PAPER

In vivo imaging with oligonucleotides for diagnosis and drug development

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Molecular imaging, the science that combines non-invasive in vivo imaging and molecular biology, has begun to use labelled oligonucleotides as radiotracers. Antisense oligonucleotides target gene expression at the RNA level, while aptamer oligonucleotides are designed to hit proteins of interest. Oligonucleotides for imaging cover a large range of applications, from the invention of new contrast agents for diagnosis to exquisite research tools for the development of new drugs.

Because they respect the integrity of living individuals, in vivo imaging techniques such as magnetic resonance imaging, single photon emission computed tomography (SPECT), ultrasound imaging, enhanced endoscopic imaging, and positron emission tomography (PET) can be repeated without harm to describe a physiological state. Constant refinement in the accuracy of these techniques has extended their field of applications from diagnostics to indicators of the physiological activity of drugs. In particular, molecular imaging methods make biochemical measurements such as drug concentration, receptor density, or cellular responses to drug challenge, easy and ethical to realise in deep tissues without the necessity for tedious and dangerous sampling. 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. Molecular imaging is the science bridging together molecular biology and in vivo imaging with the aim to detect the expression of specific genes, whether disease related or not, using non-invasive procedures in a living person.

MOLECULAR IMAGING AS A TOOL IN DIAGNOSIS AND IN THE DRUG DISCOVERY PROCESS

Among these methods, PET is today the imaging technique with the best sensitivity in terms of molecular concentrations, and with the best capacity for quantitative measurements.[1] PET is ideally suited to fulfill the requirements of modern drug discovery based on mechanistic approaches, by furnishing precious quantitative measurements of in vivo pharmacokinetics and pharmacodynamics. A recent review placed the focus on the use of PET for in vivo pharmacokinetics and pharmacodynamics in drug development and collected 36 different PET studies of pathophysiology in the fields of oncology, cardiology, and neurology.[2] Clinical PET in drug development has been used for the elucidation of pathophysiology, for the evaluation of drug pharmacokinetics, to obtain in vivo the proof of the principle of a predicted mechanism of action, to predict a response via the assessment of receptor occupancy, to define optimal dosing, and to assess the efficacy and/or the response to a treatment.[2]

PET is based on the physical principle of annihilation of a positron/electron (β+/β-) pair, in which two 511 KeV photons are emitted in opposite directions (that is, with an angle of 180°). The double coincidence in energy and direction of the annihilition photons is advantageous for imaging because it permits a simple and comparatively precise localisation of the annihilation site. Whenever two photons are detected simultaneously in two detectors calibrated around a 511 KeV energy window facing each other, the localisation of the annihilation is assigned on the line joining those detectors. In modern PET cameras, electronic coupling of rings of crystals permits the detection of all coincidence events in the volume centred by the ring and yields a 3D image of the annihilations taking place in that volume.

Popular positron emitting isotopes include Carbon-11, Nitrogen 13, Oxygen 15 and, Fluorine 18. These isotopes have in common short half lives (^{11}C=20 minutes; ^{13}N=10 minutes; ^{15}O=2 minutes ; ^{18}F=110 minues) and the capacity to be produced with very high specific radioactivities, typically 1–10 Ci/μmol (37–370 Gbq/μmol), permitting tracer dose detection in living tissues. With the progress of PET radiochemistry, during the past decades rapid and efficient labelling methods have been developed and several hundreds of PET labelled radiopharmaceuticals have been described. It is now possible to measure femtomolar (10^{-15} M) amounts of a PET radiopharmaceutical during several radioactive half lives with sub-centimetric resolution all over the body, and this has many applications in pharmacokinetics, target identification, receptor density measurements, etc. Most PET radiopharmaceuticals were designed initially for research use, however clinical applications are now booming thanks

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Abbreviations: PET, positron emission tomography; SPECT, single photon emission computed tomography; FDG, 2′-deoxy-2′-fluoro-glucose; PKA, protein kinase A; LTR, long terminal repeat; HIV, human immunodeficiency virus; SELEX, sytematic evolution of ligands by exponential enrichment; PDGF, platelet derived growth factor; VEGF, vascular endothelial growth factor; siRNA, small interfering RNA; RNAi, RNA interference

to the capacity of 2′-deoxy-2′-fluoro-glucose (FDG) labelled with ^{18}F to detect the hypermetabolic activity of proliferating cells in tumoral tissues.

