Specific binding of a hexanucleotide to HIV-1 reverse transcriptase: a novel class of bioactive molecules

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Received May 18, 2006; Revised and Accepted July 10, 2006

ABSTRACT

Short oligonucleotides below 8–10 nt in length adopt relatively simple structures. Accordingly, they represent interesting and so far unexplored lead compounds as molecular tools and, potentially, for drug development as a rational improvement of efficacy seem to be less complex than for other classes of longer oligomeric nucleic acid. As a ‘proof of concept’, we describe the highly specific binding of the hexanucleotide UCGUGU (Hex-S3) to human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) as a model target. Ultraviolet (UV) cross-linking studies and competition experiments with primer/template substrates and a RT-directed aptamer suggest site-specific binding of Hex-S3 to the large subunit (p66) of the viral enzyme. The affinity of 5.3 μM is related to hexanucleotide-specific suppression of HIV-1 replication in human cells by up to three orders of magnitude indicating that Hex-S3 exerts specific and biologically relevant activity. Experimental evidence described here further suggests a systematic hexamer array-based search for new tools for molecular biology and novel lead compounds in nucleic acid-based drug development.

INTRODUCTION

The chemical nature of nucleic acids provides the possibility for a variety of specific, biologically relevant interactions with different classes of biomolecules. Historically, this has been realized by aptamers which usually consist of 40–50 nt or more and which require specific secondary and tertiary folding for full activity. Aptamers have been selected from large sequence spaces to bind almost any kind of chemical entities including small metabolites, such as amino acids, nucleotides or antibiotics, as well as macromolecules, such as proteins or nucleic acids (1–4). Even against complex targets, such as the protozoan parasite Trypanosoma Brucei (5), the human red blood cell ghost (6), or the intact Rous sarcoma virus (RSV) (7) specific aptamers have been successfully selected. In contrast, one may consider that a sufficient number of contacts can be made to provide strong and specific binding of mono- and dinucleotides to nucleoside- and nucleotide-binding proteins. This is particularly true for some chemically modified nucleosides, which comprise an established class of approved drugs for the therapeutic clinical use (8) while oligomeric candidates are still on the way to this point.

As an extension of mono- and dinucleotides it is attractive to speculate about sequence-specific binding of very short heterooligonucleotides to a given target protein. In this case, target specificity might not occur through a combination of shape and sequence-specific interactions but rather contacts along the quasi linear oligonucleotide molecule. In this context, we ask whether specific interactions between very short non-structured oligonucleotides and a protein target do occur, and if so, the binding affinity is strong enough to be biologically relevant. As a model system for addressing this hypothesis we chose hexameric oligonucleotides and the reverse transcriptase (RT) of the human immunodeficiency virus type 1 (HIV-1). Hexanucleotides as opposed to pentanucleotides or heptanucleotides were chosen, because the sequence space of 4^6 (4096) seemed to be most appropriate to look at this concept. HIV-1 RT was chosen based on the following considerations: (i) the 3D structure is known allowing for detailed modeling studies, (ii) binding of high-affinity aptamers shows that, in principle, RT interacts with non-substrate like nucleic acids at sites that are different from the primer/template binding site, (iii) in technical terms, highly pure and active enzyme can be obtained in large amounts (9) and (iv) the availability of appropriate cell based assays for testing potential inhibitors.

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MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides used in this study were purchased from a commercial supplier (IBA, Göttingen, Germany). Their concentration was routinely determined by the ultraviolet (UV) absorption spectroscopy and the individual extinction coefficients. The integrity was controlled applying denaturing PAGE [20% (w/v) acrylamide, 7 M urea] followed by staining with Stains-All (Sigma–Aldrich, Deisenhofen, Germany). Oligonucleotides were 5’ end-labeled with T4 polynucleotide kinase (MBI Fermentas, St Leon-Rot, Germany). Briefly, 10 pmol of oligonucleotide were incubated with polynucleotide kinase and 30 μCi of [γ-32P]ATP (PerkinElmer, Boston, MA) for 30 min at 37°C. Reactions were stopped by heating the samples for 5 min at 95°C. Labeled oligonucleotides were analyzed by using a PhosphorImager after denaturing PAGE [20% (w/v) acrylamide, 7 M urea].

