FEBS Letters 528 (2002) 12–16 FEBS 26501

Minireview

Nucleic acid aptamers in cancer medicine

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Received 1 July 2002; revised 31 July 2002; accepted 1 August 2002

First published online 28 August 2002

Edited by Richard Marais

Abstract Many signalling proteins involved in diverse functions such as cell growth and differentiation can act as oncogenes and cause cellular transformation. These molecules represent attractive targets for cancer diagnosis or therapy and are therefore subject to intensive investigation. Aptamers are small nucleic acid molecules, isolated from combinatorial libraries by a procedure termed SELEX, that bind to a target molecule by providing a limited number of specific contact points embedded in a larger, defined three-dimensional structure. In some cases aptamers have the potential to inhibit the biological function of the molecule resulting in useful reagents for target validation in a variety of disease models.

Key words: Aptamer; Cancer; In vivo imaging; Oncogene; SELEX; Signal transduction

1. Introduction

Changes in the expression of key proteins of the cellular signalling pathways are at the forefront of molecular abnormalities found in cancer. An increasing number of proteins involved in cell growth, including growth factors, receptors, intracellular mediators and transcription factors have been found to be altered through multiple mechanisms of oncogene activation, such as enhanced or ectopic expression, deletions, single point mutations and generation of chimeric proteins, the latter being a consequence of chromosomal translocations.

Many cell surface receptors with an intrinsic intracellular tyrosine kinase activity can act as oncogenes and cause cellular transformation [1,2]. For example, epidermal growth factor receptor is frequently over-expressed in non-small cell lung carcinoma, bladder, cervical, ovarian, kidney and pancreatic carcinoma [1,2]. Similarly, over-expression and/or gene amplification of the human epidermal growth factor receptor-related gene (HER-2)/neu receptor is found in various types of cancers, including breast (were it occurs in 30% of early stage cases), ovarian, gastric, lung, bladder, and kidney carcinomas [1,2]. Somatic rearrangements of the Ret gene are frequently associated to the papillary histiotype of the thyroid carcinoma, and germ-line mutations of the Ret receptor cause inheritance of multiple endocrine neoplasia type 2A and 2B and familial medullary thyroid carcinoma [1–3]. Single point mutations which are frequently found in dominant oncogenes often affect members of the Ras family. Activating mutations in Ras proteins (accounting for almost 30% of all human cancers) result in constitutive signalling, thereby stimulating cell proliferation and inhibiting apoptosis [4]. The BCR/ABL oncoprotein, generated by a fusion of c-Bcr and the tyrosine kinase c-Abl, is responsible for a wide range of human leukemias [1,2].

As major molecular determinants of cancer cells, all these proteins represent primary targets in the rational approach of the cancer mechanisms. Hence, finding specific ligands capable to detect and measure their expression is a strategic objective for the diagnosis and therapy of cancer. These ligands should have the following characteristics: (i) capacity to discriminate between oncogenic and non-oncogenic forms of the proteins involved in signalling pathways; (ii) capacity to quantify the level of expression of the oncogenic forms; (iii) ideally, be usable both for in vitro and in vivo purposes; and (iv) finally, for therapeutic applications the capacity to block the activity of the oncogene product. To this goal, different types of molecules have been shown to be of potential utility for cancer diagnosis and therapy, including small chemical compounds, peptides, antibodies and nucleic acid ligands. Here we will review nucleic acid-based compounds (named *aptamers*) isolated from combinatorial libraries by an iterative in vitro selection procedure which, in the last few years, has yielded several high-affinity ligands and potential antagonists of disease-associated proteins. Aptamers are single-stranded nucleic acids that, unlike ribozymes and antisense oligonucleotides, function by folding into a specific globular structure that dictates high-affinity binding to a variety of targets. The cytoplasmic expression of aptamers, also called 'intramers', has been recently described demonstrating their striking potential as rapidly generated intracellular inhibitors of biomolecules [5]. We will discuss a number of recent non-conventional and innovative approaches to isolate specific aptamers to target critical determinants in cancer medicine.