With the advent of molecular medicine and the increased demand for improved targeting of drugs, the use of PET during drug development is rapidly growing. Leading PET centres in Europe and worldwide are now developing preclinical applications of PET in connection with the research teams of pharmaceutical companies. A new era in preclinical PET has started with the development of high resolution PET scanners dedicated to small laboratory rodents, including the many transgenic mice that are created to mimick human diseases. One reason that a sophisticated technique like PET can speed up drug discovery lies in its capacity to breach through the bottlenecks of the drug development process. These bottlenecks are attributable in great part, to the low reliability of extrapolation of in vitro data to in vivo application. With PET, it seems now feasible to test drug candidates directly in vivo, eliminating in part the risk of failure of molecules that show potent activity in vitro but exhibit redhibitory drawbacks when administered in vivo because of poor delivery. An idealistic view is that the chain of rational drug development from functional genomics to clinical applications will be complemented by screening drug libraries with PET molecular imaging, directly in vivo in transgenic or other animal models of human diseases. Naturally, molecular imaging agents capable of assessing drug targeting are also valid candidates as contrast agents for diagnostic purposes whenever the molecular determinant of a disease is worth identifying by a non-invasive procedure, hence, new PET radiopharmaceuticals aim at both the diagnostic and the therapeutic research areas.

OLIGONUCLEOTIDES AS NEW RADIOPHARMACEUTICALS

Structure and properties

Oligonucleotides are small chains of a few (Greek "oligo") nucleotides, the elementary units of nucleic acids: RNA when the sugar is ribose or DNA when it is deoxyribose. Like all nucleic acids, they are linear polymers that carry a biological information based on the nucleobases, the genetic code's four letter alphabet: adenine, guanine, cytidine, and thymidine (in DNA) or uracil (in RNA). According to the Watson-Crick model, information coded by the sequence of nucleobases along the chain can be duplicated and read by creation of reversible hydrogen bonds. Three bonds are formed between guanine (G) and cytosine (C), and two between adenine (A) and thymidine (T) or uracil (U). Hence, two chains running in opposite directions can hybridise one to another if their sequences can form G-C and A-T(U) pairs, in other words if their sequences are complementary. For example, the sequence 5′AATCTGGCT3′ binds reversibly to its complementary sequence 5′AGCCAGATT3′ to form a duplex. Duplexes can be formed between two DNA chains, two RNA chains, a DNA and a RNA chain, and between oligonucleotides and DNA or RNA. Duplex hybridisation is the basis for the capacity of nucleic acids to replicate—that is, to create multiple copies identical to the original matrix, a property that is widely applied in biotechnology with the polymerase chain reaction (PCR) in which a single sequence serves as a matrix to produce billions of identical copies. The second interesting property of nucleic acids that is present in oligonucleotides is their capacity to create myriads of different molecules by combinatorial chemistry. As the number of possible sequences of an oligonucleotide containing n nucleotides is 4^n, a very high number of molecular combinations is reached rapidly as n increases. For instance, there are over 10^{12} (4^{20}) combinations of a 20 unit oligonucleotide. Given that the sequence defines the spatial structure of the oligonucleotide, the number of possible structures present in a library of random sequence oligonucleotides raises exponentially. Finally, oligonucleotides are synthesised at the industrial level and have become comparatively cheap, and the use of oligonucleotides as in vitro diagnostic tools has grown exponentially in the past decade: they are the basic tools for major molecular biology methods such as PCR, biochip arrays, in situ hybridisation, etc.

Because of their excellent targeting capacities and easiness of synthesis in high diversity, oligonucleotides are extensively used in vitro as ligands for nucleic acids (antisense oligonucleotides[3]), proteins and small molecules (aptamer oligonucleotides[4 5]). Although the seductive idea to use oligonucleotides in vivo for therapy, diagnostic, imaging, etc[6] appeared more than 30 years ago,[7] these applications are still in their infancy and it seems that tremendous efforts are still necessary to develop these compounds as pharmaceuticals.[8] Natural oligonucleotides show poor stability, affinity and membrane passage, and it is now clear that oligonucleotides are spatially structured in vivo and that some sequences confer non-canonical biological properties to oligonucleotides. Numerous chemical modifications of the natural oligonucleotides aimed at improving their biological stability and bioavailability have been proposed and thousands of sequences tested.[9–24] Eventually, the biological activity and the putative benefit of any modification of oligonucleotides expected to exert a specific action in a living organism can only be fully appreciated through in vivo or ex vivo studies.

Labelling of oligonucleotides for pharmacoimaging

Several methods to label oligonucleotides with radioactive isotopes for imaging studies have been described, both with gamma emitters and with positron emitters (reviewed in references[25 26]).

With the aim to encompass a large panel of in vivo and ex vivo imaging techniques, we developed a method to introduce radioactive isotopes of halogens (fluorine, bromine, and iodine) in a synthon designed for its high reactivity and its capacity for stable incorporation of radiohalogens.[27] The radiosynthon can then be conjugated regioselectively with a given oligonucleotide in one step to create the appropriate radiotracer.[28–31] The objective was to develop a general non-carrier added method for labelling oligonucleotides with radioactive halogens for use in radiopharmacology. We aimed at designing a method as universal as possible to be applied to any sequence and oligonucleotide chemistry, which could be performed with the different isotopes relevant to the variety of in vivo and ex vivo imaging and analytical techniques required for the development of oligonucleotides as pharmaceuticals.

