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Synthesis and Antiviral Evaluation of Nucleic Acid-Based (NAB^{TM}) Libraries

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Abstract—Combinatorial assembly of nucleotide libraries and their antiviral evaluation against HSV-1 are described.

The discovery of safe and effective antiviral drugs presents a formidable challenge compared to bacterial and parasitic agents.[1] Indeed, very few virus-specific molecular targets have been identified that can be specifically subjected to antiviral intervention, because viral metabolic processes closely resemble host cellular processes. Nevertheless, three virus-encoded enzymes have been the targets of most 'small molecule-type' antiviral drugs—polymerases, proteases, and most recently, neuraminidase. However, the rapid emergence of resistance to antiviral drugs is a major problem and unwieldy 'cocktail regimens' often need to be employed as a desperate measure. Clearly, there exists a substantial unmet clinical need for antiviral drugs with different structures and unique mechanisms of action.[2]

Historically, antiviral drugs have been designed using both mechanism, and structure-based drug design approaches. The combinatorial methodology is emerging as a powerful contemporary drug discovery tool,[3] and consists of two steps in which: (a) biological methods are used to select and validate molecular targets, and (b) structure- and mechanism-guided drug design approaches are used, in which a library of compounds are synthesized and evaluated for their ability to interfere with the biosynthesis, structure and/or function of the target. When insufficient structure and/or function of a target is available, combinatorial methods such as "diversity oriented organic synthesis for therapeutic target validation"[4] or alternatively "combinatorial target-guided ligand assembly" have been employed.[5] An approach that seems appropriate for antiviral drug discovery, is the use of structurally diverse compounds to modulate biological pathway without regard to specific molecular target. This allows simultaneous functional validation of a target, as well as, the discovery of a lead structure that modulates the function of the target. We describe here the application of this concept for antiviral drug discovery.

Our strategy was to design a library of small molecules that mimic the repertoire of interactions that exist *amongst* nucleic acids, and proteins, as well as, that which exist *between* proteins and nucleic acids. Indeed, a number of proteins contain nucleotide-binding domains defined by the topology of protein α-helices and β-sheets. As is well known, the exquisite specificity of such interactions reside in topology-associated molecular recognition defined by local and global conformations of the ligand and receptor, and a network of hydrogen bonding, hydrophobic, ionic, and van der Waals interactions that facilitate the binding interactions.[6] Quite clearly, a nucleic acid-based (NAB^{TM}) scaffold is a logical template into which such diversity attributes could be engineered to potentially target 'hot spots' in protein–protein and protein-nucleic acid interaction surfaces.

There are many novel diversity features associated with libraries built around a NAB^{TM} scaffold: (a) the scaffold can be used to create variable spatial display of hydrogen-bonding, hydrophobic, charge-transfer, electrostatic and such other noncovalent interactions; (b) the scaffold can be conformationally rigid or flexible and by linking the individual scaffolds together, one can fashion diverse molecular topology into library members; indeed, such shape-defining motifs as circles, pseudoknots, bulges and stem loops can be incorporated into libraries to target 'hot spots' on the receptor; (c) from

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a synthetic perspective, the NAB^{TM} libraries can be assembled using well established solid-phase or solution-phase methods in nucleic acids field.[7]

Our ongoing work[8] has many objectives: (a) to design and synthesize focused NAB^{TM} libraries with different diversity attributes, (b) to evaluate the antiviral activity of NAB^{TM} libraries, (c) to identify the pharmacophore and SAR pattern that facilitate 'hit to lead' evolution, (d) to evaluate whether NAB^{TM} libraries can help rapidly identify novel molecular targets for antiviral intervention, and (e) in a broader context, explore whether NAB^{TM} diversity is biologically relevant.