Proteins

Recombinant heterodimeric wild-type HIV-1, HIV-2, equine infectious anemia virus (EIAV) RT and the p51 subunit of HIV-1 RT were expressed in Escherichia coli and purified as described before (9–11). Enzyme concentrations were routinely determined using an extinction coefficient at 280 nm of 260 (HIV-1 RT), 238 (HIV-2 RT), 223 (EIAV RT) and 124 (HIV-1 p51). The purified RTs were free of nuclease contamination. T7 RNA polymerase (12) was expressed in E.coli and purified as described (13,14). Enzyme concentration was routinely determined using an extinction coefficient at 280 nm of 140,000 M⁻¹ cm⁻¹. BSA was purchased from Promega (Mannheim, Germany).

Combinatorial screening of a random pool of hexadeoxyribonucleotides for binding to HIV-1 RT

A random library of hexanucleotides (10 nM) was mixed with HIV-1 RT (10 μM) and incubated at 37°C for 10 min in buffer containing 50 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM MgCl₂ in a final volume of 20 μl. The mixture was soaked through a nitro-cellulose filter BA85 (Schleicher & Schuell, Dassel, Germany) and rinsed with 3 ml of binding buffer. The hexamers retained on the filter were extracted from the nitro-cellulose by heating for 10 min at 95°C in 1 ml of water. The eluted hexanucleotides were subjected to poly(C) extension for the 3’ termini for 10 min at 37°C using terminal deoxynucleotidyl transferase (MBI Fermentas, St Leon-Rot, Germany). Tailed products were then precipitated in ethanol and ligated at their 5’ termini with a primer PA (TGCAGCTCCAGTAAATTAACGTG) by T4-RNA ligase (MBI Fermentas, St Leon-Rot, Germany). Reactions were stopped by heating at 95°C for 5 min. Ligated products were precipitated in ethanol and amplified by PCR in the presence of Taq polymerase (New England Biolabs, Ipswich, MA) and primers PA, PB (TGTATTGCCGCCGCTGATCTAGA) and adaptor-PB (TGTATTGCCGCCGCTGATCTAGA(G)₁₄). PCR products were subsequently cloned in the pCRII-TOPO vector (Invitrogen, Karlsruhe, Germany) and sequenced.

It should be noted that our attempts to use the RNA analogs, i.e. hexaribonucleotides as substrates for terminal deoxynucleotidyl transferase, poly(A)polymerase, and T4-RNA ligase showed extremely little product or failed. We suspect that very short RNAs are poor substrates for those enzymes.

Filter binding assay

Typically, radiolabeled oligonucleotides (1 nM) and target proteins (5–25 μM) were incubated in 30 μl of binding buffer [50 mM Tris–HCl (pH 8.0), 5 mM KCl, 5 mM MgCl₂, and 1 mM DTT] for 2 min at 25°C. An aliquot (10 μl) of this mixture was filtered under suction through a prewet nitrocellulose filter BA85 (Schleicher & Schuell, Dassel, Germany) and rinsed with 3 ml of binding buffer. Radioactivity retained on the filters was measured by scintillation counting (PerkinElmer, Boston, MA).

UV cross-linking

An amount of 250 pmol of HIV-1 RT and of 100 fmol 32P-labeled 4-thio U-modified Hex-S₃R (position 4) were pre-incubated in binding buffer (see above) for 2 min at 25°C. Samples were then transferred on a piece of Parafilm, placed on ice and irradiated for 20 min at 366 nm using a hand-held fluotest lamp (Heraeus, Hanau, Germany), followed by heating for 5 min at 95°C in 1× Laemmli loading buffer and resolved by 12% SDS–PAGE. Gels were stained with Coomassie blue, dried and exposed to a PhosphorImager screen.

CNBr cleavage of HIV-1 RT tagged with 32P-labeled 4-thio U-modified Hex-S₃R

HIV-1 RT cross-linked to 32P-labeled 4-thio U-modified Hex-S₃R was precipitated by 20% (w/v) trichloroacetic acid (TCA), followed by a washing with acetone. The pellet was then air-dried and dissolved in 100 μl formic acid (70%) containing 3.5 g/l cyanobromide (CNBr). Cleavage reaction was performed at room temperature for 24 h. Formic acid and CNBr were removed by centrifugal-lyophilization in a speed-vac. The pellet was dissolved in 50 mM Tris–HCl (pH 8.0) buffer, heated 5 min at 95°C in 1× Laemmli loading buffer and resolved by SDS–PAGE [10–20% (w/v) linear gradient]. Gels were stained with Coomassie blue, dried and exposed to a PhosphorImager screen.