Radiolabelling is performed in two steps, (a) radiolabelling of a synthon designed for high reactivity and stable incorporation of halogens and (b) regioselective conjugation of the synthon to the oligonucleotide. Performing the radiosynthesis of the synthon separately from the synthesis of the oligonucleotide permits (1) high yield incorporation in an activated C6 aromatic ring of the halogen through the use of harsh conditions that would not be compatible with the stability of the oligonucleotides; (2) purification of the synthon from other radioactive by products by HPLC, and (3) the possibility to work with any oligonucleotide obtained in house or commercially, without tedious unblocking steps to protect the oligonucleotides from unwanted reactions. The coupling reaction itself is designed to be rapid and efficient in conditions compatible with the chemical stability of the oligonucleotides and the half life of isotopes.

The family of radioprobes that can now be produced with this methodology covers a large panel of in vivo and ex vivo imaging techniques, complementing and refining studies on oligonucleotides' pharmacology: fluorine-18, a positron emitter used in PET, ($T_{1/2}$: 110 min); iodine-125, a low energy Auger electron emitter used for high resolution autoradiography, ($T_{1/2}$: 59.9 days); iodine-123, a gamma emitter used in SPECT ($T_{1/2}$

: 13.2 h); iodine-131, a gamma emitter for imaging and therapy ($T_{1/2}$: 8 days), and bromine-76, a positron emitter with a comparatively long half life ($T_{1/2}$: 16.1 h). Access to isotopes with longer half lifes allows to extend the times during which biodistribution and metabolism can be studied, in vivo with PET or SPECT or ex vivo in small animals, while low energy isotopes allow to perform high resolution imaging with autoradiography. In addition, iodine-123 and iodine-131 labelling allow for extension of oligonucleotide imaging to gammacameras.

The initial work was conducted with the natural phosphodiester backbone as template compounds. However, phosphodiester oligonucleotides are instable in vivo and cannot be used as such without incorporation into vectors with protective effects. The labelling method was hence adapted to oligonucleotides bearing modifications on their ribose phosphate backbones or nucleobases, to yield radioactive probes in sufficient quantity with high specific radioactivity.[29 30] It has been reliably and routinely applied to the most popular chemical modifications: besides phosphodiester DNA, the natural deoxyribose oligonucleotide, (1) full length internucleosidic phosphorothioate diester bonds deoxyribose oligonucleotide, the modification most favoured by industry for human antisense therapy[9]; (2) hybrid methylphosphonate/phosphodiester internucleosidic bonds deoxyribose oligonucleotides—that is, mixed backbone oligonucleotides with interesting imaging properties[15 32]; (3) 2′O-Methyl modified ribose oligonucleotide, confering resistance to nucleases and high efficiency of duplex formation with the complementary RNA[17 20]; (4) peptide nucleic acids (PNAs), a unique class of oligonucleotides that combine within the same molecule the capacity of nucleic acids to hybridise to their complementary sequence and the biostability of pseudopeptides.[18 31]

As the synthon addition does not hamper biological properties of the oligonucleotides in vitro, including hybridisation to the complementary target,[33 34] these novel radioprobes can be considered as valuable indicators of an oligonucleotide's fate in living organisms. Using the positron emitter fluorine-18 we imaged the biodistribution of oligonucleotides during several hours in vivo with PET.[34]

Nevertheless, it should be noted that our method, like all methods reported so far, modifies the oligonucleotide by addition of labelled or radiolabel chelating groups, and hence may modify its properties. For instance, it has been reported that technetium-99m chelating groups modify the accumulation and efflux of 18-mer antisense DNA against a subunit of protein kinase A (PKA) in the kidney cancer cell line ACHN.[35] The biodistribution in normal mice is also heavily influenced by the labelling method, and four hours after the injection of ^{99m}Tc labelled DNA, the percentage of injected dose per gram recovered from different organs varied by a factor 12 for two different chelators.[36] These authors concluded that imaging may depend on the method for radiolabelling the antisense, which should be adapted to the organ localisation of the target gene.