2. SELEX technology to generate nucleic acid aptamers

The starting point for the generation of an aptamer is the

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Abbreviations: RBD, Ras-binding domain; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; NES, nuclear export signal

PII: S0014-5793(02)03275-1

synthesis of a nucleic acid library (RNA, DNA or modified RNA) of large sequence complexity followed by the selection for oligonucleotides able to bind with high affinity and specificity to a target molecule, using a simple procedure called 'systematic evolution of ligands by exponential enrichment' (SELEX). Several reviews that describe the SELEX procedure in detail have appeared [6–11]. Randomization of a sequence stretch from 22 up to 100 nucleotides in length has been used to create an enormous diversity of possible sequences which in consequence generate a vast array of different conformations with different binding properties. The SELEX method includes steps of (i) incubating the library with the target molecule under conditions favorable for binding; (ii) partitioning unbound nucleic acids from those bound specifically to the selector molecule; (iii) dissociating the nucleic acid–protein complexes; and (iv) amplifying of the nucleic acids pool enriched for specific ligands. After reiterating these steps (the number of rounds of selection necessary is determined by both the type of library used as well as by the specific enrichment achieved per selection cycle), the resulting oligonucleotides are subjected to DNA sequencing. The sequences corresponding to the initially variable region of the library are screened for conserved sequences and structural elements indicative of potential binding sites and subsequently tested for their ability to bind specifically to the target molecule.

By the SELEX technology, aptamers that bind essentially any protein or molecule can be generated.

2.1. Purified proteins as targets

Binding of aptamers to a specific target molecule is conceptionally more similar to monoclonal antibodies or small molecule compounds rather than to antisense oligonucleotides or ribozymes which are designed to block translation of mRNA into proteins. Due to the fact that the specific, three-dimensional arrangements of a small number of contact points of the aptamer mediate the protein–aptamer interaction, rather than a general affinity for the sugar-phosphate backbone of the nucleic acid, aptamers can achieve high target selectivity. In addition, apart from relying on their intrinsic specificity and affinity properties, the binding characteristics of aptamers can often be influenced by the type of the experimental system used for the selection and counter selection (depletion of aptamers that bind to non-target molecules). The dissociation constants of aptamers for their targets, which extend usually from the micromolar to low picomolar range [8,12,13], are better than those obtained routinely for peptides derived from phage display experiments and can be comparable to antibody–antigen interactions.

For example, RNA aptamers have been generated that are capable of discriminating between isoforms of protein kinase C [14], a potential target in cancer medicine because of its key role in cell survival pathways (Table 1). DNA aptamers have been obtained that recognize both the native and the denatured state of ERK-2, a member of the family of mitogen-activated protein kinases which are central transducers of extracellular signals [15]. RNA ligands with high affinity for the Ras-binding domain (RBD) of Raf-1 have been isolated and shown to inhibit either Ras binding to Raf-1 or Ras-induced Raf-1 activation, but they did not affect the interaction of Ras with B-Raf, a Raf-1-related protein [16] (Tables 1 and 2).

2.2. Intact cells as targets

Aptamers that have high affinity and specificity for cells and tissues have also been produced [17], demonstrating that complex targets, including tumor tissue, are compatible with the SELEX process. Using purified proteins as targets has the advantage of easy control of the conditions to achieve optimal enrichment during the selection process. On the other hand, 'tissue SELEX' methodology could be favorable when the precise molecular target is unknown but the target is, for example, a specific type of cells. Furthermore, the selection of the aptamer in a physiological context may reduce the risk to select aptamers that target protein domains accessible only in the purified protein but inaccessible when the protein is displayed on the cell surface.

Using human red blood cell membranes as model system, DNA aptamers binding to multiple targets have been isolated simultaneously [17]. Furthermore, during the selection procedure the purified protein and the entire cells can also be alter-

Table 1
Reports some examples of aptamers able to inhibit in vitro and/or in vivo the target molecule

