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Assessment of Solution-Phase Positional Scanning Libraries Based on Distamycin A for the Discovery of New DNA Binding Agents

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Abstract—The solution-phase synthesis of two 1000-membered positional scanning libraries of distamycin A analogues is described enlisting acid/base liquid–liquid extractions for isolation and purification of all intermediates and final products. The results of their screening for functional activity (L1210 cytotoxic potency) and DNA binding affinity were compared with those derived from libraries containing the same compound members but prepared in a smaller 10-compound mixture format. The positional scanning libraries, which are substantially less demanding to prepare, allowed the accurate detection of the global observations and the clearly more potent activities, but more subtle discoveries and less distinguishable activities were not detected. This is a natural consequence of testing the larger 100-compound mixtures and the relative insensitivity of the assays to the contribution of any single, uniquely acting compound in the mixture. Thus, the disadvantages associated with the loss of some information contained within the library must be balanced against the advantages of the ease of library synthesis and judged in light of the library screening objectives.

Introduction

Few small molecule libraries have been disclosed that are designed to identify new DNA binding agents.[1] In conjunction with efforts on the rational design of minor-groove DNA binding agents,[2,3] we have been engaged in the complementary combinatorial synthesis of libraries based on distamycin A[4] capable of identifying sequence selective minor-groove DNA binding agents.[5] In these efforts, we introduced high-throughput screening technology capable of establishing relative binding affinities and absolute binding constants of single compounds, or libraries of compounds for hairpin oligo-nucleotides containing a defined sequence, or a full set of hairpin oligonucleotides constituting a comprehensive library of sequences.[6] For a single compound, this constitutes the complete definition of its DNA binding sequence selectivity in a single convenient experiment. For a defined sequence, this permits the identification of high-affinity binding agents.[6] Together, this screening of libraries of compounds against libraries of sequences permits the rapid determination of the complete DNA binding sequence selectivity of each member of a library. This screening protocol was introduced utilizing the solution-phase parallel synthesis of two prototypical libraries of distamycin A analogues produced in a format of two sets of 132 mixtures of 10 compounds (2640 compounds).[6] Herein, we report the preparation of two comparable positional scanning libraries[7,8] composed of 1000 members each and the results of their comparison examination.

Unlike solid-phase synthesis where polymer bound substrate is the stoichiometry limiting reaction partner, either the substrate or the reacting attachment group may be limiting in solution-phase chemistry. This dictates the use of mix and split synthesis for the solid phase in order to accommodate differential reaction rates, whereas the simpler protocol of mixture synthesis with limiting reagent stoichiometry may be used in solution to ensure all library members are generated.[9] The implementation of the latter only requires the ability to remove unreacted starting substrate. Although this is not possible with solid-phase synthesis, this can be accomplished by aqueous acid/base extractions[10] in the work we describe, which also serves to remove reactants, reagents, and reagent by-products providing clean products.

The synthesis of positional scanning libraries represents one of the most useful protocols for mixture synthesis. Not only is it much less time intensive than the parallel

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synthesis of individual compounds or small mixtures, but it also produces depository libraries for use in multiple screens with immediate deconvolution.[7,11–14] Thus, unlike other deconvolution protocols,[11–14] positional scanning libraries provide lead identities in a single round of testing. Despite these attributes, it was not clear how well such libraries would perform in screening for DNA binding agents relative to other formats.[15]

Library Design and Synthesis

In order to insure that the quality of information derived from the library assessment could be established, two 1000-member libraries were prepared that contain the same compounds assembled in our prior study.[6] Each positional scanning library consists of 30 sublibraries that can be divided into three sets. These sets differ in the fixed positions of a monomer subunit within the tripeptide (Fig. 1). Thus, the library was prepared by substitution of the same 10 subunits for each of the three 4-aminopyrrole-2-carboxylic acid subunits of distamycin A (Fig. 2). Included in this set was the authentic 4-aminopyrrole-2-carboxylic acid subunit of distamycin A, insuring that the natural product analogue was also among the library members. The C-terminus of the library compounds was capped as methyl or ethyl esters and the N-terminus was acylated with 4-dimethylaminobutyric acid (DMABA), a basic side chain that mimics the distamycin A amidine, providing analogues that bear functionalization and a substitution pattern established to provide DNA affinities comparable to that of the natural product.[6]

The synthesis of the library was divided into four parts (Scheme 1). First, a mixture of 100 dimers was synthesized on a 144 μmol scale by coupling the set of 10 amino acid esters **1a**–**10a** with the corresponding set of 10 BOC-amino acids **1b**–**10b** using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) and dimethylaminopyridine (DMAP) as an additive. For the preparation of sublibraries I, where the first position within the trimer is fixed with a single **A** residue, 10 portions of the dimer mixture were deprotected with HCl/EtOAc and coupled to 10 individual BOC-amino acids providing 10 sublibraries each containing a different and single **A** residue. The set of 10 sublibraries II was assembled by coupling 10 individual BOC-amino acids to a mixture of amino acid esters. Subsequent deprotection of the BOC-group and coupling to a mixture of BOC-amino acids yielded the set of 10 trimer sublibraries each containing a single and different **B** residue. Finally, the dimer mixture of 100 compounds was saponified