ANTISENSE OLIGONUCLEOTIDES

Principle

Based on the formation of a Watson-Crick hybrid between an oligonucleotide and an RNA, the antisense technology provides a simple and elegant approach to inhibit the expression of a target gene. An antisense is a short oligonucleotide whose sequence is complementary and can bind to that of its target RNA (viral RNA or mRNA), thereby inhibiting its translation (fig 1). The mechanism of inhibition is either through a steric blockage of the pre-mRNA splicing or of the initiation of translation, or through ribonuclease H mediated recognition of the mRNA-oligonucleotide duplex and ablation of the mRNA. Antisense oligonucleotides complementary to a target region of a candidate mRNA have been successfully used to inhibit protein synthesis in a number of biological

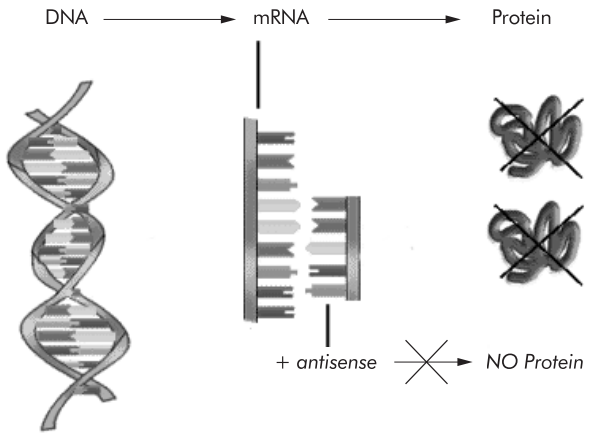

Figure 1 Antisense oligonucleotides bind to the complementary sequence of the RNA and block its transcription into a protein.

systems.[37 38] This method of gene regulation, based on the hybridisation of two nucleic acids strands through Watson-Crick base pair formation, is extremely simple to design and has many potential therapeutic application: in cancer, viral infections and in inflammatory disorders.[3 8 37–40]

Antisense oligonucleotides for gene related disease therapy

Antisense for therapy is an active field of drug development, reviews of clinical trials of oligonucleotides can be found in references[6 41] and frequent web updates.[42] Currently there are around 30 different oligonucleotides tested in about 40 different clinical trials, mostly in Phase II. So far, only one has been approved by the FDA, Vitravene for cytomegalovirus retinitis. About one half of the oligonucleotides in clinical trials are built with the phosphorothioate chemistry, 50% target cancer related genes, 25% target viral infections including hepatitis C, and three target chronic inflammatory diseases such as Crohn's disease and haemorrhagic rectocolitis.

Many of these candidate antisense drugs target haematological disorders such as chronic myeloid leukaemia (for review see Agarwal and Gewirtz[43]), by targeting specific proto-oncogenes involved in cell proliferation and neoplasic transformation: Bcr/ab1[44] c-myb,[45–47] c-myc or the tumour suppresser gene p53.[46] Other antisense strategies are based on the chemosensitisation of tumour cells by depressing anti-apoptotic genes such as Bcl-2 expression.[48] The antisense drug Genasense (Genta, Inc) is an anti Bcl2 antisense now in Phase 3 clinical trials in lymphoma,[49] and is also assayed as a chemosensitiser for dacarbazine treatment of human melanoma.[50 51] In another approach, glioma cells collected at surgery are treated ex vivo with an antisense oligonucleotide against the type I insulin-like growth factor receptor and re-implanted into the patient, inducing apoptosis and a host response.[53]

Although there are now a number of reports of antisense inhibition of human tumours, it should be emphasised that only for a very small number of patients has complete remission been observed. Many antisense oligonucleotide have been found to induce a variety of biological effects not related to their specific hybridisation to the target mRNA, including immune stimulation and other activities by oligonucleotide containing CpG motifs, release of pharmacologically active concentration of deoxyribonucleosides, or aptameric binding to proteins. Clinical efficacy of antisense on tumour growth and development is difficult to evaluate[53] and could certainly benefit from in vivo imaging evaluation methods. In some cases, side effects of antisense drugs that are not based on an antisense effect could be therapeutically useful, as suggested by a recent report showing that oligonucleotides with CpG motifs can reduce prion toxicity in mice.[54]

Improving antisense for in vivo applications

Generic molecular tools that have the capacity to adapt to any possible target or at least to a large number of different targets, such as antibodies or oligonucleotides, are often difficult to handle in vivo. This is especially true for antisense oligonucleotides, which, although they have been sometimes presented as "magic bullets", suffer from a number of major drawbacks that complicate their use in vivo, essentially (a) in vivo stability; (b) access to target sequences; and (c) non-specific interactions.[25 55]

(a) The stability issue has been addressed by the synthesis of numerous chemical alterations of the phosphate-sugar backbone. Naturally, chemical modifications that modify sensitivity to nucleases of an oligonucleotide also induce major alterations of its pharmacokinetics and targeting that in vivo imaging can measure. Another approach is to incorporate oligonucleotides into delivery agents or vectors and here again imaging is a useful assessment tool (see below).

