg-A in the two human cell lines, BT474 and U87MG, which, in contrast, was characteristic of the AR42J rat exocrine pancreatic tumour cell line and rat adrenal gland.
Figure 9. Semi-quantitative RT-PCR. A) Photographed gel with rat tissue samples separated in 2.5% agarose gel and stained with ethidium bromide. Lanes: (1) molecular weight marker (20 + 100 bp ladder), (2) heart, (3) lung, (4) liver, (5) intestine, (6) pancreas, (7) spleen, (8) kidney, (9) adrenal gland, (10) testis, (11) molecular weight marker, (12) urinary bladder, (13) muscle, (14) bone marrow, (15) parotid gland, (16) AR42J rat exocrine pancreatic tumour cell line. B) Chg-A mRNA expression percentages related to 18S-RNA expression in the same tissue with 18S-RNA expression taken as the sum of the intensity of 101 bp and 134 bp bands.

Figure 10. Organ concentrations of radioactivity (presented as SUV, mean ± S.D.) obtained 2h after i.v. injection of $^{76}$Br-PO$^{Chg}$ antisense ODN into rats.
**Bio-distribution of radio-labelled ODNs**

With the injection of labelled ODNs into rats, the aim was to examine and compare the organ distribution by knowing the quantitative accumulation in tissues.

**76Br-labelled PO\textsuperscript{Chg} ODN (paper I)**

Two hours after the injection of 76Br-PO\textsuperscript{Chg} ODN, the tracer distribution showed highest radioactivity in adrenal gland with SUV of 0.72, followed by kidney, liver, blood and heart with SUV of 0.68, 0.19, 0.07 and 0.05, respectively (Fig. 10).

**68Ga-labelled PO, PS and OMe (paper II)**

The measurement of organ radioactivity 20, 60 and 120 min after i.v. administration of labelled PO showed the highest values in the liver (average SUV of all three time points is 6.5), followed by the urinary bladder, bone marrow and spleen. Uptake in the kidney was predominant in the PS and OMe distribution patterns with an average SUV of 30 and 12, respectively.

![Figure 11. Organ concentrations of radioactivity (presented as SUV, mean ± S.E.) obtained 20, 60 and 120 min after i.v. injection of 68Ga-labelled PO, PS and OMe antisense ODN into rats (n=6 for each time point).](image)

Bone marrow, kidney, liver, spleen and urinary bladder were among the five organs with the highest SUV values in each ODN distribution pattern (Fig. 11). The SUV values of the rest of the organs were below 1.0. The lowest uptake was observed in the brain with an SUV of 0.03. On the average, PS
had higher SUV values in the tissue samples compared to that of PO (3.4 fold) and OMe (2.7 fold).

As references, $^{68}$GaCl$_3$ and DOTA-conjugated $^{68}$Ga distribution studies were performed. The $^{68}$GaCl$_3$ was retained very prominently in all organs; the SUV ranged between 3.7 and 0.5 for most of the tissues; in the brain, however, it was 0.09. The SUVs of bone marrow and lung placed second and third after blood measured 3.5 and 1.7, respectively. In contrast, $^{68}$Ga-DOTA did not accumulate extensively in the organs; in 14 out of 17 samples, no SUV value exceeded 0.37. However, the two highest radioactive concentrations were observed in the bone marrow and in the liver with an average SUV of 5.8 and 3.9, respectively, followed by the urinary bladder and kidney.

![Figure 12](image_url)

**Figure 12.** Organ concentrations of radioactivity (presented as SUV, mean ± S.E.) obtained 20, 60 and 120 min after i.v. injection of $^{68}$Ga-labelled LNA1, LNA2, Mismatched and Sense ODN into rats (n=6/time point/ODN, for LNA1 n=10).

$^{68}$Ga-labelled LNA1, LNA2, Mismatched and Sense (paper III)

On measuring tissue radioactivity 20, 60 and 120 min after i.v. injection the distribution patterns of LNA1 and LNA2 were found similar with some variance in adrenal gland, spleen and liver. The patterns of Mismatched and Sense were alike with little differences in pancreas, intestine and kidney. (Fig. 12) The kidney, liver, spleen and bone marrow were the organs of highest uptake of LNA1 and LNA2, whereas the urinary bladder was among the organs of highest uptake of Mismatched and Sense together with the kidney, bone marrow and liver but not the spleen. Looking at the neuroendocrine tissues, SUV in the adrenal gland was two-fold higher with LNA2 compared to the other three ODNs (one and half times more than with Mis-
matched). In the pancreas, SUV was also two-fold higher for LNA1 and LNA2 than for Sense; Mismatched had one and half times higher SUV than LNA1 and LNA2. Interestingly, spleen had five times higher SUV for LNA2 compared to Mismatched and Sense; LNA1 had one and half times less. The SUV values of the rest of the organs were below 1.3. The lowest uptake was observed in the brain with an SUV of 0.02.

Normalising the data by muscle SUV resulted in a much more similar pattern between Mismatched and Sense. In contrast, this led to differences on the pattern of LNA1 and LNA2, especially in the adrenal gland, spleen, urinary bladder, liver and bone marrow. LNA2 showed eight times higher ratio in the spleen, five times higher in the liver and two-fold higher in the adrenal gland compared to the other three ODNs. In the pancreas, LNA1 and LNA2 had five times higher ratio than Sense; Mismatched had only 20% less than LNA1 and LNA2.

![Figure 13. Ratios of organ SUV values between LNA2 and Mismatched, respectively, between Sense and Mismatched.](Image)

In order to allow a better comparison between organ uptake for ODNs with potential for specific hybridisation and ODN not expected to have specific hybridisation, the average SUV values per time points for LNA1, LNA2 or Sense were divided by the average SUV values per time points of Mismatched (Fig. 13). Compared to Mismatched, LNA2 had relative higher uptake in spleen and liver followed by lung and adrenal gland. However, the favourable uptake of LNA2 in adrenal gland decreased with time, whereas it increased in lung. Similar data were obtained with LNA1. In contrast, the
ratios of Sense were consistently below one for all organs except testis and muscle.

