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Solid-phase synthesis and screening of N-acylated polyamine (NAPA) combinatorial libraries for protein binding

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ABSTRACT

Inhibitors for protein–protein interactions are challenging to design, in part due to the unique and complex architectures of each protein's interaction domain. Most approaches to develop inhibitors for these interactions rely on rational design, which requires prior structural knowledge of the target and its ligands. In the absence of structural information, a combinatorial approach may be the best alternative to finding inhibitors of a protein–protein interaction. Current chemical libraries, however, consist mostly of molecules designed to inhibit enzymes. In this manuscript, we report the synthesis and screening of a library based on an N-acylated polyamine (NAPA) scaffold that we designed to have specific molecular features necessary to inhibit protein–protein interactions. Screens of the library identified a member with favorable binding properties to the HIV viral protein R (Vpr), a regulatory protein from HIV, that is involved in numerous interactions with other proteins critical for viral replication.

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The development of synthetic molecules to inhibit protein–protein interactions has made great progress in the past few years, but to date there are only inhibitors for a handful of targets in this area.[1] Most approaches to identify inhibitors of these interactions rely on rational design based upon a three-dimensional structure of the target complex.[2,3] In cases where a structure of the complex is available, there is a range of peptide mimetic scaffolds that may be used to begin targeting the interface of a protein–protein interaction.[4–6] The best inhibitors of such interactions tend to be significantly larger molecules than the average small-molecule drugs; this difference likely reflects the increased binding area involved in most protein–protein interactions as compared with the area of enzyme active sites where small molecules bind.[7] In many cases, however, there is no convenient three-dimensional structure of the complex to use as a baseline for inhibitor design. Therefore, random screening of a chemical library may be the best approach to find inhibitors of a structurally uncharacterized protein–protein interaction. While there are numerous types of chemical libraries available for screening, most consist of classes of molecules designed to bind to the active sites of enzymes.[8] Since the molecular architecture of protein–protein interactions is different from enzyme active sites, new libraries must be developed that more closely represent the types of molecular architectures associated with inhibitors of protein–protein interactions.

Recently, we introduced a scaffold we have termed N-acylated polyamines (NAPAs, Fig. 1) as a new type of molecular architecture to develop inhibitors for protein–protein interactions.[9] As illustrated in Figure 1, our general strategy is to project sidechains from the tertiary amide (R^2, R^4, R^6) and the carbon backbone (R^1, R^3, R^5) to achieve a densely functionalized array of sidechains. In our original report, we optimized the sidechains projecting from the NAPA scaffold to inhibit the protein–protein interaction between HDM and p53, and inhibitors with IC_{50} values around 2 μM were identified.[9] We report in this manuscript the methods to make a combinatorial library of these molecules and the associated procedures to screen for hits that bind to biotin-labeled proteins. These methods successfully identified a lead NAPA that binds to viral protein R (Vpr), a regulatory protein important in the life cycle of HIV-1, that is known to engage in multiple interactions with other proteins.[10] The procedures we report can be extended to make larger NAPA libraries for screening.

Although we previously developed a solid-phase synthesis of NAPAs on Rink-amide resin,[9] the original synthetic route had to be adapted to allow for efficient synthesis of a library in 96-well

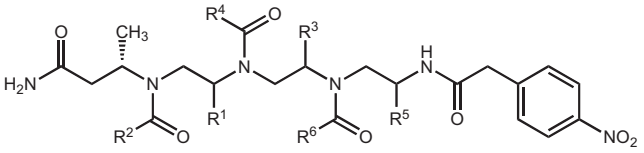

Figure 1. General NAPA structure.

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microtiter plates in a parallel format such that on-resin screening of the library could be performed. Therefore, the NAPA library was synthesized on Tentagel-NH_2 macrobeads, which swell in both water and organic solvents and are resistant to acidic cleavage. Following synthesis of the NAPA on the resin, deprotection of the sidechains under acidic conditions resulted in a Tentagel-bound NAPA that was ready for screening under aqueous conditions. The larger resin size of the macrobeads further facilitated direct visualization of library hits by a fluorescent microscope as well as allowing physical manipulation of small numbers of beads.