(b) To hit intracellular RNA target sequences, oligonucleotides should traverse cellular membranes, which they do poorly because of their low lipid solubility. In addition, RNAs are highly structured molecules exhibiting double stranded secondary structures such as stem-loops, hairpins, pseudoknots, etc, leaving comparatively little access to hybrid formation by the oligonucleotides in vivo. It has been demonstrated that, at the best, no more than 6%–12% of oligonucleotides targeting an RNA sequence are efficient at forming the duplex necessary for the antisense effect.[56] Another concern is that cellular concentration of mRNA may not be high enough to allow for its imaging by antisense hybridisation. This concentration can be comparatively high for viral RNA in infected cells, but as a rule, the mRNA coding for a given protein is less abundant than the protein itself. Abundant mRNAs such as the one coding for tyrosine hydroxylase in catecholaminergic cells (about 1800 molecules per cell[57]) are in principle detectable, while low abundance mRNAs in less than 10 copies per cell might not be.

(c) Binding of oligonucleotides to undesired sequences may pose little problems, because dissociation constants of oligonucleotides are in the nanomolar range and depend strictly on the complementarity of the two strands of the duplex. In contrast, non-specific binding to proteins has been reported, especially for the phosphorothioate derivatives,[58–60] and can induce toxic effects in some cases.[61] Studies using photoactivatable crosslinking of a phosphodiester oligonucleotide added to a cell culture showed that up to 90% was bound to a cell membrane protein of 75–79 kDa.[62 63] In the presence of serum, bovine serum albumin binds oligonucleotides with a Km in the 10^{-5} M range,[64] predominantly on site I of the protein, and this is found also with human serum albumin.[65] Several other proteins also bind oligonucleotides, and the physiological state of the cell influences binding patterns.[60]

Hence the radiotracers based on short nucleotide polymers require the invention of methods to circumvent the barriers that living organisms have evolved to protect themselves from forensic invasions. Two strategies can immediately be foreseen to reach that goal. One is to disguise the desired molecule so that the organism will not recognise it, through the use of vectors or peptides acting as Trojan horses.[66] The other is to pick the molecules with the desired properties in vivo by intelligent selection procedures that take into account the pressure of in vivo conditions on their survival and activity. Both strategies are applied to oligonucleotides in our laboratory.

Comparative pharmacoimaging of oligonucleotides

Systematically administered oligonucleotides must escape plasmatic and tissular nucleases and cross various biological membranes to reach their cellular target. Among the technological tricks explored to circumvent the efficient mechanisms by which living organisms protect themselves from an invasion by exogenously administered nucleic acids, one widely explored possibility consists in modifications of the natural oligonucleotides' backbone that enhance the biological of their stability. In an attempt to better evaluate the tracer characteristics of labelled oligonucleotides, our laboratory performed imaging studies in monkeys with 3′-end[^{18}F] labelled oligonucleotides, concentrating on the pharmacodistribution differences between phosphodiester, phosphorothioate and 2′-O-methyl RNA.[34] The same sequence, negative for any endogenous complementary sequence match, was constructed with these three chemistries,[^{18}F] labelled at its 3′ end, and imaged.[34] Metabolic analysis in plasma sampled at regular time intervals after injection showed a rapid degradation of the phosphodiester (half life in the plasma 3–5 minutes) and comparison with the unlabelled ODN indicated that metabolism was not significantly modified by labelling. In contrast, labelled phosphorothioate and 2′-O-methyl RNA remained intact in plasma during the two hours after injection. Pharmacodistribution of the radioactivity showed that the phosphodiester was eliminated both through the renal and the digestive system, while the phosphorothioate and 2′-O-methyl RNA showed only renal excretion. The phosphorothioate showed persistent accumulation in the liver (0.1 % of injected dose per ml of tissue between 20 and 80 minutes after injection) while the 2′-O-methyl RNA accumulated in the kidney (0.03% at 80 minutes after injection). The data reported in this study showed (1) that the radiolabelling method has no detrimential effect on the capacity of the antisense oligonucleotide to hybridise to its target complementary sequence; (2) that after intravenous injection of the [^{18}F]oligonucleotide it is possible to quantitatively evaluate by PET the kinetics of [^{18}F] radioactivity in any selected tissue or organ; (3) that these kinetics are highly variable with the nature of the oligonucleotide backbone; and (4) that it is possible to measure the concentration of ^{18}F labelled metabolites in the plasma during the PET measurements, opening the way to the quantitative evaluation of the tissular concentrations of [^{18}F]oligonucleotide. Overall it was demonstrated this approach was found to be relevant for the comparative evaluation of oligonucleotides in vivo.[34] The labelling of still other modified oligonucleotides is currently under way, demonstrating that this methods opens the possibility to image the pharmacodistribution and analyse the metabolites of virtually any present or future modification aimed at improving the biodisponibility of oligonucleotides. This will certainly prove useful for drug and radiotracer design and development of oligonucleotides.