\(^{68}\text{Ga}\)-labelled LNA2 with blocking of scavenger receptors by polyribonucleotides (paper IV)

The administration of poly-I and poly-A prior to the injection of \(^{68}\text{Ga}\)-LNA2 affected the uptake of the PET tracer in rat tissues. Decreased SUV could be observed with both polyribonucleotides in the adrenal gland, liver and bone marrow, whereas the pancreas, intestine and urinary bladder were affected only by poly-I, and poly-A affected kidney alone (Fig. 14).

The most pronounced reduction with poly-I was found in the liver, bone marrow and adrenal gland (50, 50 and 60% of the control, respectively), and about 80% in the pancreas, urinary bladder and intestine. In contrast, the strongest decrease with poly-A was observed in the kidney and bone marrow (66 and 75% of the control, respectively), and about 85% in the adrenal gland and liver.

Figure 14. Organ concentrations of radioactivity (presented as SUV, mean ± S.E.) obtained 60 min after i.v. injection of \(^{68}\text{Ga}\)-LNA2 antisense ODN (n=12 /time point). Shortly (1 min) before, treated animals received poly-I (10 mg/kg) or poly-A (10 mg/kg), control animals received phosphate buffered saline only (1 ml/kg).

When data was normalised by plasma SUV, the reduction effect became more obvious and extended to more tissues but still in favour of poly-I. More than 60% blocking effect was found with this polyribonucleotide in the liver, bone marrow and adrenal gland, 40% in the pancreas, urinary bladder and
intestine, and about 30% in the testis, kidney, parotid gland and muscle. Poly-A was able to reach 50% blocking effect in the kidney, 40% in the bone marrow, 30% in the adrenal gland and liver, and 20% in the pancreas.

Figure 15 shows the data normalised by plasma SUV in relation to control. The one-sided t-test revealed that the blocking effect of poly-I was highly statistically significant (p<0.0001) in the pancreas, intestine, testis, adrenal gland, liver and bone marrow; whereas that of poly-A at similar probability (p<0.0001) was observed only in the kidney.

Figure 15. Organ concentrations of radioactivity normalised by plasma and expressed in relation to the control (presented as SUV ratio, mean ± S.E.). The signs indicate those tissues in which poly-I and poly-A had statistically significant blocking effect: *(p<0.0001), †(p<0.005), ‡(p<0.01), §(p<0.05).

Whole-body autoradiography

The objective of whole-body autoradiography was to compare the two biodistribution results and to obtain more detailed information about the tracer accumulation in tissues.

$^{68}$Ga-labelled PO, PS and OMe (paper II)

In order of decreasing magnitude in levels of radio-labelled PO taken up by various tissues, liver and urinary bladder obtained the highest uptake followed by, kidney and spleen, and thereafter the lung, and finally skin and bone. For radioactive PS, labelling of the kidney cortex was predominant.
together with the urinary bladder (Fig. 16). The radioactive distribution pattern in the liver was uneven – a high uptake in some areas but low in other areas. The low magnitude of uptake in the liver corresponded almost with that in the parotid gland, the lung and spleen. Uptake in skin was lowest, though higher than the background radioactivity. OMe showed similar distribution pattern to PS, but the uptake in the kidney cortex was noticeably less.

One hour after the $^{68}$GaCl$_3$ injection, the most pronounced uptake was observed in the liver and spleen. Radioactivity in the lung was also considerable. Uptake in the kidney, bone marrow, lymph nodes and vessels, and skin was much lower but well above the background. In contrast, the DOTA-conjugated $^{68}$Ga was slightly taken up by most of the organs. The highest uptake was seen in the urinary bladder and some parts of the liver and lung (small pockets of radioactivity distributed all over these organs). According to decreasing magnitude of radioactivity, the next tissue was kidney cortex. Uptake in the liver and lung, spleen, skin and bone marrow was slightly above the background.

![Image](ex-vivo-autoradiograms-obtained-20-min-after-i-v-injection-of-68-ga-labelled-ps-and-lna2-antisense-odn)

**Figure 16.** *Ex vivo* autoradiograms obtained 20 min after i.v. injection of $^{68}$Ga-labelled PS and LNA2 antisense ODN.

$^{68}$Ga-labelled LNA1 and LNA2 (paper III)

The highest radioactive signal of $^{68}$Ga-LNA1 was observed in the kidney cortex and urinary bladder. With radioactivity decreasing in magnitude, liver
was the next tissue, followed by the spleen, lung, skin and bone. In the liver, areas were observed where uptake was higher or lower. Uptake in the lung, heart, bone and skin was much lower but well above the background.

The most pronounced uptake with $^{68}$Ga-LNA2 at both 20 and 60 minutes time points was observed in the kidney cortex and urinary bladder. Radioactivity in the liver and spleen was also considerable but lower in intensity (Fig. 16). According to decreasing magnitude of radioactivity, the next tissues were lung, bone marrow and parotid gland. Uptake in the heart and skin was the lowest.

**Plasma residence of $^{68}$Ga-labelled ODNs**

Knowing the SUV of plasma and the approximate percentage of intact ODN at a certain time point, non-metabolised labelled ODN in plasma expressed in SUV can be calculated (Table 6). The data demonstrates the overall advantage of PS over Mixmer, OMe and PO modifications and LNA2 seems to be favourable over LNA1.

<table>
<thead>
<tr>
<th>ODN type</th>
<th>20 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>2.52</td>
<td>0.54</td>
</tr>
<tr>
<td>LNA2</td>
<td>1.10</td>
<td>0.09</td>
</tr>
<tr>
<td>LNA1</td>
<td>0.53</td>
<td>0.04</td>
</tr>
<tr>
<td>OMe</td>
<td>0.46</td>
<td>0.12</td>
</tr>
<tr>
<td>PO</td>
<td>0.20</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Calculated as plasma SUV*intact ODN%

**Metabolite analysis**

Metabolite analysis was performed to obtain knowledge about the *in vivo* and *in vitro* stability of the $^{68}$Ga-labelled, modified ODNs.