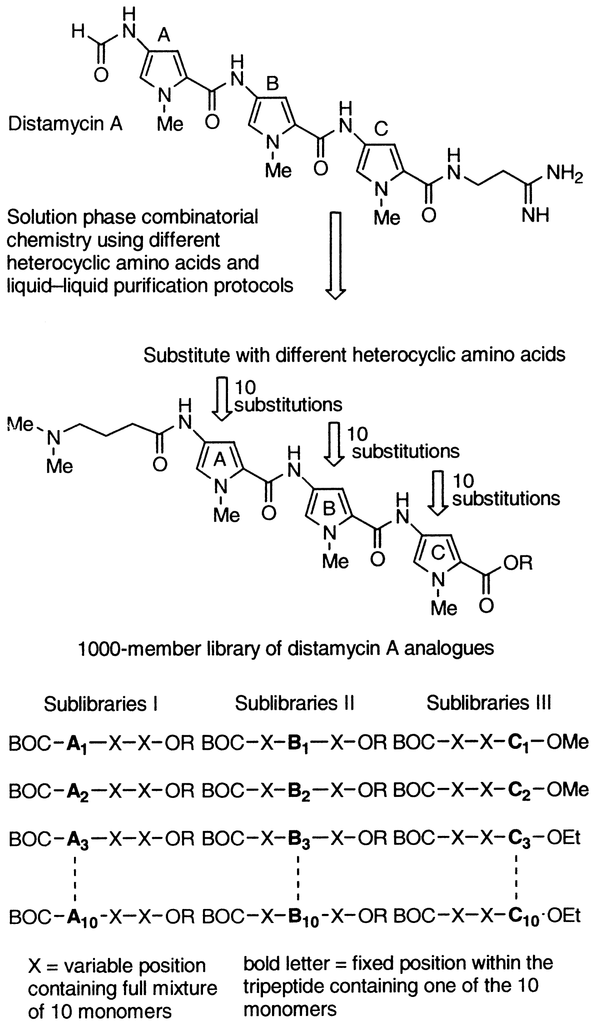


Figure 1. Design of the positional scanning library.

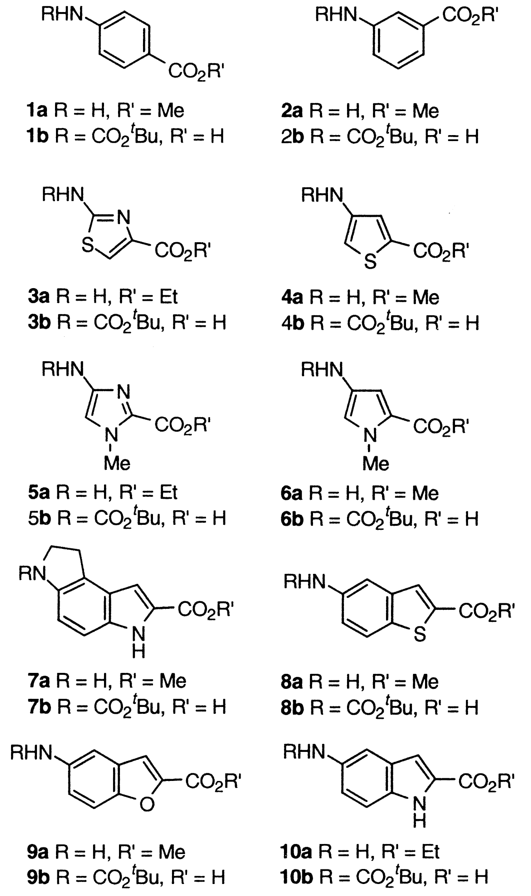
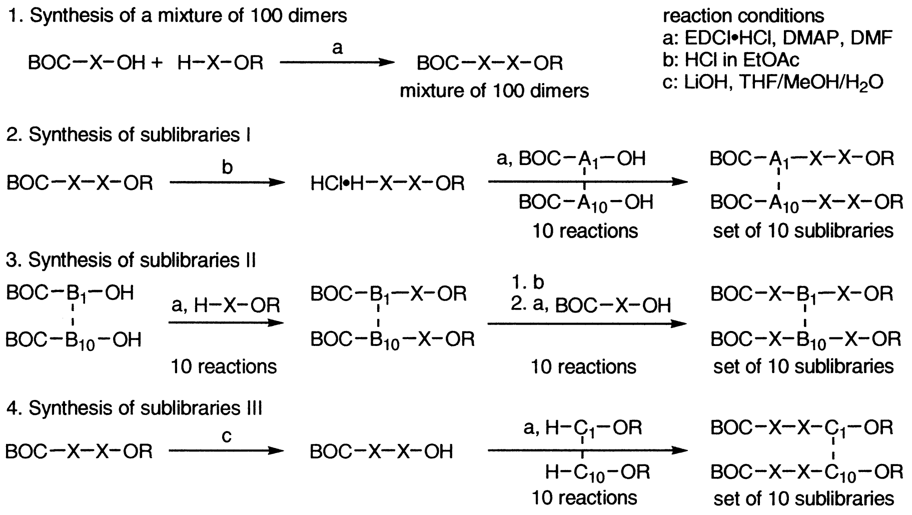


Figure 2. Amino acid monomer units used in the preparation of libraries.