Evaluation of delivery vectors for oligonucleotides

Chemical modifications may decrease sequence specificity and/or activity of oligonucleotides, and have little or no effect or may even be deleterious for membrane passage.[67] Another line of research is to incorporate oligonucleotides into synthetic vectors acting as Trojan's horses that would at the same time protect them against nucleasic attack and direct them inside cells.[68] Synthetic vectors are human tailored on the basis of their known physicochemical properties and can be repeatedly administered. Complexes of DNA with cationic lipids (lipoplex)[69] result in the respective condensation of both entities by way of electrostatic interactions. One of the crucial impediments for successful systemic oligonucleotides or DNA transfer with lipoplex exhibiting a positive global charge seems to be their inactivation because of non-specific binding with anionic serum proteins. In this respect, efforts undertaken to develop synthetic vectors for nucleic acids delivery remain gratuitous as long as their efficiency in improving the bioavailability and resistance to degradation of the active ingredient cannot be demonstrated in vivo. We have recently applied the PET technology to whole body quantitative imaging of an antisense octoeikosinucleotide directed to a highly

conserved sequence of the dimerisation site of the LTR (long terminal repeat) region of HIV[70] encaged in a unique formulation process allowing to prepare stable and homogenous lipoplex particles, called Neutraplex, exhibiting a negative global charge.[71] We showed (1) that PET imaging yields in vivo quantitative pharmacokinetics information that is ideal to evaluate the modifications induced by vectors in the biodistribution and organ bioavailability of the oligonucleotide; (2) that competitive hybridisation pictures the capacity of synthetic vectors to enhance in vivo stability; (3) that the combination of these two techniques is able to demonstrate that carefully tailored anionic vectors of the Neutraplex type dramatically ameliorate the in vivo delivery of an oligonucleotide.[71]

In summary, whatever difficulties exist with in vivo applications of antisense oligonucleotides, such a powerful molecular tool is likely to bear fruits in the in vivo diagnostic and perhaps therapeutic fields. Even though the enthusiasm of venture capitalists for the antisense technology is today at a low level, more and more technological improvements of these chemically versatile compounds are becoming available. Antisense oligonucleotide imaging with PET offers the unique opportunity to visualise the efficiency of these improvements directly in vivo and should be an important advantage in evaluating their potential.

APTAMER OLIGONUCLEOTIDES

Principle

Combinatorial approaches are a modern alternative to the rational conception of ligands. Very large libraries of candidate molecules can be randomly synthetised and screened simultaneously to identify those with the wanted property. Combinatorial libraries of oligonucleotides may thus yield selective ligands for specific targets called aptamers ("adaptable oligomers"[4 5]). Aptamers are selected by a generic method termed SELEX (Sytematic Evolution of Ligands by EXponential enrichment), which combines the use of three basic properties of oligonucleotides:

(1) *The possibility to create very large families of combinatorial molecules*. The number of different molecules in a library of oligonucleotides with a window of randomised sequence *n* residues in length (that is, in which any of the four bases (A, T, G, C) is introduced randomly at any position) is 4^n. Under 1 µmol scale solid phase DNA synthesis it is possible to obtain about 10^{14} to 10^{15} individual sequences.

(2) *The possibility to bind to small molecules and protein motifs.* Like any biopolymer, oligonucleotides fall in a three dimensional arrangement that can make contact with other molecules. The binding of an aptamer to its target molecule is based on the complementarity of their respective 3D structure and not on the formation of a Watson-Crick duplex between complementary sequences. Nevertheless, the 3D structure of oligonucleotides depends on their sequence of bases rather than on the sugar phosphate backbone. Aptamers can achieve high target selectivity with dissociation constants in the micromolar to low picomolar range[72–74] comparable to antibody-antigen interactions.

(3) *The capacity to be readily multiplied unchanged.* Amplification of libraries of oligonucleotides is achieved by flanking the window of random sequence with known fixed sequences that are complementary to the primers used for PCR. This permits easy handling and amplification of libraries containing up to 10^{15} different molecules.

The SELEX strategy was designed to select, from a random library of oligonucleotides, those molecules with the desired binding property for a designed target (fig 2).[4 5 72–78] Single stranded DNA oligonucleotides, or RNA oligonucleotides with an additional transcription step may be used. SELEX is basically an iterative cycle of the following actions in sequential order: (1) incubation of the random sequence oligonucleotide library with a decoy—that is, a system in which all the elements of the true target binding system (support, linkers, buffer, etc), except the specific target aimed at, are present. This is the *counter-selection* step; (2) separation of unbound oligonucleotides from those bound to the decoy, which are discarded; (3) incubation of the unbound pool with the true target. This is the *selection* step; (4) separation of oligonucleotides bound to the target from those unbound, which are discarded, and recovery of the bound pool; (5) amplification of the bound pool, yielding a library enriched in sequences binding to the target but not to the decoy. This pool is then submitted to the next selection/amplification cycle. Once affinity saturation of binding to the target is achieved, ususally after 5–20 cycles of selection/amplification, the enriched library is cloned and sequenced. Isolated individual oligonucleotides are then screened for sequences of potential binding sites and tested individually for their ability to bind specifically to the target molecule.