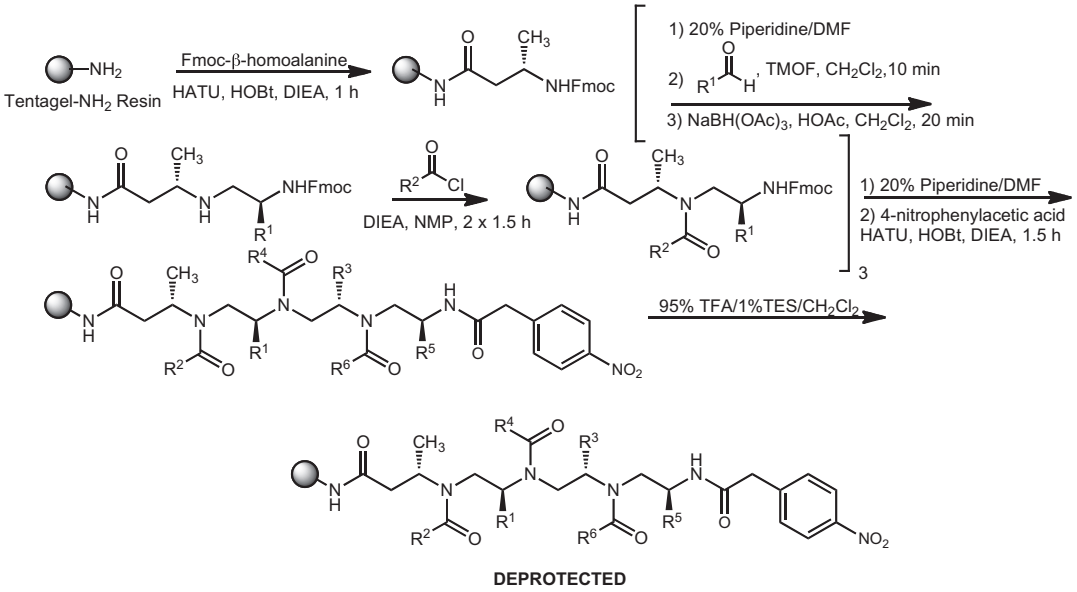
Following our previously described procedures,[9] NAPAs were synthesized using a series of reductive alkylations, acylations, and Fmoc deprotections on Tentagel resin (Scheme 1). In contrast to the previous procedure, which used β-alanine as the first residue, a β-homoalanine was used to reduce over-alkylation for the first reductive amination in the NAPA synthesis (Supplementary data). Aldehydes used for reductive amination were made via lithium aluminum hydride reduction of the Weinreb amides of Fmoc-amino acids.[11] For the reductive amination step, imine formation between a primary amine on the resin and the Fmoc-amino aldehyde was promoted by excess trimethylorthoformate (TMOF),[12] and the intermediate imine was immediately reduced with a solution of $NaBH(OAc)_3$ in acetic acid.[13] Success of the reductive amination was determined by a positive chloranil test, which confirms the presence of a secondary amine on the Tentagel resin.[14]

The resulting secondary amine was then acylated with the desired acid chloride in *N*-methylpyrrolidone (NMP) and an excess of diisopropylethylamine (DIEA). Completion of this reaction was monitored by a negative result for the same chloranil amine test. The final amine was capped with 4-nitrophenylacetic acid, which aided in HPLC visualization when the hits from the screens of the library were resynthesized. All these steps were performed in parallel using a 96-well microtiter plate.