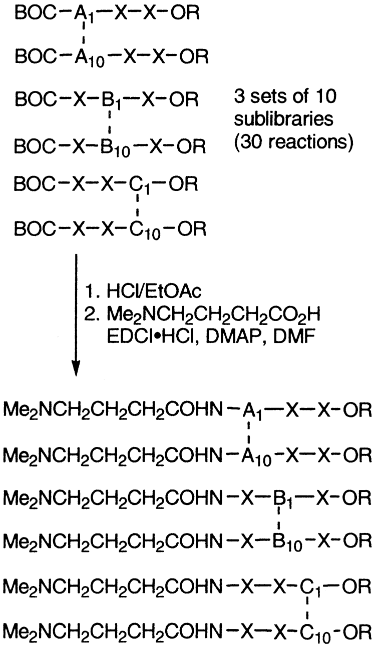
Scheme 1. Synthesis of the BOC-trimer positional scanning libraries.

with LiOH, divided into 10 portions, and coupled with 10 individual amino acid esters (**C** residue) to give the third set of sublibraries III. The entire library containing 1000 compounds was synthesized conducting 43 reactions.

The 30 positional scanning sublibraries were also converted into their corresponding dimethylaminobutyric acid (DMABA) derivatives as shown in Scheme 2. Yields of the BOC- and DMABA-trimer libraries are given in Table 1.

Cytotoxic Activity

Evaluation of the original library in a cellular functional assay for L1210 cytotoxic activity revealed two structurally-related BOC-trimers, BOC-A_9-B_9-C_5-OEt (**11**, 29 nM) and BOC-A_8-B_9-C_5-OEt (**12**, 68 nM), which exhibited uniquely potent activity.[6] Both were identified in a single deconvolution of a potent sublibrary of 10 compounds, which exhibited activity 10-fold more potent than any other sublibrary and > 100 times more potent than 90% of the mixtures. While it cannot be excluded that additional unidentified members of the library exhibit comparable activity, the uniquely potent activity of the sublibrary containing **11** and **12** and the dependable performance of the small mixture testing reflecting the composite activity of the components suggest that **11** and **12** are at least 10 times more potent than any other library member. The testing of the 30 positional scanning libraries (Fig. 3 and Table 2) also revealed the identity of **11** and **12** (Fig. 4), but required the preparation of more candidate structures in the deconvolution of the activity, 16 compounds in total. Notably, both **11** and **12** are 1000 times more potent than distamycin A.



Scheme 2. Conversion of BOC-trimer into DMABA-trimer libraries.

The most potent residues identified in the scanning library were A_9, B_9 and B_1, and C_{10}, C_2, C_6 and C_5. Importantly, the combination of the most potent residues, BOC-A_9-B_9-C_{10}-OEt, was not a compound that exhibited potent cytotoxic activity. Moreover, none of the alternative 14 possible combinations exhibited cytotoxic activity that approached that of **11** and **12**. Only **13**

Table 1. Yields of BOC- and DMABA-trimer library synthesis

	Yield (%) BOC-trimers			Yield (%) DMABA-trimers		
Subunit number	A_{1-10}	B_{1-10}	C_{1-10}	A_{1-10}	B_{1-10}	C_{1-10}
1	77	77	45	54	12	42
2	72	72	71	32	54	54
3	68	68	40	43	22	47
4	77	77	70	50	33	57
5	92	92	51	39	29	64
6	79	79	73	13	62	76
7	99	99	81	26	41	65
8	76	76	58	53	45	46
9	71	71	58	55	39	65
10	69	69	64	49	38	79

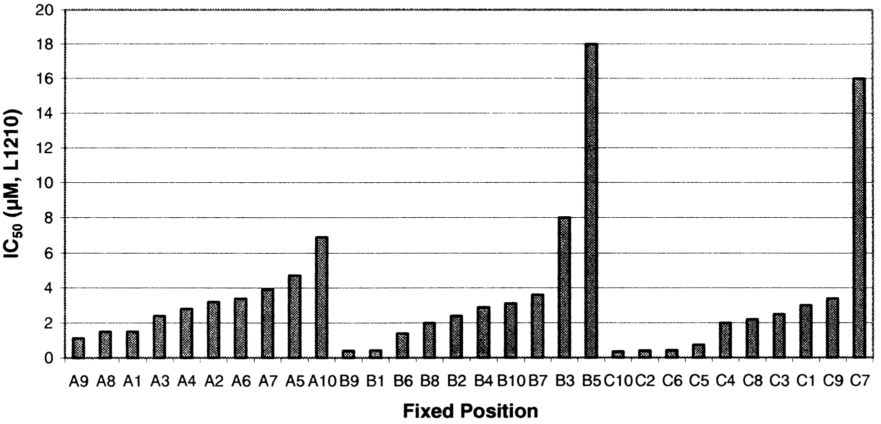


Figure 3. Cytotoxicity (L1210) for BOC-trimer scanning libraries. Smaller numbers indicate more potent cytotoxic activity.