A typical small molecule-type NAB^{TM} library is shown in Figure 1, and represents multiple elements of diversity. It is pertinent to mention that, and although not shown here, several diversity elements can be linked in a variety of ways to generate a virtual library of millions of compounds that would occupy a significant chemical space.[9]

To have a library amenable to parallel synthesis, and help validate our stated goals, we prepared representatives that possess some of the key diversity attributes enunciated above. Our libraries **1**–**5** consist of di-, tri- and tetranucleotides that carry modifications at the backbone, sugar, and nucleobase (Fig. 1). Key elements that contribute to diversity deserve mention: (a) the two backbone modifications, phosphorothioates and phosphoramidates provide desirable metabolic stability to compounds when used in cell-based assays; the former also could potentially participate in electrostatic interactions, while the latter could facilitate hydrophobic and hydrogen-bonding interactions with the target receptor, (b) the dominant furanose modification was the substitution of a 2′-OMe group in place of a 2′-hydrogen in the deoxyribofuranoside ring; as is well known, the 2′-substituent can act as a 'conformational switch',[6] that transforms the furanose ring pucker from the 2′-*endo* to 3′-*endo* thereby affecting the global conformation of the individual library members, (c) the nucleobase modifications included both the replacement of the parent heterocyclic moiety, as well as, substitution on the heterocycle that provided additional hydrophobicity to the library members, and (d) all linkages were 3′ to 5′. Clearly, a repertoire of diversity attributes could be captured in a representative NAB^{TM} library with a molecular weight range of 400 to 1200.

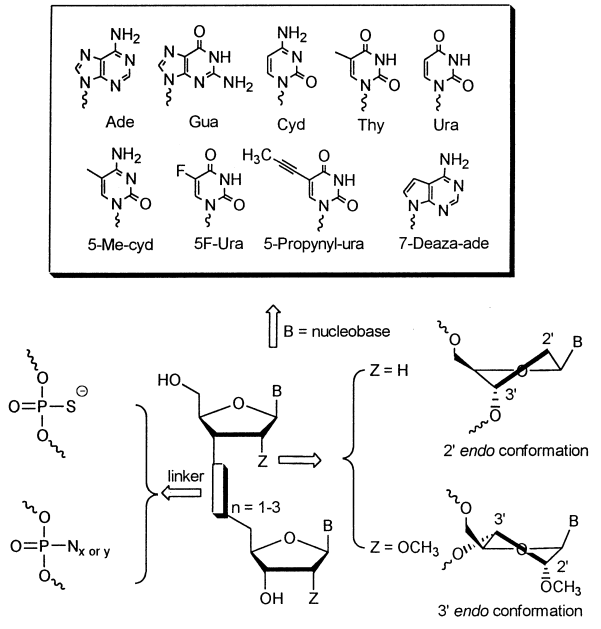


Figure 1. General structure of a simple NAB^{TM} library depicting diversity elements.

It is pertinent to mention that in the past, selected dinucleotides and modified oligonucleotides have been prepared in most cases as mixtures.[10] However, a systematic diversity-directed synthesis of NAB^{TM} libraries as individual compounds and their antiviral evaluation have not been carried out. We report here the synthesis and antiviral evaluation of NAB^{TM} libraries against herpes simplex virus (HSV-1) using cell-based assays.

Synthesis of Libraries

Library 1

A 64-member dinucleoside phosphorothioate library 1 (Table 1) was assembled on solid support using commercially available deoxy-ribonucleoside and 2′-OMe ribonucleoside phosphoramidite building blocks in conjunction with the corresponding controlled-pore-glass (CPG)-linked nucleosides.[11] The assembly was carried out in a parallel synthesis mode (DMT-off, 10 to 15 μmol scale). Following the assembly, each solid support was treated with aqueous ammonium hydroxide (28%, 55 °C) to remove nucleobase-, and phosphate-protecting groups and to cleave the products off the support. The products were purified and evaluated as described in the experimental.

Library 2

Using eight of the commercially available monomers and nucleoside-bound-CPGs, potentially 512 trinucleotide phosphorothioates could be assembled as a mixture of R_p, S_p diastereomers. We prepared a representative 64-member library (Table 2) using phosphoramidite chemistry. Following work up and extraction, the products were obtained 85 to 95% pure as determined by reversed-phase HPLC.