Modeling and docking studies

Rigid-body docking studies of HIV-1 RT (Brookhaven Protein Data Bank accession nos: 1HMV, 1RTH, 1RTD and 1HVU) and Hex-S₃ were performed with the program Hex 4.2 (15). Molecular graphics images were produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (16).

Virus replication assay

Hexamers were co-transfected by calcium phosphate co-precipitation with pGJ3-Luci and pczVSV-G in 1 × 10⁶ 293T cells as described in detail recently (17). In brief,
60 min thereafter medium was exchanged and the cells were incubated for 40 h followed by filtration (0.45 μm). The cleared supernatant (0.2–0.3 ng of p24 core antigen per ml) was used to infect a number of 2 × 10^4 293 cells in a 96-well format for 24 h in the presence of polybrene (8 μg/ml). Cell viability was monitored by the conversion of fluorescein diacetate (FDA) (18) and the expression of luciferase was measured in a Fluoroskan Ascent FL (Thermo Labsystems, Dreieich, Germany) after addition of 50 μl of the following buffer: 28 mM Tricine (pH 7.8), 500 μM ATP, 250 μM coenzyme A, 250 μM luciferin, 33 mM DTT, 200 mM EDTA, 15 mM MgSO_4, 1.5% (v/v) Triton X-100 and 5% (v/v) glycerol. All reagents were purchased from Sigma–Aldrich (Deisenhofen, Germany).

RESULTS AND DISCUSSION

Binding of hexanucleotides to HIV-1 RT

First, we performed a combinatorial selection approach in solution at physiological ionic strength and temperature to search for DNA hexanucleotides binding to RT. Sequence analysis of a number of 35 cloned hexamers revealed seven different species with divergent nucleotide sequences (data not shown) which is consistent with the view that at the experimental conditions and stringency of selection used here a number of divergent hexanucleotides can bind at similar strength. The best-binding species in this screening procedure included Hex-S1 (CGACCG) and Hex-S3 (TCGTGT) (A. Mescalchin and W. Wünsche, unpublished data). To further analyze their interaction with RT a filter binding assay was used (19). As positive controls we included a well-characterized high-affinity RNA aptamer (19–21), a DNA aptamer (22) and a DNA/DNA primer/template substrate (23). As negative controls we included hexanucleotides (Hex-0, Hex-1) chosen from the group of non-binders. Since we used DNA as well as RNA backbones as controls in this study, we further decided to investigate Hex-S1, Hex-S3 and Hex-S4 and their hexameric controls as RNA (lower script ‘R’) and DNA (lower script ‘D’) in filter binding assays (Figure 1A). Here we find no binding of the negative controls Hex-0 and Hex-1 but measurable binding of all selected hexamers. The RT-directed DNA aptamer was set 100 arbitrary units binding in Figure 1A. Remarkably, all RNA and DNA versions of the same sequence show comparable binding to RT (Figure 1A). Since Hex-S3 shows strongest binding, we performed a more detailed analysis with this species. Its binding affinity to RT was reflected by an apparent K_d of 5.3 (±0.5) μM (Figure 1B). By stepwise replacing the original nucleotides of the adenosine-free Hex-S3_R for an adenosine, important contributions of the central positions 3–5 opposed to the terminal nucleotides at positions 1, 2 and 6 could be identified (Figure 1C). This clearly indicates that binding affinity and specificity require bases in the central portion of the molecule but not necessarily at the termini. In order to test whether this is also true for the hexameric backbone, i.e. simply a phosphodiester/sugar backbone, we measured binding of the two 5’- and 3’-shortened pentameric derivatives of Hex-S3_R. Under the experimental conditions used here we did not observe any significant binding of both Hex-S3_R-derived pentameric and shortened versions thereof (CGTGT and TCGTG, Supplementary Figure 1a). From those observations we conclude that a hexameric backbone and the stretch GTG are necessary for efficient binding of Hex-S3 to RT.