Aptamer	Target molecule	Aptamer activity		Diagnostic or therapeutic application	Ref.
		in vitro	in vivo		
PKC-6 PKC-10	protein kinase CβII	inhibition of the enzyme autophosphorylation	nd	nd	[14]
9A	Raf-1 RBD	inhibition of the Ras-induced Raf-1 activation	nd	nd	[16]
NX1838	$VEGF_{165}$	inhibition of VEGF binding to VEGF receptor	inhibition of the VEGF-induced vascular permeability	administration in humans in phase-I clinical trials	[22]
PDGF-B aptamer	PDGF-B	inhibition of PDGF binding to PDGF receptor	reduction of tumor interstitial fluid pressure	administration in tumor-bearing rats	[29,30]
NX21909	neutrophil elastase	inhibition of the enzyme	inhibition of lung injury and neutrophil influx	imaging of inflammation	[24]
Toggle-25	thrombin	inhibition of plasma clot formation and platelet activation	nd	nd	[32]
XAP	anti-NES antibody	inhibition of binding to the NES receptor	inhibition of the Rev-dependent export	nd	[35,36]
AL6-A	angiogenin	inhibition of the ribonucleolytic activity of angiogenin	nd	nd	[38]

Where determined, the diagnostic or therapeutic effect of the aptamer is reported. nd, not determined.

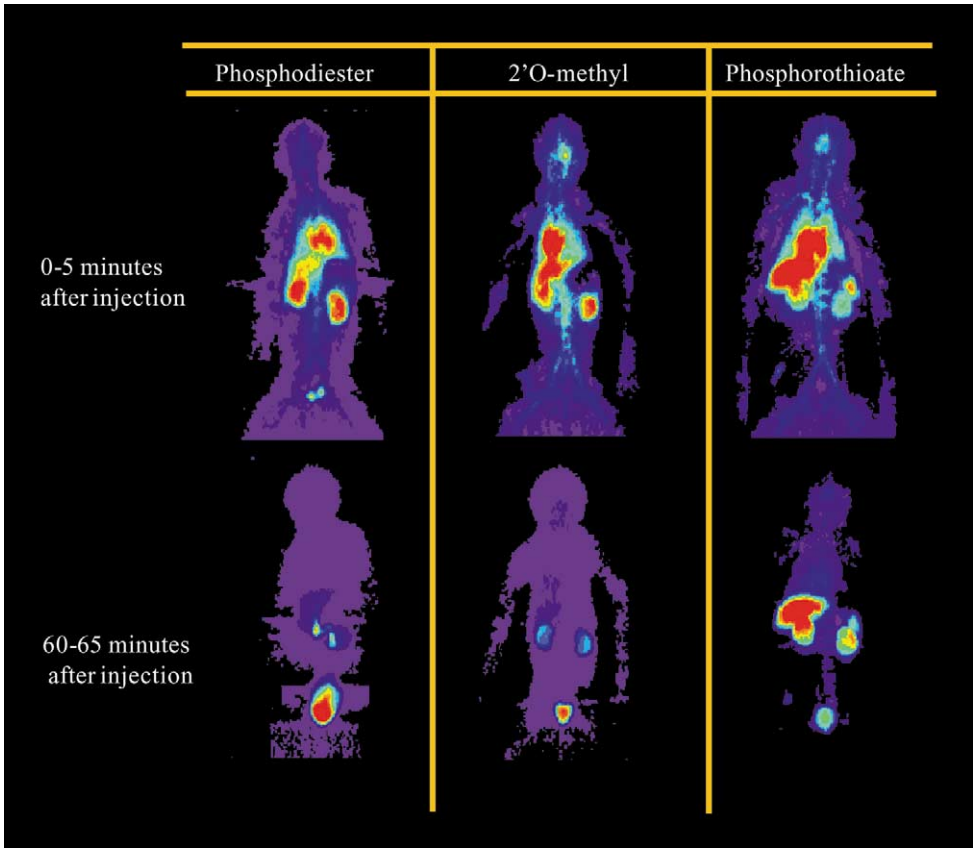

Fig. 1. Direct in vivo evaluation of oligonucleotide's pharmaco-distribution by quantitative whole body imaging. An octadeca-oligonucleotide with an orphan sequence was synthesized in three different sugar-phosphate backbones and 3′-end labelled with ^{18}F. Positron emission tomography was used to visualize in vivo the tissue distribution of the radioactivity following a tracer dose intravenous administration in male adult baboons. Pharmaco-imaging of aptamers is extremely effective for the quantitative evaluation of their pharmacokinetic parameters.

nated as recently described for the isolation of RNA aptamers against tenascin-C, an extracellular matrix protein over-expressed during tissue remodelling processes including tumor growth [18]. As stated in Section 1, tumor-targeting agents also need to differentiate between normal and malignant forms of the same tissue, a degree of specificity that can be obtained with aptamers. A fluorescence-based SELEX procedure was applied against transformed endothelial cells as a complex target to detect microvessels of rat experimental glioma, a fatal brain tumor which is highly vascularized. A secondary selection scheme, named deconvolution SELEX, was carried out to facilitate the isolation of ligands for components of interest within the targeted mixture [19].