Library 3

The parallel assembly of tetranucleotides posed a special challenge because a 4096-member tetranucleotide library (representing an 8×8×8×8 array) could be assembled. In order to have a library amenable to parallel synthesis, three nucleotide positions were fixed with the fourth position being degenerate. Thus, we first assembled each of the 64 trinucleotides on CPG by parallel synthesis, and each CPG-bound trinucleotide was reacted with an equimolar mixture of dA, dC, dG, and T nucleoside phosphoramidites (Scheme 1). In this way, 256-member library was assembled (Table 3).

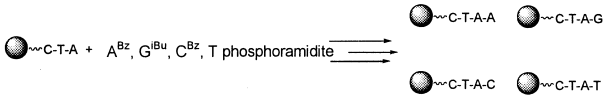
Table 1. Dinucleoside phosphorothioate library

3′AA	3′AC	3′AG	3′AT	3′A<u>A</u>	3′A<u>C</u>	3′A<u>G</u>	3′A<u>U</u>
3′CA	3′CC	3′CG	3′CT	3′C<u>A</u>	3′C<u>C</u>	3′C<u>G</u>	3′C<u>U</u>
3′GA	3′GC	3′GG	3′GT	3′G<u>A</u>	3′G<u>C</u>	3′G<u>G</u>	3′G<u>U</u>
3′TA	3′TC	3′TG	3′TT	3′T<u>A</u>	3′T<u>C</u>	3′T<u>G</u>	3′T<u>U</u>
3′<u>A</u>A	3′<u>A</u>C	3′<u>A</u>G	3′<u>A</u>T	3′<u>A</u><u>A</u>	3′<u>A</u><u>C</u>	3′<u>A</u><u>G</u>	3′<u>A</u><u>U</u>
3′<u>C</u>A	3′<u>C</u>C	3′<u>C</u>G	3′<u>C</u>T	3′<u>C</u><u>A</u>	3′<u>C</u><u>C</u>	3′<u>C</u><u>G</u>	3′<u>C</u><u>U</u>
3′<u>G</u>A	3′<u>G</u>C	3′<u>G</u>G	3′<u>G</u>T	3′<u>G</u><u>A</u>	3′<u>G</u><u>C</u>	3′<u>G</u><u>G</u>	3′<u>G</u><u>U</u>
3′<u>U</u>A	3′<u>U</u>C	3′<u>U</u>G	3′<u>U</u>T	3′<u>U</u><u>A</u>	3′<u>U</u><u>C</u>	3′<u>U</u><u>G</u>	3′<u>U</u><u>U</u>

A, C, G, and T correspond to deoxyribonucleosides, whereas <u>A</u>, <u>C</u>, <u>G</u>, and <u>U</u> correspond to 2′-OMe-ribonucleosides. All internucleotidic linkages are phosphorothioates.

Table 2. Trinucleoside phosphorothioate library

3′AAA	3′ACA	3′AGA	3′ATA	3′AAC	3′ACC	3′AGC	3′ATC
3′CAA	3′CCA	3′CGA	3′CTA	3′CAC	3′CCC	3′CGC	3′CTC
3′GAA	3′GCA	3′GGA	3′GTA	3′GAC	3′GCC	3′GGC	3′GTC
3′TAA	3′TCA	3′TGA	3′TTA	3′TAC	3′TCC	3′TGC	3′TTC
3′AAG	3′ACG	3′AGG	3′ATG	3′AAT	3′ACT	3′AGT	3′ATT
3′CAG	3′CCG	3′CGG	3′CTG	3′CAT	3′CCT	3′CGT	3′CTT
3′GAG	3′GCG	3′GGG	3′GTG	3′GAT	3′GCT	3′GGT	3′CTT
3′TAG	3′TCG	3′TGG	3′TTG	3′TAT	3′TCT	3′TGT	3′TTT



Scheme 1.

Library 4

A representative base-modified library of 24 members were assembled as di-, tri-, tetra-nucleoside phosphorothioates (Table 4) using the corresponding commercially available phosphoramidite monomers.