We designed a NAPA library that was trimeric in amino acid composition, as we felt that this represented a reasonable size for inhibitors of protein–protein interactions. Within this type of NAPA library, there are a total of six positions that may be randomized: three positions for amino acid incorporation and an additional three positions for acylation. One advantage of the NAPA library is the rapid diversity that may be achieved at short oligomer lengths; for example, in the current trimeric library, the number of molecules in the library equals the number of different amino acids used to make the library raised to the third power times the number of different acylating groups also raised to the third power. Monomers used to build the library were selected to maximize diversity, and included a selection of hydrophilic, hydrophobic, charged, and aromatic sidechains.

We began with smaller test libraries to ensure the feasibility of the synthesis and screening. In developing these libraries, we identified two specific problems: (1) sterically hindered acyl chlorides (such as isobutyryl chloride) were not very reactive with the secondary amines in the acylation steps and, (2) while histidine could be incorporated into the library, it resulted in false positives in the screens for protein binding. Therefore, the main library was constructed from the four amino acids and two acid chlorides listed in Table 1, resulting in a library of 512 $[(4)^3 \times (2)^3]$ different NAPA molecules. To identify different NAPAs within the library, we used a one-letter amino acid code for the amino acid-derived sidechains (K, Y, J, or F_f) in addition to a numbered code for the acid chlorides (2 or 3). For example, a NAPA consisting of three tyrosine residues and three hydrocinnamoyl residues would be referred to as Y2Y2Y2.

To screen for protein binding after completion of the library synthesis, we modified a procedure originally reported by Kodadek and coworkers[15] that relies on the use of a streptavidin-coated quantum dot (Qdot 605) to signal the presence of a NAPA that interacts with a biotinylated protein target (Fig. 2). The use of quantum dots was necessary since TentaGel resin exhibits autofluorescence over a broad range of wavelengths that makes visualization of standard fluorescent dyes difficult. Fluorescence emission of quantum dots occurs at wavelengths that do not overlap with TentaGel's autofluorescence.[16] The first step of the screening protocol involved manually transferring a few beads from each well of the 96-well microtiter plates that were used for synthesis to a 384-well microtiter filter plate that would be used for screening. After transfer, the NAPA-containing Tentagel resin was swelled in water, then washed with an appropriate buffer, and then a biotinylated protein target was added. After thorough washing to remove any unbound protein, a solution of streptavidin-coated quantum dots (Qdot 605) was added, followed by additional washes. The plate was then examined using a fluorescent micro-



Scheme 1.

Table 1
Amino aldehydes and acid chlorides used to make the NAPA library

Aldehyde or acid chloride	Label
Tyrosine aldehyde	Y
Lysine aldehyde	K
Norvaline aldehyde	J
4-Fluorophenylalanine aldehyde	F_f
Hydrocinnamoyl chloride	2
Isovaleryl chloride	3

scope equipped with a triple bandpass filter to determine if protein binding to members of the NAPA library had occurred. In this scenario, beads that appeared bright red were deemed to have a NAPA bound to the protein target while the beads that appeared green were considered to have a NAPA that was not able to bind the protein target. Therefore, the NAPAs on the bright red beads were deemed preliminary 'hits' and resynthesized for validation and further binding studies.

A protein derived from HIV (viral protein R, or Vpr) was selected for screening against this NAPA library. Vpr is a 96 amino acid protein (14 kDa) consisting of three α helices connected by two loops.[17] There are approximately 200 copies of Vpr per virion, and it has a variety of functions, including the regulation of the transcription of the HIV-1 long terminal repeat, nuclear translocation of the HIV-1 preintegration complex, and induction of cell cycle arrest.[10] Investigations into the roles of Vpr in the replication cycle of HIV are ongoing, and molecules that selectively bind to this protein could be useful in such studies.[18] Currently, there are only a few small molecules that inhibit Vpr activity in cell-based assays.[19,20] For this study, synthetic Vpr containing a single biotin group at the N terminus was purchased from Dr. Ulrich Schubert and Dr. Peter Heinklein (University of Erlangen-Nürnberg, Germany).