$^{68}$Ga-labelled PO, PS and OMe (paper II)

After the extraction of radioactivity from plasma with phenol-chloroform, the recovery (expressed as mean ± S.D.) was 23 ± 13%. The analyses of plasma samples showed that PS was the most stable as intact PS was found at both 20 and 60 min time points with PAGE and with HPLC (Fig. 17). Degradation was slightly noticeable after 20 min (<10%) but increased considerably after 60 min (<50%). The metabolites represented a high molecular weight fraction.

In contrast, PO and OMe were degraded faster giving both high and low molecular weight fractions. Metabolites of PO were predominant at both time points; nevertheless, intact compound (<15%) could still be detected in plasma after 20 min. Considerable intact OMe was found in 20 min (<30%) and 60 min (<21%) samples, however, degradation was apparent already 20 min after injection.
The three methods (PAGE, UV-radio-HPLC and TLC) applied for metabolite analysis complemented each other and thus provided evidence for the observations. TLC showed increased amount of low molecular weight metabolites of PO and OMe as time elapsed, while there was no sign of low molecular weight fraction of PS even after 60 min.

68Ga-labelled LNA1 and LNA2 (paper III)

The metabolite analysis was carried out both in vivo by injecting the labelled LNA1 or LNA2 antisense ODNs into rats and in vitro by mixing these tracers with rat plasma. The recovery of radio-labelled ODNs from plasma using phenol-chloroform-TE extraction was 81 ± 6% (expressed as mean ± S.D.), which corresponded to the yield reported by KÜHNAST et al. (114).

A) LNA2

B) LNA2 – in vitro

Analysis of the in vivo radio-chromatograms displayed the presence of intact LNA2 in both 20 and 60 min time point samples (Fig. 18A); whereas intact LNA1 could be found only in the 20 min time point sample. Radio-metabolites of both 68Ga-ODNs could also be observed already in the 20 min
time point samples (~20%), but increased considerably in the 60 min time point samples (~80%).

In contrast, the in vitro incubation with plasma revealed that $^{68}$Ga-labelled LNA1 and LNA2 were stable at 37°C even after 120 minutes of incubation (Fig. 18B); although slight degradation could also be observed (~27%) beside the intact ODNs (~65%). When incubating at 4°C, the ODNs remained entirely intact.

Figure 19. A) $^{68}$Ga-LNA2 uptake of cell lines incubated for 10, 20, 30, 60, 120 and 180 min in medium with or without serum. B) Uptake in cell lines incubated for 2 h with different concentrations (2-66.7 μM) of $^{68}$Ga-LNA2 in medium with or without serum.
68Ga-LNA2 uptake by cell lines

The tumour cells formed multi-cellular spheroids about 1 day after seeding. Within the next day the characteristic rounded shape with a rim of viable cells and a central necrosis was observed.

The relative 68Ga-LNA2 tracer uptake increased in each cell line as time elapsed; however, serum-free medium facilitated the uptake (Fig. 19A). The accumulation profile of BT474 and U87MG showed an initial quick phase in the first 20-60 min that slowed down but continued to increase steadily; whereas, AR42J had a continuous linear uptake over time. The most noticeable difference could be observed between the serum- and serum+ uptake in U87MG in favour of the former. When MTSs were incubated in the presence of increasing concentration of 68Ga-LNA2, it also led to a two phase curve in BT474 and U87MG, which was noticeable under serum-free condition with a decrease between 10-30% of max, followed by an almost constant but slightly decreasing phase until max concentration (Fig. 19B).

Figure 20. Uptake in cell lines when the cells were incubated for 60 min with poly-I or poly-A prior to the administration of 68Ga-LNA2 in medium without serum.

Blocking the scavenger receptors prior to incubation with the tracer led to a reduced relative uptake in each cell line, which is characterised by an initial quickly decreasing phase that was followed by an almost constant phase at higher concentrations of blocking polyribonucleotides (Fig. 20). Poly-I showed higher blocking effect than poly-A in each cell line.
Additional study – PNA

The objectives
The objective of this project was the sequence-specific detection of nucleic acid of human endogenous retroviruses (HERV) in the brain of live animals and humans using peptide nucleic acid and PET. Our genomes are full of these viruses and although they are normally harmless, they have been implicated in the brain diseases multiple sclerosis and schizophrenia. To start with, our Chg-A model gene was chosen since Chg-A is widely expressed in the central nervous system (187). Additionally, a sequence for the human c-fos proto-oncogene and transcription factor was chosen.

Table 7. PNA antisense sequences and PO sense sequences used

<table>
<thead>
<tr>
<th>Designation</th>
<th>Target</th>
<th>Sequence</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Chg-A</td>
<td>5’-GTGTCCCCCTTTTGTC-3’</td>
<td>16</td>
</tr>
<tr>
<td>PNA&lt;sup&gt;F&lt;/sup&gt;</td>
<td>c-fos</td>
<td>5’-AAGCCCGAGAACATCA -3’</td>
<td>16</td>
</tr>
<tr>
<td>PNA&lt;sup&gt;C-NLS&lt;/sup&gt;</td>
<td>Chg-A</td>
<td>5’-GTGTCCCCCTTTTGTC-PAKKKRKV</td>
<td>16</td>
</tr>
<tr>
<td>PNA&lt;sup&gt;F-NLS&lt;/sup&gt;</td>
<td>c-fos</td>
<td>5’-AAGCCCGAGAACATCA -PAKKKRKV</td>
<td>16</td>
</tr>
<tr>
<td>sense&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Chg-A</td>
<td>5’-TGACAAAAAGGGGACAC -3’</td>
<td>16</td>
</tr>
<tr>
<td>sense&lt;sup&gt;F&lt;/sup&gt;</td>
<td>c-fos</td>
<td>5’-TGATGTCTCTCGGGCTT-3’</td>
<td>16</td>
</tr>
</tbody>
</table>

NLS = nuclear localisation signal; PKKRRKV = amino acid sequence

Oligonucleotides
Table 7 summarises the ODNs used in the project. The *antisense ODNs* consisted of a full peptide nucleic acid (PNA) backbone with DOTA already conjugated to the 5’ end of the ODNs for <sup>68</sup>Ga-labelling. In order to improve penetration over blood-brain barrier, an oligopeptide sequence (NLS) was also attached to the 3’ end of the PNA ODNs. In contrast, *sense ODNs* were made up of PO backbone and were used only for hybridisation in solution.