Library 5

In the phosphoramidate dinucleotide library (Table 5), an additional diversity element was incorporated at the backbone. A 192-member dinucleoside phosphoramidate library was assembled by parallel synthesis using H-phosphonate chemistry[12] in conjunction with a series of amines. The requisite dinucleoside H-phosphonates (5′-DMT-off) were assembled on CPG using the corresponding commercially available H-phosphonates and the CPG-bound nucleosides in conjunction with 1-adamantanecarbonyl chloride as the activator. The CPG-bond nucleoside H-phosphonates were converted to phosphoramidates by reported procedure.[13]

Antiviral Evaluation

The libraries **1–5** were evaluated in antiviral assays against HSV-1. A number of compounds, induced 30 to 60% inhibition of HSV-1-induced plaques at a dose of 25 μM when compared with acyclovir (EC_{50} of 6 μM). Structure–activity relationship studies towards exploration of lead compounds, through virtual library mapping and focused library preparation, are being planned. Full details will be reported in due course.

In conclusion, we have prepared and evaluated different classes of NAB^{TM} libraries and demonstrate that they represent biologically relevant chemical diversity for drug discovery.

Assembly of the libraries 1–4

The library synthesis (5 to 15 μmol scale) was performed using standard automated DNA synthesis protocols (DMT-off). Oxidative sulfurization was effected using 3*H*-1,2-benzodithiole-3-one-1,1-dioxide.[14] After synthesis, the CPG was dried using N_2, and transferred to 5-mL centrifuge tubes. Aqueous ammonium hydroxide (28%, 4 mL) was added and the mixture was heated at 55 °C for 3–6 h. The resulting suspension was cooled and centrifuged. The solution was evaporated in a Speed Vac. Each resulting product was dissolved in water (3 to 5 mL), and extracted with ethyl acetate (2×1 mL). The aqueous layer was evaporated to remove

Table 3. Tetranucleoside phosphorothioate tetranucleotide library

3′AAA9	3′ACA9	3′AGA9	3′ATA9	3′AAC9	3′ACC9	3′AGC9	3′ATC9
3′CAA9	3′CCA9	3′CGA9	3′CTA9	3′CAC9	3′CCC9	3′CGC9	3′CTC9
3′GAA9	3′GCA9	3′GGA9	3′GTA9	3′GAC9	3′GCC9	3′GGC9	3′GTC9
3′TAA9	3′TCA9	3′TGA9	3′TTA9	3′TAC9	3′TCC9	3′TGC9	3′TTC9
3′AAG	3′ACG9	3′AGG9	3′ATG9	3′AAT9	3′ACT9	3′AGT9	3′ATT9
3′CAG9	3′CCG9	3′CGG9	3′CTG9	3′CAT9	3′CCT9	3′CGT9	3′CTT9
3′GAG9	3′GCG9	3′GGG9	3′GTG9	3′GAT9	3′GCT9	3′GGT9	3′GTT9
3′TAG9	3′TCG9	3′TGG9	3′TTG9	3′TAT9	3′TCT9	3′TGT9	3′TTT9

9: represent a mixture of dA, dC, dG, and T.

Table 4. Base modified phosphorothioate library

3′AC^{-5Me}	3′CC^{-5Me}	3′GC^{-5Me}	3′TC^{-5Me}	3′CTGC^{-5Me}	3′CTGC^{-5Me}
3′AA$^{-7Deaza}$	3′AA$^{-7Deaza}$	3′CA$^{-7Deaza}$	3′GA$^{-7Deaza}$	3′TA$^{-7Deaza}$	3′AA$^{-7Deaza}$G
3′AU^{-5F}	3′CU^{-5F}	3′GU^{-5F}	3′TU^{-5F}	3′CU^{-5F}G	3′CTGU^{-5F}
3′AU^{-P}	3′CU^{-P}	3′GU^{-P}	3′TU^{-P}	3′CU^{-P}G	3′CTGU^{-P}

C$^{-5Me,}$ 5-Methyl-dC; A$^{-7Deaza}$, 7-Deaza-dA; U^{-5F}, 5F-dU; U^{-P}, 5-propyne-dU. A, C, G, and T are deoxyribonucleosides; A, C, G, and U represent 2′-OMe-ribonucleosides.