Initially, screens were performed using 175 nM protein and incubating it with the NAPA beads for 2 h at 4 °C. Surprisingly, a large number of the wells had very bright red beads while all the control wells were green. The brightest wells from this initial screen, 80 in all, were re-plated into another 384-well plate and re-screened with 75 nM protein for 30 min at room temperature. This time there were about 10-fold fewer hits and the controls were again green. The two hits with the highest fluorescence intensity were chosen, K2K2Y2 and K2K3Y2, and resynthesized. The resynthesis followed similar procedures, except that Rink-amide resin was used as the solid support and the acid chlorides were coupled to the secondary amine in dichloromethane instead of NMP (acylation proceeds more quickly in dichloromethane, but it is not used for this step in the library synthesis due to chemical incompatibility with the filter plate). K2K2Y2 was synthesized cleanly with one major HPLC peak, whereas K2K3Y2 produced a large amount of a by-product, which, by mass, consisted of the desired molecule plus an additional isovaleryl group. Therefore, K2K2Y2 was selected for additional studies (Fig. 3).

To verify that K2K2Y2 did indeed bind to Vpr, we performed experiments using isothermal titration calorimetry (ITC). Using a Vpr concentration of 1000 μM, a pronounced binding curve was observed as the NAPA was titrated into the protein solution. From these data, the binding of K2K2Y2 was found to have a K_d of 25 μM, indicating modest binding of the NAPA to Vpr (Fig. 3). Similar screens were conducted with two other biotinylated proteins, MDMX[21] and FKBP52.[22] For both proteins, hits from the library were identified (K3K2K2 for MDMX, and Y3K2J3 for FKBP52) and resynthesized, but unfortunately neither NAPA showed any activity in binding assays to these proteins.

In conclusion, we have developed the procedures to make and screen a NAPA library for new protein-binding ligands. The scaffold can be used to generate very diverse libraries with short oligomer lengths. From our initial studies on the NAPA library, we were able