3. In vivo applications of the aptamers

To be of practical use in vivo aptamers must possess defined molecular properties, for instance adequate stability in the biological situation in which it will be employed, or sufficient systemic clearance in the case of aptamers used as imaging reagents.

3.1. Stability of the aptamers

One of the limitations in the use of aptamers in animal models of disease and in humans is the reduced stability of natural nucleic acids in biological media. Major efforts have therefore been directed towards improving the stability of aptamers by a variety of approaches. Initially, attention is focused on 'post-SELEX modifications', i.e. the substitution of nucleotides with the corresponding 2′-fluoro, 2′-amino or 2′-*O*-alkyl variants [9,11,20]. However, due to the fact that folding rules for single-stranded oligonucleotide regions change when these modifications are introduced, the binding properties of an aptamer selected in the presence of standard nucleotides might be completely different when the same sequence is synthesized with nucleotides containing a different 2′-substituent [19–21]. To circumvent this limitation selections can be performed directly in the presence of 2′-modified nucleotides, as long as the modified nucleotides are accepted by T7 RNA polymerase for the in vitro reaction steps of the selection [21,22].

An interesting application of the SELEX process is based on the selection of RNA aptamers binding to the mirror-image of an intended target molecule (e.g. an unnatural D-amino acid peptide), followed by the chemical synthesis of the mirror-image of the selected sequence. As a consequence of molecular symmetry, the mirror-image aptamer (made from L-ribose) binds to the natural target molecule. Because of the substitution of the natural D-ribose with L-ribose, the mirror-image aptamer (referred as a Spiegelmer) is totally stable. For example, Spiegelmers that bind to gonadotropin-releasing hormone I, a decapeptide associated with several malignant diseases, have been isolated and characterized [23].

3.2. Systemic clearance of the aptamers

Due to their relatively small size (8–15 kDa) in comparison

to antibodies (150 kDa), aptamers should be better suited for rapid tumor penetration and blood clearance, two excellent characteristics for contrast agents in imaging. A notable example of aptamer plasticity is represented by the use of the DNA aptamer inhibitor of the human neutrophil elastase for in vivo imaging of inflammation [24] (Table 1). The aptamer isolated by 'blended SELEX' (see below) was labelled with Technetium-99m and used for imaging in a mouse model. Remarkably, a better signal-to-noise ratio was achieved by the aptamer compared to the rat anti-elastase antibody. The application of aptamers for in vivo imaging is especially promising due to the very wide range of possibilities available to introduce changes in their structure through defined chemical modifications that will modify their pharmacokinetic properties [25] (Fig. 1). For instance, the clearance rates of aptamers can be altered 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 [26]. There is no experimental evidence so far for aptamers being immunogenic, a very useful property for reagents that need to be administered repeatedly to the same individual for therapy or diagnostic when studying disease progression.

For diagnostic uses an aptamer should recognize and bind to a specific target, for therapeutic uses the aptamer has in addition to be a function-blocking compound and to directly interrupt the disease process. Aptamers have been reported to act as direct antagonists of the biological function of proteins involved in tumor development by competing with the natural ligands for binding to the same protein domains. Vascular endothelial growth factor (VEGF) is a central positive regulator of angiogenesis whose expression is elevated in most solid tumors and in several pathological conditions [27]. Nuclease-resistant RNA aptamers specific for the $VEGF_{165}$ isoform potently inhibit the binding of VEGF to the human receptor in vitro, and one of them (the NX 1838 aptamer) significantly reduces intradermal VEGF-induced vascular permeability in vivo [22]. NX 1838 is currently the first therapeutic aptamer to be administered to humans in phase-I clinical trials (Tables 1 and 2).