The labelling procedure was performed with pre-concentrated <sup>68</sup>Ga-eluate in a similar manner as described under radio labelling as it was used for peptides. Table 8 summarises the specific radioactivity for PNA ODNs.

Table 8. Specific radioactivity of <sup>68</sup>Ga-PNA (mean ± S.E.) after labelling

<table>
<thead>
<tr>
<th>ODN type</th>
<th>MBq/nmol</th>
<th>MBq/mg</th>
<th>SRA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNA&lt;sup&gt;C&lt;/sup&gt;, PNA&lt;sup&gt;C-NLS&lt;/sup&gt;</td>
<td>1.66 ± 0.78</td>
<td>360.98 ± 177.89</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>PNAF, PNA&lt;sup&gt;F-NLS&lt;/sup&gt;</td>
<td>2.63 ± 0.65</td>
<td>535.26 ± 149.78</td>
<td>0.08 ± 0.03</td>
</tr>
</tbody>
</table>

SRA* is the average of the normalised specific radioactivity calculated as follows: spec. radioactivity (MBq/nmol) divided by the corresponding <sup>68</sup>Ga radioactivity from the generator multiplied by the amount of ODN used for the labelling. It reflects the incorporation efficiency of the labelling.
Hybridisation in solution

Hybridisation in solution was performed as described under 2.3, except that the $^{68}$Ga-labelled antisense PNA$^C$ or PNA$^F$ ODN was dissolved in H$_2$O.

An increasing radioactive signal of $^{68}$Ga-antisense-PNA-ODN was observed, proportional to the increase of concentration of sense ODN; however, ethidium bromide was unable to stain either PNA or PNA-PO hybrid, therefore the hybrid band and antisense reference band on the autoradiogram could not be confirmed on the stained gel. Additionally, PNA having neutral backbone was hardly able to move in the gel under charged conditions of the gel electrophoresis.

MicroPET

*In vivo* imaging was performed to find out whether PNA passes the blood-brain barrier. Male Sprague-Dawley rats (10-14 weeks, average weight 456 g) received a bolus of 5-10 MBq of $^{68}$Ga-labelled PNA$^C$ or PNA$^F$ (with or without NLS tail) in the tail vein with saline as the vehicle (Research Animal Ethics Committee permission C2/05).

Neither PNA nor PNA with NLS tail was able to cross blood-brain barrier irrespective of the sequence adopted (Fig. 21).

![Figure 21. MicroPET image over the transaxial rat brain after i.v. injection of PNA$^C$-NLS. Radioactivity distribution was recorded for 60 min using a microPET R4 tomograph (Concorde Microsystems) (188).](image)

Bio-distribution of PNA ODNs

After the microPET scan, the animal was sedated with CO$_2$-O$_2$ mixture and samples of blood, plasma (200 µl), heart, lung, liver, kidney, pancreas, spleen, intestine, adrenal gland, urinary bladder, testis, brain, muscle, bone, bone marrow, skin, and parotid gland were excised, weighed, and measured in a calibrated well-counter for 30s. The radioactivity concentration was expressed as SUV (Standardised Uptake Value).

The measurement of organ radioactivity after 60 min incubation time resulted in similar bio-distribution patterns between PNA$^C$ and PNA$^F$, as well as, between PNA$^C$-NLS and PNA$^F$-NLS ODNs (Fig. 22). The PNA ODNs without tail showed the highest SUV in the urinary bladder, whereas uptake in the kidney was predominant in the patterns of ODNs with NLS tail. Considerable amount of radioactivity was observed in the plasma with all PNA ODNs, where PNA$^C$-NLS showed twice higher SUV than the other three ODNs. Noticeable radioactivity was also found in the bone marrow (SUV
2.8) and SUV was above 1.0 in the spleen and liver with this ODN. The rest of the SUV in the distribution patterns was below 1.0. The lowest uptake was observed in the brain with an SUV of 0.03; nevertheless, PNA$^C$-NLS displayed 0.07.

Figure 22. Organ concentrations of radioactivity obtained 60 min after i.v. injection of $^{68}$Ga-labelled PNA or PNA-NLS ODN specific for rat Chg-A or human c-fos mRNA. The rats received anaesthesia. C = Chg-A, F = c-fos, NLS = nuclear localisation signal.


Discussion

The initial objective of the present study was to develop radio-labelled ODNs for in vivo monitoring of gene expression, i.e. in vivo hybridisation, utilising PET methodology. An essential prerequisite was to establish the radio-labelling of ODNs with a radionuclide of sufficiently long half-life to be able to monitor hybridisation.

As a suitable radionuclide, $^{76}$Br was selected because of its 16.2 h half-life, which is rather long allowing studies of organ kinetics for up to two days. However, despite its favourable half-life, it decays only by 55% via positrons and additionally generates high-energy gamma rays; therefore, it is less compatible for human health. In addition, $^{76}$Br is not optimal for PET imaging because its cascade gamma radiation and low noise equivalent count rate causes degradation in image quality and quantitative accuracy (189).

As an alternative, the $^{68}$Ga radionuclide decays by 89% via positrons and by 11% EC; its $\beta^+$ max energy of 1.9 MeV provides good resolution, and its half-life of 68 min might be enough to follow biological processes for 3-4 h. Further advantages are that $^{68}$Ga is produced more easily using a generator and the labelling procedure is simpler using a metal chelator and microwave activated complexation. In addition, the labelling is reliable and reproducible. Yet, the major drawback of $^{68}$Ga is the 3-4 h time frame that might be too short to study specific hybridisation in vivo with a necessity to allow entry into the cells, hybridisation to the target, and more importantly, release of non-hybridised ODN from the cells. Proof of this drawback was shown in paper III where non-hybridisation specific uptake was observed in tissues of the rat while studying antisense, mismatched and sense sequences. However, we still believe that $^{68}$Ga-labelled ODN can be used for evaluation of a number of key issues, such as distribution to different organs, metabolism and specific non-hybridisation related uptake mechanisms.