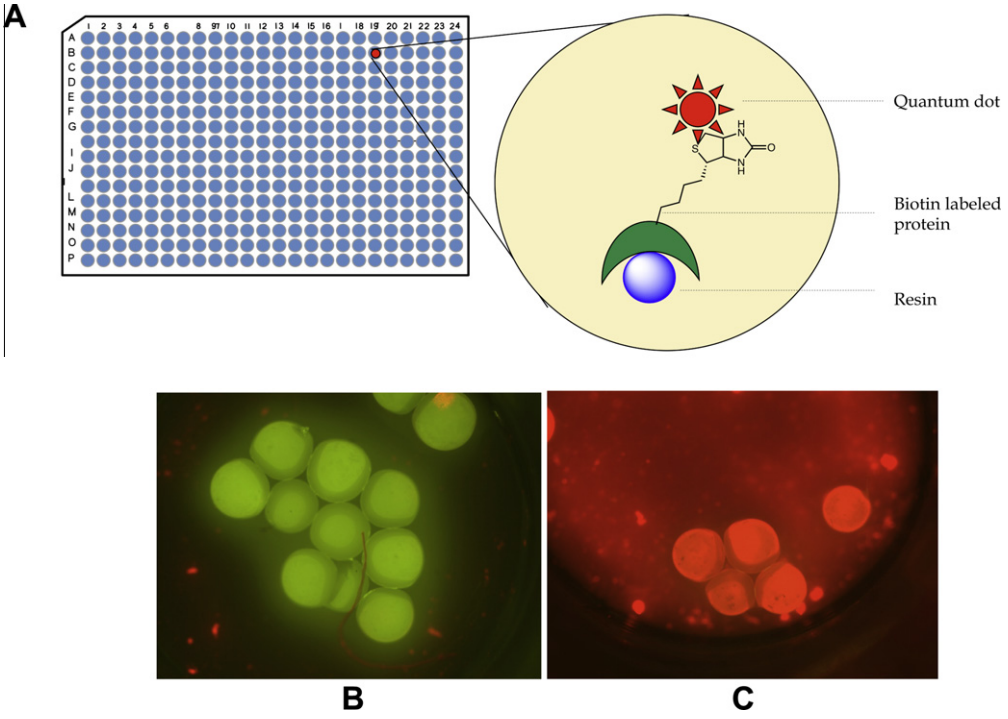


Figure 2. (A) Schematic for screening of NAPA library. Red-colored beads derive from retention of the streptavidin-labeled quantum dot and is therefore indicative of binding of the NAPA to the protein target. (B) Green bead color under a fluorescent microscope indicating no retention of streptavidin-labeled Qdot 605 and therefore no NAPA binding to the protein target. (C) Red bead color observed under a fluorescent microscope indicating a NAPA-protein-binding interaction.

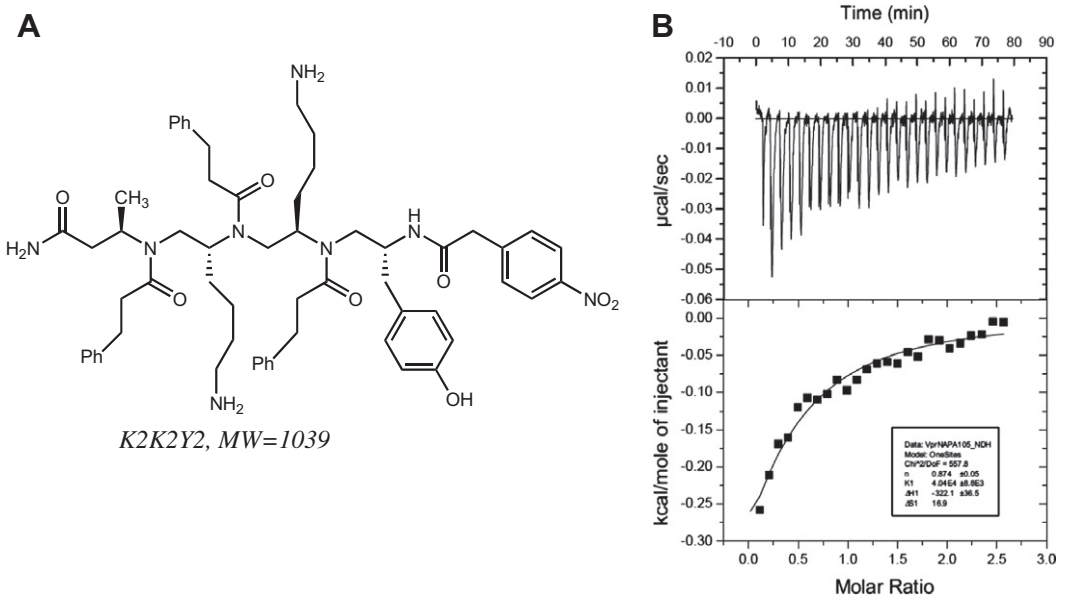


Figure 3. (A) Chemical structure of K2K2Y2. (B) Isothermal titration data (ITC) of K2K2Y2 binding to Vpr.

to identify a lead candidate, K2K2Y2, that binds to the protein Vpr. Due to the variety of complex roles this protein plays in the HIV life cycle, there are a large number of protein–protein interactions involving Vpr and, at the present time, there is no readily available in vitro assay to test whether K2K2Y2 directly inhibits a protein–protein interaction involving Vpr. Confirmatory tests of the biological activity will be the subjects of future collaborative research. While we were not successful at finding NAPA binders for other protein targets, we believe this could be an indication that our library lacks promiscuity in its interactions with proteins. The manual aspects for synthesis and screening of the previous NAPA library preclude expanding this technique to larger libraries at this time. In future studies, we plan to automate the synthesis and screening process so that larger NAPA libraries may be examined across a range of protein targets.

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Supplementary data

Supplementary data (synthetic procedures and data for all assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.054.

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