The platelet-derived growth factor (PDGF) receptor is expressed in the stroma of a broad range of solid tumors [28]. An antagonistic PDGF-B aptamer coupled to polyethylene glycol was used to specifically inhibit the action of PDGF in a rat model of renal disease [29] and to reduce, by systemic treatment, tumor interstitial fluid pressure in an experimental rat colon carcinoma [30] (Table 1).

4. Non-classical approaches to generate aptamers

Recently, the basic SELEX technology has been modified to improve the direct use of aptamers in medicine. For example, the 'blended SELEX' process allows to confer high-affinity binding to a weak, covalent inhibitor of an enzyme. An RNA library attached covalently to an inhibitory compound was used to select for a secondary, stabilizing contact for the target molecule [24].

Modified DNA aptamers have been generated through a covalent methodology called 'photochemical SELEX' [31] capable of photocross linking to recombinant basic fibroblast growth factor (bFGF), a potent inducer of neovascularization, and to distinguish it from the related VEGF and PDGF (Table 2).

Table 2
Reports some examples of aptamers able to discriminate between the selector target protein (*italic*) and non-target-related proteins

Aptamer	Target	K_d (nM)
9A	*Raf-1 RBD*	152 ± 23
	B-Raf RBD	285 ± 63
06.15	*bFGF*	0.016
	VEGF	16
	PDGF	16
NX 1838	*human* $VEGF_{165}$	0.049
	mouse $VEGF_{164}$	0.090
	human $VEGF_{121}$	$>1\times10^3$

The K_d values of the aptamer–protein complexes are reported.

The alternating use of two related target molecules during the selection (named toggling) creates ligands able to bind to both targets as recently reported for selection of RNA aptamers that bind to both human and porcine thrombin [32] (Table 1).

Finally, it has been shown that RNA molecules can form surfaces that functionally mimic those of proteins [33]. The strategy based on the so-called anti-idiotype approach uses antibodies directed against interfaces of protein–protein interactions to isolate from combinatorial libraries, mimics of one of the interaction domains. This strategy was used to isolate aptamers binding to a neutralizing antibody directed against the insulin receptor [34]. Furthermore, an antibody raised against the HIV-Rev nuclear export signal (NES) has been used to select 'export aptamers' mimicking the NES. These export aptamers bind to the NES receptor in vitro and inhibit Rev-dependent export in vivo [35,36] (Table 1). The mimic selection has now been improved further by combining the anti-idiotype approach with a strategy related to the 'blended SELEX' to isolate aptamers mimicking the substrate of the mitogen- and stress-activated protein kinase MSK1. The obtained 'bi-functional' substrate mimic binds stably to the kinase and inhibits specifically kinase activity in vitro [37].

5. Concluding remarks

The encouraging results obtained with aptamers combined with their intrinsic properties and the versatility of the SELEX procedure should allow the use in diagnostic and even therapeutic applications. The aptamers' ability to discriminate between two closely related targets, i.e. between a cancerous cell and an untransformed cell of the same tissue type, makes these specific and stable ligands exquisitely suited as imaging reagents for non-invasive diagnostic procedures. For therapeutic applications, aptamers might have to be modified, for example by linking them to other molecules, in order to increase their tumor-targeting ability in vivo and, ultimately, to force their action to inhibit the biological function of the target. Furthermore, the intracellular delivery of natural and modified aptamers is presently at a promising, but still preliminary stage.

It is noteworthy that in addition to use aptamers in cancer medicine, in the past few years aptamers have been identified as powerful antagonists of proteins which are associated with a number of other diseases [9]. The therapeutic and diagnostic uses of aptamers will ultimately rely on the identification of

molecular targets that are part of a complex macromolecular machinery that eventually is involved in the disease process.

Acknowledgements: The authors would like to thank their colleagues working within the OLIM (Oligonucleotide Ligand Imaging) Consortium at: CEA/INSERM, Orsay France; NOXXON Pharma, Berlin, Germany; CSIC/University of Barcelona, Spain; CGM/CNRS, Gif sur Yvette, France; University of Torino, Italy; IEOS/CNR, Naples, Italy, under EU contract number QLG1-CT-2000-00562.

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