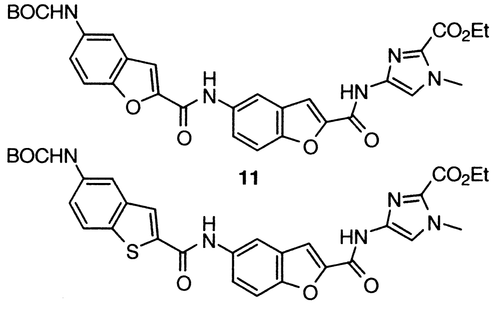
Table 2. Cytotoxic activity of candidate compounds

Compound	IC$_{50}$ (μM, L1210)
BOC-A$_9$-B$_9$-C$_{10}$-OEt	1.4
BOC-A$_9$-B$_9$-C$_2$-OMe	>100
BOC-A$_9$-B$_9$-C$_6$-OMe	1.8
BOC-A$_9$-B$_9$-C$_5$-OEt (**11**)	0.029
BOC-A$_9$-B$_1$-C$_{10}$-OEt	2.7
BOC-A$_9$-B$_1$-C$_2$-OMe	>100
BOC-A$_9$-B$_1$-C$_6$-OMe	>100
BOC-A$_9$-B$_1$-C$_5$-OEt (**13**)	0.42
BOC-A$_8$-B$_9$-C$_{10}$-OEt	3.4
BOC-A$_8$-B$_9$-C$_2$-OMe	51
BOC-A$_8$-B$_9$-C$_6$-OMe	46
BOC-A$_8$-B$_9$-C$_5$-OEt (**12**)	0.069
BOC-A$_8$-B$_1$-C$_{10}$-OEt	5.2
BOC-A$_8$-B$_1$-C$_2$-OMe	>100
BOC-A$_8$-B$_1$-C$_6$-OMe	>100
BOC-A$_8$-B$_1$-C$_5$-OEt	3.7

(BOC-A$_9$-B$_1$-C$_5$-OEt) exhibited respectable cytotoxic activity (IC$_{50}$ = 0.42 μM) and this compound was still 15-fold less active than **11**.

The distinctions between the activities of the positional scanning libraries are small and smaller than those of the 10-compound mixtures prepared by parallel synthesis.[6] This is a consequence of testing mixtures of 100 compounds (positional scanning) versus 10 compounds (parallel synthesis) where the impact of any single compound in the mixture is diminished. Nonetheless, the two potent compounds in the library of 1000 were detected. Thus, the ease of the synthesis of the initial positional scanning library relative to the small mixture library assembled by the more time consuming parallel synthesis is offset by the less distinct biological discrimination observed in the assay of the library and the increased effort required in the deconvolution.

The evaluation of the second positional scanning DMABA-trimer library provided observations that were analogous to those detected with the small mixtures prepared by parallel synthesis.[6] In general, the DMABA-trimers were less active than the corresponding BOC-trimers (Fig. 5). The exceptions, as detected in the previous studies with the small mixtures, tend to be the residues which convey insolubility to the resulting compounds (e.g. C$_7$). Presumably, the protonated side chain of the DMABA-trimers offsets this insolubility contributing productivly to characteristics that enhance their activity. Only one DMABA-trimer mixture was deconvoluted in our prior work.[6] Although it was the most potent, 10 mixtures exhibited comparable activity, IC$_{50}$ < 1 but > 0.1 μM. Aside from deconvolution of the most potent of these mixtures, IC$_{50}$ = 0.42 μM, no effort was made to deconvolute the remaining mixtures, even though they all would contain compounds with similar activities. This deconvolution provided DMABA-A$_8$-B$_4$-C$_7$-OMe (**14**) with an IC$_{50}$ = 0.46 μM. The examination of the second positional scanning library did not suggest this compound as a candidate lead (Fig. 5). The most potent residues identified were A$_9$ and A$_5$, B$_8$, and C$_{10}$. The preparation and testing of the two candidate structures DMABA-A$_9$-B$_8$-C$_{10}$-OEt (**15**) and DMABA-A$_5$-B$_8$-C$_{10}$-OEt (**16**) revealed IC$_{50}$'s of 0.32 and 3.2 μM, respectively. Thus, although **14** was not identified, a structure (**15**) of comparable activity was identified. Notably, both **14** and **15** are 100 times more potent than distamycin A (IC$_{50}$ = 42 μM), and its close tripyrrole analogue **17** (IC$_{50}$ = 44 μM) (Fig. 6).



11

12

Figure 4. Structure of the two most cytotoxic compounds within the libraries.