As further support to the necessity of longer exploration time, feedback mechanisms may stimulate the production of additional mRNA, which was concluded from the results of a 24 h long cell culture incubation study (190, 191). If this hypothesis is correct, the half-lives of radionuclides might be a limiting factor for the application of antisense ODNs as radiopharmaceuticals for PET imaging, unless they are replaced by such long-lived radionuclides as $^{66}$Ga, $^{64}$Cu or $^{89}$Zr with half-lives of 9.5 h, 12.8 h and 3.2 days, respectively.

Hybridisation abilities

The labelling method should not perturb the property of molecules with respect to selective hybridisation. When evaluating the effect of labelling by hybridisation in solution, neither the chemical modification of the ODN backbone or the sugar moiety, nor the $^{68}$Ga-labelling did influence the hy-
bridisation abilities of the ODNs. In contrast, investigation with PCR resulted in amplified products only with PO ODN.

A desired PCR product is amplified if (a) the ODN primers are able to bind complementarily to the template sequence (annealing) and (b) the DNA polymerase enzyme can attach and start copying the template (extension). The results suggest that PS is likely to bind to the target sequence; however, the primer extension fails because an ODN carrying PS modifications is a strong inhibitor of DNA polymerases in a linkage number-dependent manner (192). Longer oligomers are more potent inhibitors than shorter ones, and the inhibitory effect can be avoided by decreasing the number of PS linkages at the backbone. OMe was also demonstrated to inhibit polymerisation by sterically blocking the AMV reverse transcriptase (193) and failed to be a reverse primer in the PCR reaction. OMe and PS tend to bind to the enzyme rather than to the template (58, 194).

It is our belief that RT-PCR is an attractive method to identify the selective binding of modified ODNs to target mRNA. However, PCR is not applicable for modified ODNs due to restriction of the enzyme.

Chg-A in rat tissues

Chg-A mRNA was mainly present in rat tissues that are known to contain neuroendocrine cells. With in situ hybridisation and immunohistochemistry, Chg-A was demonstrated in adrenal medulla, adrenohypophysis, thyroid gland, pancreatic islets, endocrine gut, gastric endocrine cells and many parts of the central nervous system but not in the liver, kidney, parotid gland, adrenal cortex or exocrine pancreas (187, 195-197). Leydig cells in the testis was also found to express numerous neuroendocrine markers, among them Chg-A (198). The rat AR42J cell line, beside its exocrine properties, also displays some neuroendocrine features such as Chg-A mRNA expression, as shown by HOFSLI et al. (184).

For mRNA quantification, the use of 18S-RNA proved to be a reliable internal standard with little expression differences between tissues (182, 199). Having a consistent expression is an important property for a housekeeping gene because its expression level is presumed to be proportional to the total amount of mRNA being examined.

Bio-distribution

Before the oligo-PET imaging method can be applied in humans, it is essential to obtain information about the quantitative accumulation in the target organs versus non-targeted tissues with respect, especially, to the chemical modifications of ODNs.

Bio-distribution patterns in almost all tissues seemed to vary with the nature of the type of modification and the length of ODN. High kidney uptake was observed with $^{76}$Br-PO$^{\text{Chg}}$ ODN (30-mer), PS and OMe, whereas PO accumulated predominantly in liver. Since the selected PO, PS and OMe
ODNs (17-mer) were chosen to be specific for human mRNA, they do not have any biological targets in rats and their tissue distribution reflects their non-specific interactions and elimination. LNA1 and LNA2 mixmer ODNs (20-mer) resembled the distribution of PS ODN, which has been described by others (68). Accumulation in kidney was less compared to PS but uptake in spleen, liver and adrenal gland was higher, especially with LNA2. Nevertheless, the positioning of the LNA modifications also resulted in differences in the distribution pattern, which could reflect their different uptake, metabolism and elimination from tissues since the differences were seen mainly in non-neuroendocrine tissues. LNA2 entered tissues most effectively and obtained the lowest SUV ratio in kidney and urinary bladder indicating higher retention in the body.

Our previous work (145) investigating PSChg ODNs of different length (6, 12, 20 and 30 bp) reinforces the dependence of distribution pattern on length of ODNs. The highest amount of a 17-mer PO was found in the liver, while a 30-mer PO ODN displayed the highest accumulation in the kidney, but on the contrary, kidney came first for a 17-mer PS while liver was number one for a 30-mer PS, irrespective of sequence. In addition, the distribution pattern of a 17-mer PS in the present study was similar to a 20-mer PS in our previous study. The dependence of length of sequence on the distribution might be explained by the fact that increasing size of the ODN may further hamper the passage through cell membrane, especially in kidney and adrenal gland and may rather end up in liver and spleen. Scavenger receptors and other mechanisms could prove to be dependent upon the length of the ODN.

In relation to possible specific uptake, primarily in the adrenal gland, LNA2 showed the highest SUV followed by LNA1, POChg and PSChg. When exploring further with Mismatched and Sense control sequences, the patterns of these two ODNs were alike but differed from those of LNA1 and LNA2 antisense ODNs indicating that the replacement of the two nucleotides in the middle of the antisense sequence was enough to affect the uptake process in rat tissues. The fact that normalised SUV ratio in adrenal gland stayed constant by two-fold in favour of LNA2 over LNA1, Mismatched and Sense ODNs suggests that the antisense sequence has an advantage in this neuroendocrine tissue, but not as much in non-neuroendocrine tissues. Nevertheless, the uptake of ODNs seems not to be dominated by the presence of target Chg-A mRNA in these tissues but rather by some other phenomena, probably involving certain membrane transporters giving advantage to the antisense sequence. In addition, it has been reported that non-specific accumulations of 99mTc-labelled sense, scrambled or random control ODNs are sequence dependent in cell culture (200).