Binding of derivatives of Hex-S3 to RT

Next we analyzed the effect of chemical modifications of Hex-S3_R on binding to RT. The similar binding strength of the DNA and the RNA derivatives Hex-S3 (Figure 1A) suggests that the 2’-OH does not substantially contribute to binding. Further binding studies with several derivatives of Hex-S3 revealed that the 5’-phosphate is not important for binding whereas fully 2’-Ometh derivatives and a LNA derivative at position 1 show decreased binding affinity (Table 1). Phosphorothioate derivatives of Hex-S1, Hex-S3, Hex-0 and Hex-1 all show increased binding to RT accompanied by a dramatic loss of sequence-specificity (Table 1). Moreover, a 4-thio U at position 4 of Hex-S3 used later for UV cross-linking studies had no effect on binding (Table 1).

It is noteworthy that all possible eight derivatives of Hex-S3 elongated by 1 nt at either terminus show an ~3-fold stronger binding to RT while still being specific for the target, i.e. for HIV-1 RT versus HIV-2 RT (Supplementary Figure 1b). However, extension to a 12mer or 18mer is accompanied, besides further increase in affinity, by a progressively increased loss of specificity (Supplementary Figure 1c). This observation is consistent with the view that the binding mode of Hex-S3, i.e. binding to a specific pocket on the protein surface, changes at increasing chain length in such a way that it resembles natural substrates of this polymerase.

Site-specific binding of Hex-S3 to RT

A specific interaction of Hex-S3_R with HIV-1 RT, as implied by the findings given above, would suggest a defined binding site on the protein. In order to examine this possibility we performed UV cross-linking with a 32P-radiolabeled 4-thio-uridine derivative of Hex-S3_R followed by CNBr treatment of RT. This experiment clearly shows that only the large subunit p66 is cross-linked (Figure 1D) and of the fragments obtained, specifically two are labeled. The two fragments were isolated from the gel and their N-terminal amino acid sequence was determined. One of them (42–183 amino acid, indicated by green color in Figure 1E) carries approximately one-third and the second one (231–356 amino acid, indicated by red color in Figure 1E) about two-thirds of the label (Figure 1D). Further information on the binding site of Hex-S3_R comes from competition experiments with a 18/36mer DNA/DNA primer/template (23), a RNA aptamer (19,20), as well as the non-nucleoside inhibitor S-TIBO (24). TIBO has been shown to stabilize an open conformation of RT, i.e. rotation of the p66 thumb domain of ~30–40° generating a large nucleic acid binding site (25,26), whereas the RNA aptamer induces a closed conformation (20,26). These competition studies are surprising in the sense that primer/template and aptamer compete for hexamer binding but not vice versa. S-TIBO increased hexanucleotide binding by ~2-fold (data not shown). Taking these experimentally derived constraints into consideration, we performed rigid-body docking studies of HIV-1 RT and Hex-S3 (Figure 1E, Supplementary Figure 2). According to
this speculative model, Hex-S3 binds the p66 subunit at the bottom of the thumb domain pointing towards the nucleic acid binding cleft of the viral polymerase. Binding of this hexanucleotide ligand requires the thumb domain to be in an upright position. Furthermore, the interaction of RT with natural primer/template substrates is not hampered, whereas access of Hex-S3 to its binding site is sterically blocked in the presence of substrate. This would explain the results of
Table 1. Percentage of binding of derivatives of hexanucleotides to HIV-1 or HIV-2 RTs at 1 μM protein concentration

<table>
<thead>
<tr>
<th>Name</th>
<th>HIV-1 RT</th>
<th>HIV-2 RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hex-000</td>
<td>0.3 ± 0.0</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-100</td>
<td>0.4 ± 0.1</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-S100</td>
<td>5.8 ± 0.6</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-S300</td>
<td>8.1 ± 1.0</td>
<td>nd</td>
</tr>
<tr>
<td>4-thioU-Hex-S3R</td>
<td>9.8 ± 0.5</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-000 -2'OMe</td>
<td>2.0 ± 0.0</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-100 -2'OMe</td>
<td>0.9 ± 0.2</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-S100 -2'OMe</td>
<td>0.5 ± 0.1</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-S300 -2'OMe</td>
<td>2.0 ± 0.1</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-S300 -LNA</td>
<td>4.3 ± 0.4</td>
<td>nd</td>
</tr>
<tr>
<td>Hex-000 -PS</td>
<td>19.1 ± 2.1</td>
<td>10.6 ± 1.9</td>
</tr>
<tr>
<td>Hex-100 -PS</td>
<td>20.8 ± 2.9</td>
<td>12.9 ± 0.7</td>
</tr>
<tr>
<td>Hex-S100 -PS</td>
<td>18.3 ± 6.0</td>
<td>6.6 ± 1.7</td>
</tr>
<tr>
<td>Hex-S300 -PS</td>
<td>52.8 ± 9.8</td>
<td>24.9 ± 3.6</td>
</tr>
</tbody>
</table>