DNA binding properties

The positional scanning DMABA-trimer libraries were screened enlisting the ethidium bromide displacement

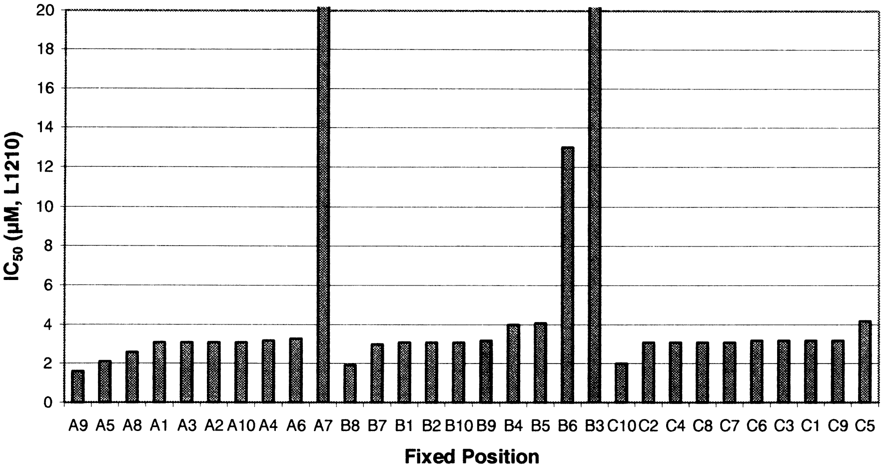


Figure 5. Cytotoxicity (L1210) for DMABA-trimer scanning libraries. Smaller numbers indicate more potent cytotoxic activity.

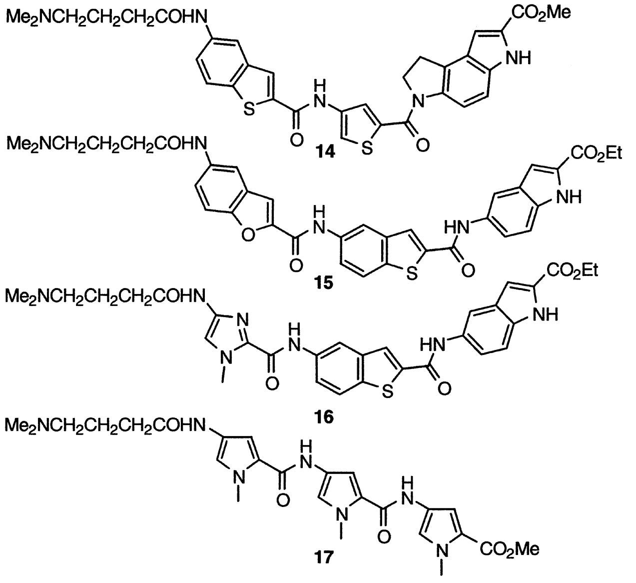


Figure 6. Structures of DMABA-trimers.

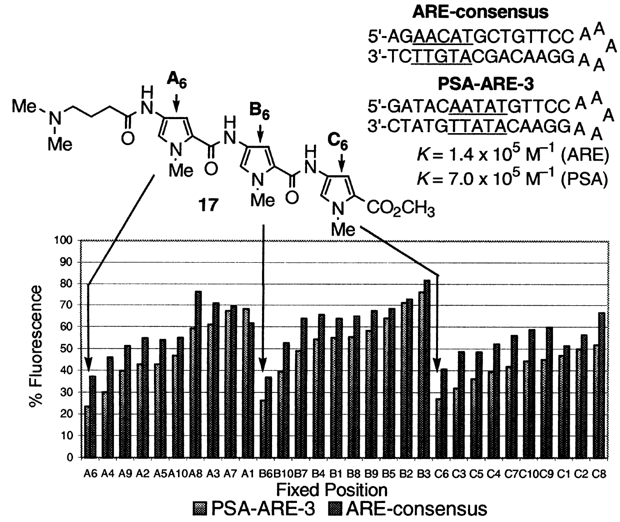


Figure 7. Ethidium bromide displacement assay for DMABA-trimer libraries (99 µM). DNA at 0.88×10^{-5} M, ethidium bromide at 0.44×10^{-5} M. Smaller numbers indicate greater DNA binding affinity.

DNA binding assay[6,16] with two hairpin oligonucleotides that were used in our prior study.[6] They constitute the dimer androgen receptor binding consensus sequences, ARE-consensus[17] and PSA-ARE-3,[18] and targets for chemotherapeutic resistant prostate cancer.[19] The latter contains a 5 base-pair AT-rich sequence known to bind distamycin A, while the former contains the same sequence interrupted by a single GC base-pair. The screening of the library, which entails measurement of the loss of fluorescence derived from compound binding and displacement of prebound ethidium bromide, identified A_6, B_6, and C_6 as the most effective residues for binding to the PSA-ARE-3 hairpin containing the 5 base-pair AT-rich site as well as the ARE-consensus hairpin. However, binding to the latter sequence was less effective (Fig. 7). This constitutes the identification of DMABA-A_6-B_6-C_6-OMe (**17**), the direct distamycin A analogue, in the 1000-member library as the most effective agent. This successful identification of **17** from the positional scanning library is tempered by the fact that it did not identify as candidate binders DMABA-A_8-B_9-C_5-OEt (**18**) or DMABA-A_{10}-B_8-C_6-OMe (**19**), which were identified in our prior study.[6] In addition, since the decreases in affinity for binding to the GC interrupted ARE-consensus hairpin were rather small and uniform, the ability to detect compounds including **19** that bound both sequences[6] equally well was not possible. This is a natural consequence of testing the larger 100 compound mixtures and the relative insensitivity of the assay to the contribution of any single, uniquely acting compound in the mixture. Thus, the more global observations are accurately detected with the positional scanning library and a useful lead structure with defined properties was identified. However, more subtle discoveries within the library were not identified. Thus, the disadvantages associated with the loss of their detection and this information contained within the library must be balanced against the advantages of the ease of synthesis of the parent libraries and judged in light of the objectives of the library synthesis. Typically, the positional scanning libraries would be most effective for lead identification and would be less suitable for lead optimization.