Whole-body autoradiography is a complement to quantitative distribution because it provides qualitative images with more detailed information about tissues in vivo. The autoradiograms confirmed the bio-distribution results; moreover, it supplied detailed information about the elevated uptake in kid-
ney cortex with PS, OMe, LNA1 and LNA2 ODNs. It was reported that this is due to significant uptake in Bowman’s capsule, in the proximal convoluted tubule lumen, and within intra-tubular epithelial cells (97, 201). The data suggests that PS ODNs are filtered by the glomerulus and then reabsorbed by the proximal convoluted tubule epithelial cells (120). It was also suggested that LNA-DNA mixmers and OMe ODNs accumulate in the kidney cortex due to renal clearance but they do not enter the cells of kidney (141). It is reported that PS may be taken up by the glomerular endothelial cells possibly via a receptor-mediated process (202). In liver, ODNs may accumulate in hepatic Kupffer cells.

In rats, the salivary gland has been found among the high accumulating organs (121). Accumulation of PS in the bone marrow is also reported in several studies (97, 118, 202-204) and it seems to be related to megakaryocytes, myeloid and macrophage cells, rather than to lymphocytes. The accumulation in the bone marrow might explain some of the toxic effects of antisense ODNs (205). This uptake could also be related to the fact that gallium resembles iron (206). Organs of the reticuloendothelial system (RES), such as liver, spleen, kidney and lungs represent most likely clearance sites, at least following systemic administration of ODN complexes (103).

Plasma residence
The data suggest an advantage of PS ODN ahead of LNA2 and LNA1 because of a longer bioavailability of intact PS molecule for the tissues. However, the long residence in plasma is probably because PS binds to plasma proteins, such as albumin, reducing significantly the bioavailability for transfer to tissues. In contrast, LNA exhibit much less protein binding, therefore it is present in plasma mainly as free molecule being ready for uptake by tissues. Transfer of LNA-DNA mixmer from plasma to tissues seems to be very rapid. It was reported that only 20% of the injected dose remained in mouse serum 30 min after injection (68).

Metabolite analysis
In our initial work, we could only recover 23% of radio-labelled PO, PS and OMe ODNs. However, by adopting extraction procedures according to KUHNAST et al. (114), we were able to improve the extraction up to 81% with radio-labelled LNA1 and LNA2. Thus the PO, PS and OMe metabolite data may only represent a qualitative and not a quantitative assessment, although, the results are in agreement with literature (58, 110, 114, 115).

It was reported that the terminal positioning of LNA nucleotides is important as the 3’ terminal LNA residue slows the 3’→5’ exonucleolysis while an LNA group in the second position from the 3’ terminal gives complete resistance to degradation. In contrast to this, we found that LNA1 having an LNA group in such a second position was metabolised quicker compared to LNA2, which had a 3’ terminal LNA. It is possible that our measurements in
plasma were dominated by metabolites because within the experimental time most of the intact compound had already entered the tissues due to reported rapid plasma clearance of LNA-DNA mixmer. In support of this hypothesis, radioactivity was just enough for analysis at the 60 min time point. The extensive plasma protein binding of PS ODN might explain why intact PS was detected in plasma 2 h after injection. The LNA radio-chromatograms at 20 min time point displayed additional peaks to the left next to the peak of intact LNA1 and LNA2, which might result from binding to nucleic acids or to small peptides in the plasma.

Uptake of ODN by rat tissues

Blocking of scavenger receptors by poly-I or poly-A could reduce the uptake of $^{68}$Ga-LNA2 in rat organs in a tissue specific manner, as described in the literature (92, 175, 177), suggesting that “liver”-type (SR-AI/II) scavenger receptors are more widely expressed in rat compared to “kidney”-type receptors but their manifestation levels vary from tissue to tissue. It has been shown that most probably the capability of aggregate formation can be one explanation why only poly-I can effectively block class A type scavenger receptors. It was concluded that antisense ODNs which could not form aggregates are free from clearance by scavenger receptors (173). However, we are not aware of whether $^{68}$Ga-LNA-DNA mixmer is able to form such aggregates or not.

Interestingly, spleen seems to be less sensitive to poly-I and poly-A, although splenic uptake was also found to be saturable. Besides, spleen usually has a high non-specific ODN uptake. At high dose (60 mg/kg), however, both poly-I and dextran sulphate reduced PS ODN uptake not only in liver but also in spleen (92). This indicates a class A type I/II scavenger-receptor-mediated uptake by spleen, as well, presumably through macrophages. If this is true, the scavenger receptor-mediated uptake by macrophages of the RES system could partly explain the non-hybridisation specific tissue distribution observed in the present study. In addition, RES organs were reported to represent effective clearance sites (103). The fact that kidney, liver, spleen and bone marrow were among the tissues of high uptake suggests that ODNs could end up in the endosomes of the macrophages of the RES system, and additionally in the endosomes of liver and kidney endothelial cells and thereby participating in the clearance and elimination of ODNs. In support, it was found that not only liver but also spleen and bone marrow accumulated a bi-cholesterol-conjugated PS ODN. These tissues together accounted for >95% of the clearance (175).

However, the above mentioned observations can not be taken as definitive evidences of the role of scavenger receptors because several other receptors and uptake mechanisms are also inhibited by the blocking agents poly-I, poly-A and dextran sulphate. Nevertheless, the extensive data available suggest that scavenger receptors may be involved in uptake by macrophages and
liver endothelial cells since SR-AI/II expression is normally restricted to these cells (177).

In contrast, PNA, due to the neutral charge, was hardly able to enter rat tissues and the permeability-improving peptide, NLS, did not facilitate membrane passage either, although plasma contained extensive amount of PNA. A similar high accumulation in kidney with low levels of PNA in other organs has been published (146, 207). Using the permeation protein, a somewhat increased uptake in liver and muscle has been reported (148).