Abbreviations: -2'OMe, 2'-O-methyl, fully modified; -LNA, locked nucleic acid, 1 nt modified at 5'-terminus; -PS, phosphorothioate, fully modified; nd, not determined. Indicated are mean value ± standard deviation.

The competition experiments described above (Supplementary Figure 2).

Protein target specificity of Hex-S3

So far, we showed that hexanucleotide binding to RT is highly specific for the nucleotide sequence. Next we investigated whether Hex-S3 specifically binds to HIV-1 RT as a target. For this matter, filter binding assays were performed with (i) the small subunit p51 of HIV-1 RT, (ii) HIV-2 RT, which is highly homologous to the HIV-1 RT (60% amino acid identity plus 13% amino acid homology), (iii) EIAV RT, (iv) T7 RNA polymerase and (v) BSA (Figure 1F). The weak or absent binding of Hex-S3 to all of those control proteins suggests a high degree of target specificity which is particularly striking when considering the close structural and functional relationship among the polymerases tested here, particularly the RT of HIV-2 (27).

Biological activity of Hex-S3R in a cell culture model for HIV-1 replication

Regarding the development of potential hexanucleotide-derived drugs, we analyzed whether Hex-S3 is able to exert biological effects, i.e. to negatively interfere with the replication of HIV-1 in human cells by using a recombinant proviral construct as described in detail elsewhere (17). In this setup, packaging cells are transfected with a luciferase-harboring defective HIV-1 genome and plasmid-encoded packaging functions which give rise to the release of infectious recombinant particles that are subsequently used to infect recipient cells in which luciferase gene expression monitors infectivity. In this system, we co-transfected hexanucleotides in the first step and observed a strong, concentration-dependent decrease of luciferase activity in newly infected 293T cells using Hex-S3 but not in case of the control Hex-1 (Figure 2). Regarding an assessment of the particular step of the viral life cycle that could be affected by Hex-S3, we performed a number of additional experiments. In summary we find the following: (i) if one co-transfects hexamers together with packaging functions of HIV-1 using the single-round replication systems described, then clearly less viral particles, as monitored by a p24-specific enzyme-linked immunosorbent assay (ELISA), are released from transfected cells when Hex-S3 is used versus a control hexamer (Hex-1). In spite of this, when equal amounts of these viruses (produced in the presence of Hex-S3 or a control hexamer) are subsequently applied to infect 293T cells, there is no difference between the observed reporter gene activity. This strongly suggests that the Hex-S3-specific inhibitory step occurs in packaging cells rather than during early steps of infection. In depth in vitro biochemical analyses of the polymerase activity of RT in the presence of either hexamers does not indicate a difference that could explain the observed dose-dependent inhibition of HIV-1 in cell culture. Those analyses included the following characteristics of RT: fidelity, processivity, RNase H activity and DNA- and RNA-dependent DNA polymerase activity (data not shown). All of the experimental data, however, are compatible with the view that the packaging efficiency of HIV-1 in the presence of Hex-S3 is substantially suppressed in a concentration-dependent fashion. This view has been described more in detail recently for the inhibition of HIV-1 by a RT-directed RNA aptamer (21). Several scenarios are feasible. To address but a few it is quite possible, that a compound, which does not interfere with the enzymatic activities of the viral polymerase blocks assembly of infectious particles simply by physical interaction with RT. There are mutations of RT known supporting this view (28,29). Alternatively, selective packaging of the tRNA primer could be affected (30).