Conclusions

The screening of two prototypical positional scanning libraries and their comparison with prior results obtained on a library composed of mixtures of 10 compounds prepared by parallel synthesis was conducted. In a cellular assay for functional activity (L-1210 cytotoxic activity), the two potent members of the library were identified but required more extensive deconvolution and were deduced from activities that exhibit less distinction. Notably, the combination of most effective residues revealed in the assay did not correspond to the most potent compound or even an effective compound. This is a natural consequence of testing 100-compound mixtures where the impact of any single compound is relatively small. The performance in a DNA binding assay was just as revealing. The close distamycin A analogue **17** was identified as the most effective binder to a hairpin oligonucleotide that contains the PSA-ARE-3 consensus sequence and a five base-pair AT-rich site. However, the distinctions in the assay were small and subtle discoveries tucked into the library were not detected, including additional effective binders and those which bound both the PSA-ARE-3 and ARE consensus sequence equally well. Nonetheless, the combined use of solution-phase mixture synthesis and positional scanning is simple and technically nondemanding even for a large library being less demanding than the parallel synthesis of individual compounds or solid-phase split and mix synthesis[20] with or without tagging.[21] Unlike iterative,[11,22] surf,[23] or recursive deconvolution,[12] positional scanning can be conducted upfront for depository libraries subjected to multiple screening assays. Thus, the loss of resolution in the testing data, which does not preclude identifying effective leads, must be balanced against the ease and ultimate breath of the library synthesis and its appropriateness judged in light of the screening objectives.

Experimental

Synthesis of dimer mixtures. Solutions of a mixture of the 10 BOC-protected acids **1–10b** (each 160 μmol, 1.1 equiv) and the 10 amino esters **1–10a** (each 144 μmol, 1 equiv) in DMF (20 mL) were treated with EDCI (3.2 mmol, 22.2 equiv) and DMAP (3.6 mmol, 25.0 equiv). The resulting solutions were stirred for 12–16 h, then the DMF was removed under reduced pressure and the resulting oil was taken up in EtOAc (20 mL) and washed with 10% aqueous HCl (3×20 mL) and saturated aqueous $NaHCO_3$ (3×20 mL). The resulting solutions were dried (Na_2SO_4), filtered, and concentrated to dryness providing the mixture of dimers that was used without purification.

General procedure for preparation of sublibraries I. BOCNH-A_{1-10}-CONH-X-CONH-X-OR. Ten individual portions of the mixture of dimers BOCNH-X-CONH-X-OR (144 μmol, 1.0 equiv) were dissolved in 4.0 N HCl/EtOAc (1 mL), and the mixtures were stirred at 25 °C for 2 h. The solvent was removed under a stream of N_2 and the residues were dried in vacuo for 4 h. Each sample was dissolved in DMF (1.0 mL) and treated with one of the 10 BOC-carboxylic acids **1–10b**, followed by EDCI (67.5 mg, 352 μmol, 2.2 equiv) and DMAP (48.9 mg, 400 μmol, 2.5 equiv). The solutions were stirred for 16 h at 25 °C. The mixtures were poured into EtOAc (20 mL) and washed with 10% aqueous HCl (3×20 mL), followed by saturated aqueous $NaHCO_3$ (3×20 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated in vacuo to afford the final trimers (68–99%).

General procedure for preparation of sublibraries II. BOCNH-X-CONH-B_{1-10}-CONH-X-OR. Ten single BOC-amino acids **1–10b** (160 μmol, 11 equiv) and a mixture of 10 amino acid esters **1–10a** (each 14.4 μmol, 1 equiv) were dissolved in DMF (1.5 mL) and treated with EDCI (75 mg, 390 μmol, 24.4 equiv) and DMAP (49 mg, 400 μmol, 25 equiv). The solutions were stirred for 16 h at 25 °C. The mixtures were poured into EtOAc (20 mL) and washed with 10% aqueous HCl (3×20 mL), followed by saturated aqueous $NaHCO_3$ (3×20 mL). The organic phases were dried (Na_2SO_4), filtered, and concentrated in vacuo to afford 10 mixtures of dipeptides BOCNH-B_{1-10}-CONH-X-OR. Each of these 10 mixtures was dissolved in 4.0 N HCl/EtOAc (1 mL), and the mixtures were stirred at 25 °C for 2 h. The solvent was removed under a stream of N_2 and the residues were dried in vacuo for 4 h. Each sample was dissolved in DMF (1.5 mL) and treated with a mixture of the 10 BOC-carboxylic acids **1–10b** (each 15.0 μmol, 1.04 equiv) followed by EDCI (69 mg, 360 μmol, 24 equiv) and DMAP (44 mg, 360 μmol, 24 equiv). The solutions were stirred for 16 h at 25 °C. The mixtures were poured into EtOAc (20 mL) and washed with 10% aqueous HCl (3×20 mL), followed by saturated aqueous $NaHCO_3$ (3×20 mL). The organic phases were dried (Na_2SO_4), filtered, and concentrated in vacuo to afford the final sublibraries II (68–99%).