The central nervous system is inaccessible to many molecules, especially those that are larger than 500 Da, polar or hydrophilic, because they cannot penetrate the tight junctions of the blood-brain barrier (208). Recent studies indicate that this barrier is much more dynamic than it was assumed but still most of the naked antisense ODNs cannot cross it. However, Tyler et al. demonstrated that 0.007% of the intra-peritoneal injected PNA dose (10 mg/kg) at 2 hours could be detected in the brain of Sprague-Dawley rats where it reduced the expression of its target gene (209, 210). Also, after an i.v. injection, the brain contained low levels, 60 ng PNA/g (207). Uptake can be significantly enhanced by membrane-penetrating peptides at least at concentrations above 3-5 μM, and it displayed gene downregulation activity upon forming stable hybrids with complementary sequences (37, 211, 212).

Uptake of ODNs by cells
The results in cell spheroids indicate that scavenger receptors or similar receptor mediated uptake systems have a role in the uptake process of the cells used. However, the cellular uptake, as the serum-free time and concentration curves revealed, does not consist of only a saturable phase but also of another that rather has a passive diffusion-like characteristic. The two human cell lines showed similar characteristics, while the rat cell line showed slight signs of saturation. Serum-free incubation medium was more favourable as it provided more available free (non protein-bound) 68Ga-LNA2, which was taken up by the cells. Additionally, serum could also contribute to saturation of uptake mechanisms.

ZHANG et al. published saturation with 35S- and 99mTc-labelled antisense PS ODNs during a 24 h time span (104, 190), additionally, the same researchers demonstrated the steady accumulation of a 15-mer 99mTc-labelled full LNA over 24 h, whereas the accumulation of the sense ODN levelled off to a decrease after 8 h (213). At 24 h, 4.5x10^7 LNA molecules were calculated to accumulate per cell. NAKAMURA et al. reported that accumulation of a 99mTc-labelled antisense PS ODN decreased in the cells with the increase of labelled ODN but the available targets in the cells also influenced the results: the more target mRNA that was present, the steeper the concentration curve became, resembling much more a line than a bent curve (150). A saturation curve similar to ours was found with a cell line expressing less target mRNA. It was concluded that antisense ODN was able to saturate
target mRNA because the accumulation of antisense continuously decreased reaching the concentration curve of sense ODN at higher concentrations when antisense showed only non-specific accumulation. However, the concentration curve of sense ODN followed the curve of antisense and was also much steeper in high target expressing cells indicating the involvement of another factor that seems to depend on the cell type. Finally, BISSEN et al. demonstrated monophasic and saturable binding of a $^{32}$P-labelled PO ODN to receptors on liver endothelial cells and Kupffer cells (172).

We found it interesting that the two human cell lines showed saturable uptake characteristics as if they had a target, while AR42J, which indeed expresses the target Chg-A mRNA, only slightly did so. This saturable curve resembles very much the concentration curve of that cell line that had lower target expression in the paper of NAKAMURA et al. (150). Therefore, we believe that the criteria of a successful specific hybridisation need to be strengthened. According to the existing criteria suggested by Hnatowich’s research group, the main indications for specific hybridisation include higher accumulation of antisense ODN compared to a control (sense, random or scrambled); furthermore, if it should be concentration dependent. At present, criteria 1, 2 and 4 (described under existing criteria in the introduction) have been achieved both in vitro and in vivo by studying different cell lines that contain or do not contain target and applying different control sequences (132, 149-151). These experiments showed the clear advantage of antisense over control sequences in target cells, whereas in non-target cells or cells with much lower expression of the target, the uptake of antisense and sense were similar. These experiments are few and we are of the opinion that the proof of specific hybridisation is significantly more complicated and needs much further exploration. This view is based on the existence of specific uptake mechanisms, which may by themselves be sequence dependent and, expressed differently in various cell types and may be saturated by an increased concentration of the probe. Moreover, providing evidence by the in vivo approach appears to be a much more difficult task. To the best of our knowledge, the only one xenograft model experiment published, which resulted in higher accumulation of antisense versus sense in the tumour, was made after a direct intra-tumoural injection, while following an i.v. injection, however, no difference between the two ODNs was observed (149).

Future perspectives
In the antisense field, major improvements have been achieved by the development of modified nucleotides that provide high target affinity, enhanced biostability and low toxicity. Improved methods are now available for identifying base sequences that effectively target particular mRNA, new methods of radio-labelling have been developed, and carriers to improve cellular accumulations are under investigation. In antisense therapy, the gapmer design and modified ODNs with less off-target effects provide hope. Moreover it
appears that novel ODN modifications may rival siRNA molecules in gene downregulation. Furthermore, siRNA molecules have recently also entered clinical trials (154).

Yet, many hurdles have to be overcome and problems have to be solved until imaging with antisense ODNs, i.e. \textit{in vivo} hybridisation is accomplished. The major remaining challenges are regarded to be the efficient delivery of ODNs into the cytosol or nuclear compartment of cells, extensive exploration of the conditions of improved targeting and the sufficient evidences of specific hybridisation \textit{in vivo}. We strongly believe that the breakthrough is only a question of time and that for example, modern molecular biology methods would make it possible to construct a target gene that can be regulated and switched on and off intentionally, thereby providing a more unambiguous proof for antisense mechanism. One such attempt has already been performed (214).

Despite the noticeable decline in the antisense imaging field, the third generation ODNs – LNA, PNA and PMO – mean a great hope as they are not fully explored yet. Successful results from thorough experiments will definitely encourage and stimulate further research, especially if they could be reproducible. It is envisaged that similar to the scenario with monoclonal antibody-based therapy in which the initial hope was replaced by disappointment and eventually by success in selected areas (153), the concept of imaging with antisense ODNs may one day triumph and play a role in the clinic to the benefit of the patients.
Conclusions

We have shown in this thesis that the radio-labelling carried out for the purpose of PET applications by conjugating \( ^{76}\text{Br}\)bromobenzoate or DOTA chelator through 5’-aminohexyl group did not result in change in the hybridisation ability of ODNs regardless of (i) the type of radionuclide (\( ^{76}\text{Br} \) or \( ^{68}\text{Ga} \)), (ii) ODN modification (PO, PS, OMe, LNA, and PNA), (iii) sequence (Chg-A, K-\text{ras}, c-fos) or (iv) length of ODN (30, 20, 17, 16 bp). Therefore, these types of labelling methods do not seem to endanger the ODNs from being representative for ODN in their external detection of distribution and tissue uptake.