Since the viral target is RT in this study, we investigated the well-established nucleoside inhibitor AZT and the non-nucleoside inhibitor S-TIBO in the same cell system. Both suppress RT by different modes of action, however, they show IC<sub>50</sub> values here (AZT, 79 nM; S-TIBO, 101 nM; Supplementary Figure 3) that are somewhat lower when compared with IC<sub>50</sub> values in infectious test systems. If one
considers Hex-S3 as a ‘lead’ compound then the gap between the effective drugs AZT and S-TIBO and Hex-S3 [IC$_{50}$ = 1.8 (±0.2 µM), data not shown] is surprisingly narrow.

**Stability of hexanucleotides versus Hex-S3-derived 24mers**

Further, we tested the serum stability of Hex-S3 and of chemically modified versions including the phosphorothioate-, LNA- and 2’-OMe derivatives and compared it with oligonucleotides of common length of 24 nt (data not shown). All chemically modified hexamers behave as one would derive from well-known data of longer ones. It is noteworthy, however, that the unmodified hexamers show an increased nuclease resistance when compared with 24mers, which is particularly true for the RNA version (Supplementary Figure 4).

**Binding of RT to hexamers covalently attached to a trial array**

To test whether an array-based approach is feasible we studied the differential binding of HIV-1 RT to hexamer species (for sequences see Figure 1A), which have been covalently attached via linkers to a solid surface. In order to monitor binding of RT we used a site-directed fluorescently labeled RT (p66/p51$^{K287C}$Alexa$^{488}$) which was shown earlier to pre-binding of RT we used a site-directed fluorescently labeled (for sequences see Figure 1A), which have been covalently attached to a trial array.

Figure 3. Binding of RT to hexamers and controls covalently attached to a trial array. Oligonucleotides were printed such that they were positioned at the terminus of a 40 atoms tether covalently bound to the matrix. The density was ~1 fmol of oligonucleotide per spot (top panel). The arrays were incubated with fluorescently labeled RT, washed and fluorescence intensity was measured with a fluorescence imager. A quantification of representative experimental data is shown in the bottom panel and the corresponding $K_d$ values in solution are shown on top of the bars. The nucleotide sequence of the DNA 45mer is d(T20CTGTACAGGTAGCAATGGCAGGTGC).

CONCLUSION

Taken together, this work strongly suggests that hexanucleotides may be considered as an as yet unexplored class of compounds with a number of highly promising characteristics for drug development. Based on the results shown for Hex-S3, we propose hexanucleotides in general may provide high specificity and sufficient binding affinity for a given target and, thus, represent attractive lead compounds. It is noteworthy that binding of a protein target by short oligonucleotides was described for the integrase (32,33) and the envelope protein gp120 of the HIV-1. In case of gp120 this was related to inhibition of gp120-mediated cell fusion (34). However, binding affinity in those cases was not due to specific primary nucleotide sequences but rather to the presence of an acridine moiety (33), a 6-oxo-cytosine residue, or the ability of phosphorothioate-modified oligonucleotides to form higher quadruplex structures (34).

Several strategies can be envisaged to further increase the binding affinity and, hence, the biological activity of Hex-S3. First, rational drug design in the classical sense could be based on docking studies with the known 3D structure of HIV-1 RT. Second, saturation transfer difference (STD) NMR (35) could be exploited to determine the critical residues for binding of Hex-S3 to RT, which in turn can be chemically modified potentially leading to increased binding strength. Third, systematic pools of unmodified or chemically modified hexanucleotides can be tested for specific binding to RT or any other target of interest. This may exploit the array-based technology, briefly described above. This enables one, in principle, to perform an extremely rapid and systematic search for hexamers that bind to a given target sufficiently specific and tight, thereby representing a first lead.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR online.
ACKNOWLEDGEMENTS

The authors thank Axel Rethwilm for the self-inactivating HI-Virus vector system, Thomas Peters for helpful discussions, Paul Rothwell for fluorescence-labeled RT and Patrick Zeltz (BF Biolabs, Freiburg, Germany) for excellent advice in the use of arrays. This work was supported by the ESF, the Possehl-Stiftung (Lübeck) and EC-grant LSHG-CT-2003–503480. Funding to pay the Open Access publication charges for this article was provided by the K-DDTM, Lübeck, Germany.

Conflict of interest statement. None declared.

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