General procedure for preparation of sublibraries III. BOCNH-X-CONH-X-CONH-C_{1-10}-OR. The mixture of dimers BOCNH-X-CONH-X-OR (190 μmol, 1.0 equiv) was dissolved in THF/MeOH/H_2O (20 mL, 2:1:1), LiOH (760 μmol, 4 equiv) was added and the mixture was stirred at 25 °C for 18 h. The solvent was removed under reduced pressure and the residue was acidified with 10% aqueous HCl. The dipeptide acids were extracted with EtOAc (3×20 mL), the combined organic layers were washed with 10% aqueous HCl and water, dried (Na_2SO_4), filtered, and concentrated in vacuo. Ten individual portions of the mixture of BOCNH-X-CONH-X-OH (182 μmol, 1.14 equiv) were dissolved in DMF (2.0 mL) and treated with one of the 10 amino acid esters **1–10a** (each 160 μmol, 1 equiv) followed by EDCI (76.7 mg, 400 μmol, 2.5 equiv) and DMAP (48.9 mg, 400 μmol, 2.5 equiv). The solutions were stirred for 16 h at 25 °C. The mixtures were poured into EtOAc (20 mL) and washed with 10% aqueous HCl (3×20 mL), followed by saturated aqueous $NaHCO_3$ (3×20 mL). The organic phases were dried (Na_2SO_4), filtered and concentrated in vacuo to afford the final sublibraries III (45–81%).

General procedure for preparation of DMABA-trimer libraries. Each of the BOC-trimer sublibraries (0.007 mmol, 1 equiv) was dissolved in 4.0 N HCl/EtOAc

(1 mL), and the mixtures were stirred at 25 °C for 2 h. The solvent was removed under a stream of N_2 and the residues were dried in vacuo for 4–8 h. Each sample was treated with dimethylaminobutyric acid as a 0.1 M solution in DMF (150 μL, 0.015 mmol, 2 equiv) followed by EDCI as a 0.1 M solution in DMF (180 μL, 0.018 mmol, 2.5 equiv) and DMAP as a 0.1 M solution in DMF (219 μL, 0.022 mmol, 3.0 equiv). The solutions were stirred for 12–16 h at 25 °C. The solvent was removed under a stream of N_2 and the residues were taken up in H_2O (10 mL) and extracted with EtOAc (4×10 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated in vacuo. The resulting solids were slurried in Et_2O (1 mL) and centrifuged. The pellets were again slurried in Et_2O (1 mL) and collected by filtration to afford the desired DMABA-trimers (22–100%).

General procedure for preparation of individual BOC-trimers. The individual dipeptides BOCNH-X-CONH-Y-OR (X = **1**, **9**; Y = **2**, **5**, **6**, **10**) (1.0 equiv) were dissolved in 4.0 N HCl/EtOAc (1 mL), and the mixtures were stirred at 25 °C for 2 h. The solvent was removed under a stream of N_2 and the residues were dried in vacuo for 4 h. Each sample was dissolved in DMF (1.0 mL) and was treated with a BOC-carboxylic acid (**8b**, **9b**), followed by EDCI (2 equiv) and DMAP (2.5 equiv). The solutions were stirred for 16 h at 25 °C. The mixture was poured into EtOAc (10 mL) and washed with 10% aqueous HCl (3×10 mL), followed by saturated aqueous $NaHCO_3$ (3×10 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated in vacuo to afford the final trimers (37–99% yield).

BOCNH-9-CONH-1-CONH-10-OEt. (9.1 mg, 79%); ^1H NMR (DMSO, 400 MHz) δ 10.75 (s, 1H), 10.12 (s, 1H), 9.50 (s, 1H), 8.15 (s, 1H), 8.01–7.96 (m, 5H), 7.76 (s, 1H), 7.63 (d, 1H, J = 9.1 Hz), 7.57 (d, 1H, J = 9.1 Hz), 7.47 (d, 1H, J = 8.8 Hz), 7.42 (d, 1H, J = 8.8 Hz), 7.15 (s, 1H), 4.34 (q, 2H, J = 7.0 Hz), 1.50 (s, 9H), 1.35 (t, 3H, J = 7.0 Hz).