The bio-distribution pattern appeared to be varying according to the modification and length of ODNs. Nevertheless, PS and antisense LNA ODNs displayed similarity. By evaluating five different antisense ODN modifications, we can conclude that LNA2 ODN proved to be the most favourable regarding the most extensive accumulation in tissues, stability and longer plasma persistence. It possess therefore a great potential for further exploration of the hurdles towards \textit{in vivo} imaging of hybridisation possibly with other types of LNA-DNA constructions, as well.

The bio-distribution of PO, PS and OMe ODNs specific for point mutationally activated human K-\text{ras} reflected their various metabolism and non-specific binding in rats. Similarly, bio-distribution of antisense LNA-DNA mixmer ODNs complementary to rat Chg-A mRNA displayed a non-hybridisation specific distribution with high accumulation observed in the kidney, liver, spleen and bone marrow. As indicated in the summary in the introduction, this further demonstrates how the accomplishment of the detection of gene expression \textit{in vivo} is difficult. Nevertheless, this may also indicate that we have not found the right sequence since we managed to test only one for Chg-A.

As one specific aspect of the antisense problematics, we managed to reveal that scavenger receptors and/or other saturable processes unrelated to hybridisation may play a roll in tissue uptake and in clearance of antisense ODNs through these organs. These processes may be sequence dependent suggesting that proof of \textit{in vivo} hybridisation through imaging needs much more elaborate evaluations than just comparison of sense and antisense sequences and proving dose-dependency.

Med avbildningsmetoder kan man få en bild av anatomiska strukturer, t.ex. genom dator-tomografi, eller av fysiologiska mekanismer om man använder positron emission tomografi (PET). Med hjälp av en specifik spårsubstans som är märkt med en isotop (t.ex. $^{11}$C, $^{15}$O, $^{18}$F eller $^{68}$Ga) och injiceras intravenöst i kroppen, kan man avbilda t.ex. var vissa molekylära receptorer finns, eftersom spårsubstansen (receptor ligand) binder specifikt i de vävnader där målreceptormolekyler finns. Bilder får man fram genom att placera patienten (eller djuret) i en PET kamera som fångar den strålning som sänds ut från vävnader när radio-isotoper på spår molekylerna sönderfaller. Sedan räknar en dator ut varifrån en kroppen strålningen kom och gör detaljerade bilder av var spårsubstansen befann sig.

I avhandlingen har PET tekniken utnyttjats för att undersöka hur läkemedel som blockerar genuttryck fördelas respektive om genuttryck kan avbildas.

En aktiv gen innebär att genens information översätts till ett protein med hjälp av en informationstransporterande molekyl som heter messenger RNA. Den här processen, genuttrycket, kan spåras genom att designa och syntetisera en oligonukleotid (OND), som är komplementär till ett specifikt mRNA och specifikt känner igen och binder till det. För att användas vid PET undersökningar måste OND märkas med lämplig radionuklid.

Naturliga ODN degraderas snabbt i kroppen, därför har man utvecklat olika modifierade ODN för att öka molekylens stabilitet och bindningsförmåga. Några av dessa är phosphorothioate (PS), locked nucleic acid (LNA) eller peptide nucleic acid (PNA).

Syftet med avhandlingen var att utvärdera och karakterisera olika modifierade antisense ODN för avbildning av genuttryck med PET. Först behövde vi säkerställa att märkningen inte förändrar den biologiska förmågan av oligonukleotiden att binda till avsett mRNA. Det visade sig att alla typer av
ODN modifikationer som vi studerade behöll förmågan att känna igen specifika sekvenser och binda till dem.

Fördelning i kroppen efter injektion av spårsubstansen i råttor visade sig bero på typen av modifikation och längden av ODN. Vi drog slutsatsen att en LNA-DNA mixmer (omväxlande LNA och DNA nukleotider i sekvensen) var den mest lovande bland de fem olika modifikationer som vi undersökte. Sammantaget visade LNA-DNA hög distribution till olika vävnader, relativt hög stabilitet och lång vistelse i blodet, och hade störst potential för vidare studier, syftande till att närmare utforska de mekanismer som hitintills har försvårat avbildningen av genuttryck.

I våra studier framgick dock att fördelningen av LNA-DNA mixmer i råttor var oberoende av om det specifika mRNA:t fanns i vävnaden. Oligonukleotiden anrikades mest i njure, lever och mjälte, vilka är organ associerade med elimination, möjligen genom vita blodkroppar som finns i dessa organ. Vi kunde visa att blockering av specifika receptorer som finns på dessa celler leder till minskat upptag i organen. Dessutom demonstrerade vi i celldlings-försök att andra mättnadsbara mekanismer har en roll i upptag av ODN och att dessa kan vara sekvens-beroende.

Det har gjorts många framsteg i antisense forskningsområdet det senaste decenniet men mycket återstår fortfarande. Vi måste uppnå effektiv leverans av antisense ODN till celler och vi måste utveckla tillförlitliga bevis för att antisense ODN verkligen binder till target mRNA inne i celler i kroppen. Vi tror att det bara är en tidsfråga innan verklig avbildning av genuttryck i reproducerbara studier presenteras och att dessa kan öka intresset för denna forskning inom ett område som är begränsad till några få forskningsgrupper. I framtiden kan moderna molekylärbiologiska metoder användas för att skråddarsy en gen som avsiktligt kan slås på och av, vilket skulle kunna användas för att bevisa att antisense ODN binder till sitt mål- mRNA i en levande organism.
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Uppsala, September 2007
“Do you think you have built a house from where you are entitled to view the world with satisfaction at the top of your carrier? Don’t you know that you always are a wanderer, and everything you do represents the movement of a wanderer who is advancing on the way? You travel between cities, aims, the stages of life and changes that go on for ever, and if you take a rest, you can do it neither securely nor permanently. As the wanderer, who drops himself down in the shadow of an apple tree for half an hour on his way. You should know this when you work out a plan. The meaning of your journey is not the aim but the wandering. You don’t live in situations but you do live on the way.”

Sándor Márai
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