The difficulties in adapting oligonucleotides to the in vivo context differ somewhat for aptamers and for antisense. Target accessibility is much less of a problem for aptamers than for antisense. Many aptamer targets are extracellular proteins readily accessible in vivo and comparatively abundant in regard to the low concentrations of intracellular RNA targets for antisense. However, in vivo stability of oligonucleotides is an issue for the aptamers, because the modifications introduced in the sugar-phosphate backbone to improve oligonucleotide stability are generally not compatible with the polymerases used during the amplification steps of the SELEX procedure. Modifications that increase resistance to nucleases may be introduced after selection of the aptamer sequence, but then there is great risk that the folding pattern and the binding properties of the aptamer will be changed.[79–81] Hence, post-selection modifications require a tedious systematic testing of the influence of every modified residue in the sequence both on stability and binding. A limited number of modifications of the 2′ carbon of ribose confer increased resistance to the oligonucleotides and are compatible with the T7 RNA polymerase, allowing to use 2′-Fluoro- and 2′-amino-2′-deoxynucleoside oligonucleotides directly during the SELEX process.[81 82]

Another approach is to substitute natural D-ribose with L-ribose to create totally stable aptamers in a mirror image configuration termed Spiegelmers. While L-ribose is not acepted by T7 polymerase, the selection of natural D-ribose aptamers binding to the mirror image of the target, such as for instance a D-aminoacid peptide, followed by the chemical

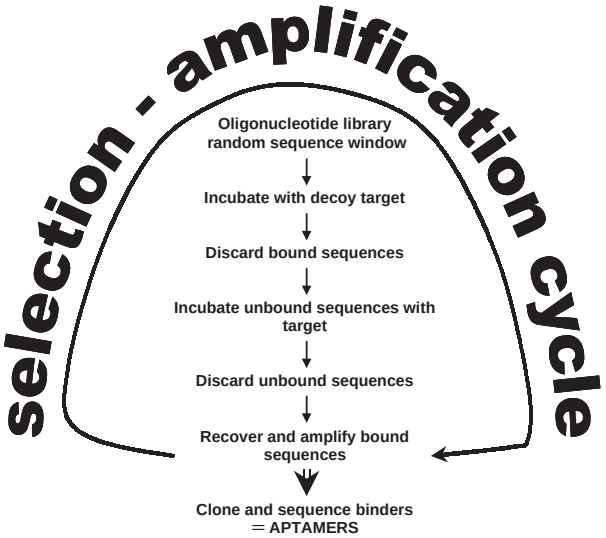

Figure 2 Principle of SELEX, the selection-amplification cycle to obtain oligonucleotide ligands (aptamers) against a designed target.

synthesis of the mirror image of the selected sequence, yields by virtue of molecular symmetry a Spiegelmer that binds to the natural target molecule (that is, the L-aminoacid). Spiegelmers that bind to GnRH I have been recently isolated and characterised with this mirror image SELEX.[83]

Applications

SELEX has proved successful against a variety of targets, such as proteins, small molecules, RNA, and has a high potential in the fields of therapy,[84 85] diagnosis,[86] and biotechnology.[87] Aptamers rival antibodies in terms of affinity for their biological target, with the advantage that they are smaller, cheaper, and easier to engineer. Hence, they have quickly become valuable research tools[88] and many therapeutic and diagnostic applications have been envisaged.[86 88 89] In contrast with antibody production, aptamers can be generated against any small molecule or protein target using a completely synthetic method, and their binding characteristics depend on the experimental system used during the selection process. The selectivity of aptamers can thus be oriented through the choice of pertinent counter-selection/selection targets, as shown by reports of aptamers capable of discriminating between isoforms of protein kinase C,[90] or of interfering with Ras binding to Raf-1 but not to B-Raf, a Raf-1 related protein.[91] Conversely, the discrimination capacity can be reduced and aptamers have been obtained that recognise ERK-2 both in its native and denatured forms.[92]

Moreover, the SELEX process is not limited to the use of purified proteins as targets but can be applied to complex heterogeneous targets such as cells, organelles or even tissues.[93] Using a combination of proteic and cellular targets (*blended* SELEX), RNA aptamers were selected against tenascin-C, an extracellular matrix protein over-expressed during tumour growth.[94] This approach may also help to identify molecular hallmarks of cell surfaces, as shown with human red blood cell membranes,[93] or to differentiate between quiescent and proliferating states of the same endothelial cells.[79] In this latter study, *deconvolution* SELEX was carried out to identify the membrane protein that was one of the targets of the aptamers in the selected binding pool, and was found to be specifically expressed during endothelial cell proliferation.[79] Selecting aptamers in a physiological context in which cell surface proteins are displayed in their native state paves the way to in vivo applications of aptamers. Until now only limited studies have been realised in vivo,[95] but is is remarkable that aptamers have entered in therapeutic trials just eight years after their invention was conceived.[96]