Table 5. Dinucleoside library

3′AA-Nx	3′AC-Nx	3′AG-Nx	3′AT-Nx	3′AA-Ny	3′AC-Ny	3′AG-Ny	3′AT-Ny
3′CA-Nx	3′CC-Nx	3′CG-Nx	3′CT-Nx	3′CA-Ny	3′CC-Ny	3′CG-Ny	3′CT-Ny
3′GA-Nx	3′GC-Nx	3′GG-Nx	3′GT-Nx	3′GA-Ny	3′GC-Ny	3′GG-Ny	3′GT-Ny
3′TA-Nx	3′TC-Nx	3′TG-Nx	3′TT-Nx	3′UA-Ny	3′UC-Ny	3′UG-Ny	3′UT-Ny

Nx corresponds to N_1, N_2, N_3, N_4, N_5, N_6; and Ny corresponds to N_5, N_6; A, C, G, T correspond to deoxyribonucleosides, A, C, G, U correspond to 2′-OMe-rA, rC, rG, rU nucleosides. All internucleotidic linkages are phosphoramidates.

traces of ethyl acetate and the residue taken up in ultra pure water and filtered through 0.2 μm filter. Lyophilization gave the products as a white foam.

Assembly of the library 5

The requisite dinucleotide H-phosphonates were assembled on solid support using standard H-phosphonate chemistry. Following washing, the dry CPG was transferred to 5 mL centrifuge tubes and a solution of amine in CCl_4 (10%, 3–4 mL).[13] The mixture was shaken for 20 to 30 min. Following washing, the CPG was treated with 28% aqueous NH_4OH (55 °C, 3–6 h). The suspension was cooled and centrifuged. The solution was evaporated to dryness in vacuo, the residue dissolved in H_2O (5 mL), and extracted with ethyl acetate (2 mL). The aqueous layer was evaporated, residue taken up in ultra pure water and filtered.

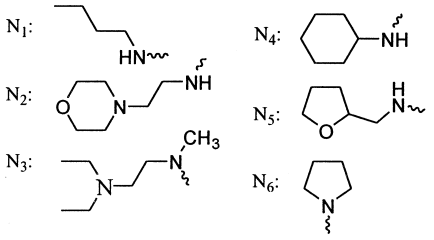
Analysis of the library

Reversed-phase HPLC analysis of the libraries was performed on a Waters 600 system equipped with a photodiode-array UV detector 996, autosampler 717, and Millennium® 2000 software, using a Radial-Pak® liquid chromatography cartridge [8 mm I.D., 8NVC18]. Mobile phase: Buffer A: 0.1 M NH_4OAc; Buffer B: 20% A/80% CH_3CN, v/v: Gradient: 100% A, 0–3 min; 40% A, 40 min; 100% B, 49 min. Product purity ranged from 85 to 95%. Yields were estimated to be 65 to 90% on the basis of A_{260} units.

Spectral characterization of representative library members

Before spectral acquisition, the representative library members (10% of the library size) were further purified by ion-exchange column (DEAE-5PW Resin, Buffer A: H_2O, Buffer B: 0.5 M NaCl) followed by desalting (C18 column, Buffer A: H_2O, Buffer B: 20% CH_3CN in H_2O) to give individual library members of 95–99% purity as determined by reversed-phase HPLC.

^{31}P NMR analysis of selected library members revealed clear signals at δ 58–59 ppm, and 13–14 ppm, characteristic



of phosphorothioate and phosphoramidate linkages respectively. Other peaks in the ^{31}P NMR spectra constituted less than 3% of the total area corresponding to the desired product peaks. Additionally, the ES-MS of selected library members were consistent with the expected molecular weights corresponding to the assigned structures.

Antiviral assays against HSV-1:

Vero cells (African green monkey kidney cells) (ATCC) were infected with HSV-1 at an MOI of 0.005 in 96-well plates in Dubelco's modified Eagle's (DMEM) medium. The plates were maintained at 37 °C for 3 h. The compounds were added at 25 μM concentration, following which the plates were incubated at 37 °C for 24 h. The media was removed, and the cells were fixed with 10% formal saline for 10 min. The cells were stained with 0.1% crystal violet, incubated at room temperature for 30 min, and then washed with distilled water. The viral plaques were counted, and the antiviral effect estimated as a percent reduction in the number of plaques compared with untreated control. Acyclovir was used as the positive control (EC_{90}, 6 μM).

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