Therapeutic aptamers

Effective aptamer strategies have been developed for in vivo therapeutics. Aptamers can compete with the natural ligands of their target proteins and thus antagonise their biological function. Antagonistic aptamers to the platelet derived growth factor beta chain (PDGF-B) induced a significant reduction of mesangioproliferative changes in rats with progressive glomerulonephritis.[97] Injection of antagonistic aptamers against PDGF-B in rats with PROb colon carcinomas decreased interstitial hypertension in the tumours.[98] Aptamer NX 1838 significantly reduced vascular endothelial growth factor (VEGF) induced vascular permeability in vivo[99] and is currently in phase-I clinical trials in humans.[100]

Aptamers as contrast agents for diagnosis

The molecular weight of aptamers (10–15 kDa) is one order of magnitude lower than that of antibodies (150 kDa), hence they exhibit higher tissue penetration and faster blood clearance, two critical parameters for imaging agent. The ability of an aptamer binding to human neutrophil elastase to image inflammation was compared with the reference antibody in vivo in a rat reverse passive Arthus reaction model.[101] The aptamer performed better than the reference antibody to achieve a peak target to background ratio. The conclusion of that pionneer study was that aptamer ligands were useful in diagnostic imaging, and could offer significant advantages over monoclonal antibodies. The tenascin aptamer has now been labelled with technetium-99m and used for imaging in a mouse model.

The use of aptamers for in vivo imaging is especially promising because of the very wide range of possibilities available to introduce changes in their structure through defined chemical modifications that will modify their pharmacokinetics properties.[34] For instance, the clearance rates of aptamers can be changed to keep them in circulation by anchoring them to liposome bilayers, by coupling them to inert large molecules such as polyethylene glycol or to other hydrophobic groups.[102] The discrimination and targeting capacities of aptamers suit them exquisitely as imaging agents for non-invasive diagnostic procedures. In this respect, *escort* aptamers are a budding concept in which the aptamer oligonucleotide may be used to deliver an active drug, radionuclide, toxin, or cytotoxic agent to the desired site for diagnostic tests and therapy.[78]

OTHER APPLICATIONS OF OLIGONUCLEOTIDES

Triple helix forming oligonucleotides for anti-gene radiotherapy

Under certain conditions, an oligonucleotide can associate with a DNA double helix through Hoogsten hydrogen binding to form a triple helix and block gene expression.[103] Targeted radiotherapy with oligonucleotide has been proposed by using sequence specific triplex forming oligonucleotides carrying Auger-electron-emitters such as iodine-125[104] to induce localised radio damage to specific sites in the genome.[105]

Oligonucleotides as immune modulators

The immune stimulation induced by specific oligonucleotide sequences containing unmethylated CpG motifs has already been mentioned. The induction of an immune response by oligonuclotides may find useful applications to protect against infection or trigger immunotherapeutic responses for cancer, allergic or non-conventional infectious diseases.[54]

Silencing interfering RNA

Small interfering RNAs (siRNAs) mediate RNA interference (RNAi), a process in which target mRNAs are degraded.[106] RNAi is a evolutionarily conserved gene silencing mechanism that uses 21-nt to 23-nt double stranded RNA as a signal to trigger the degradation of homologous mRNA.[107] Naturally occurring siRNAs are generated by an RNase processing reaction from long dsRNA, but chemically synthesised siRNA duplexes with overhanging 3′ ends are active in plants and animals including mammals[108] and humans.[109 110] Most recently it has been shown that effective gene silencing can be performed in vivo in mice, opening the way for an exciting new method to target gene expression with oligonucleotides.[111 112]

CONCLUDING REMARKS

The application of oligonucleotides to in vivo imaging is promising because of the very wide range of targets that these biopolymers can aim at: RNA, proteins, cell surface determinants of diseases, etc. The ease with which their backbone can be modified for in vivo applications while keeping the sequential and to some extent the structural information that they hold warrants future use in vivo. Two decades of intensive nucleic acid chemistry have now created myriads of chemical modifications, which in turn need to be explored for their pharmacokinetics properties.[34]

Molecular imaging has taken in the past two years a sense analogue to molecular biology—that is, the part of biochemistry that deals with nucleic acids. The ambition of molecular

imaging is to image specific patterns of gene expression in tissues, non-invasively and in vivo and become a key to help drug development in the post-genomic era. More than a few validated radiopharmaceuticals, this ambition demands that generic methods for the measurement of gene expression, similar to those that have been developed by molecular and cell biologists in vitro, be adapted to in vivo imaging. Recent demonstrations that PET can image non-invasively the expression of reporter genes introduced artificially in mice supports the feasibility of these approaches. Future applications of oligonucleotides for in vivo imaging will certainly make important contributions to that exciting goal.

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