BOCNH-9-CONH-1-CONH-2-OMe. (9.2 mg, 99%); ^1H NMR (DMSO, 400 MHz) δ 10.77 (s, 1H), 10.40 (s, 1H), 9.50 (s, 1H), 8.48 (s, 1H), 8.08 (d, 1H, J = 8.5 Hz), 8.01–7.98 (m, 5H), 7.77 (s, 1H), 7.69 (d, 1H, J = 7.0 Hz), 7.63 (d, 1H, J = 8.8 Hz), 7.52 (d, 1H, J = 8.2 Hz), 7.49–7.46 (m, 1H), 3.88 (s, 3H), 1.50 (s, 9H).

BOCNH-9-CONH-1-CONH-6-OMe. (9.0 mg, 70%); ^1H NMR (DMSO, 400 MHz) δ 10.73 (s, 1H), 10.27 (s, 1H), 9.49 (s, 1H), 8.01 (s, 1H), 7.95 (s, 4H), 7.75 (s, 1H), 7.62 (d, 1H, J = 8.8 Hz), 7.53 (d, 1H, J = 2.0 Hz), 7.48 (dd, 1H, J = 2.0 Hz, J = 8.8 Hz), 6.95 (d, 1H, J = 2.0 Hz), 3.86 (s, 3H), 3.75 (s, 3H), 1.50 (s, 9H).

BOCNH-9-CONH-1-CONH-5-OEt (13). (8.7 mg, 70%); ^1H NMR (DMSO, 400 MHz) δ 10.73 (s, 1H), 9.49 (s, 1H), 8.07 (d, 2H, J = 8.8 Hz), 8.01 (m, 1H), 7.93 (d, 2H, J = 8.8 Hz), 7.6 (s, 2H), 7.62 (d, 1H, J = 8.8 Hz), 7.47 (dd, 1H, J = 2.2 Hz, J = 8.8 Hz), 4.29 (q, 2H, J = 7.0 Hz), 3.96 (s, 3H), 1.50 (s, 9H), 1.31 (t, 3H, J = 7.0 Hz).

BOCNH-8-CONH-9-CONH-10-OEt. (19.3 mg, 81%); ^1H NMR (DMSO, 400 MHz) δ 10.60 (s, 1H), 10.48 (s, 1H), 9.56 (s, 1H), 8.29 (s, 2H), 8.18–8.16 (m, 2H), 7.92 (d, 1H, J = 8.8 Hz), 7.79–7.73 (m, 3H), 7.62 (dd, 1H, J = 1.2 Hz, J = 9.2 Hz), 7.53 (dd, 1H, J = 1.2 Hz, J = 8.7 Hz), 7.45 (d, 1H), 7.17 (s, 1H), 4.34 (q, 2H, J = 7.0 Hz), 1.51 (s, 9H), 1.35 (t, 3H, J = 7.0 Hz).

BOCNH-8-CONH-9-CONH-2-OMe. (20.3 mg, 77%); ^1H NMR (DMSO, 400 MHz) δ 10.78 (s, 1H), 10.61 (s, 1H), 9.56 (s, 1H), 8.51 (s, 1H), 8.30 (s, 1H), 8.28 (s, 1H), 8.15–8.10 (m, 2H), 7.92 (d, 1H, J = 8.8 Hz), 7.85 (s, 1H), 7.82–7.72 (m, 3H), 7.56–7.52 (m, 2 H), 3.88 (s, 3H), 1.51 (s, 9H).

BOCNH-8-CONH-9-CONH-6-OMe. (18.2 mg, 71%); ^1H NMR (DMSO, 400 MHz) δ 10.76 (s, 1H), 10.59 (s, 1H), 9.55 (s, 1H), 8.27 (m, 2H), 8.15 (s, 1H), 7.92 (d, 1H, J = 8.8 Hz), 7.77 (d, 1H, J = 9.1 Hz), 7.72 (d, 1H, J = 9.1 Hz), 7.69 (s, 1H), 7.54 (m, 2 H), 7.02 (s, 1H), 3.87 (s, 3H), 3.76 (s, 3H), 1.51 (s, 9H).

BOCNH-8-CONH-1-CONH-10-OEt. (8.2 mg, 49%); ^1H NMR (DMSO, 400 MHz) δ 10.68 (s, 1H), 10.13 (s, 1H), 9.56 (s, 1H), 8.31 (s, 1H), 8.15 (s, 2H), 8.02 (d, 2H, J = 8.8 Hz), 7.93 (d, 1H, J = 8.5 Hz), 7.92 (d, 2H, J = 8.8 Hz), 7.59–7.54 (m, 2H), 7.42 (d, 1H, J = 8.8 Hz), 7.15 (s, 1H), 4.34 (q, 2H, J = 7.0 Hz), 1.51 (s, 9H), 1.35 (t, 3H, J = 7.0 Hz).

BOCNH-8-CONH-1-CONH-2-OMe. (17.4 mg, 99%); ^1H NMR (DMSO, 400 MHz) δ 10.72 (s, 1H), 10.41 (s, 1H), 9.56 (s, 1H), 8.48 (s, 1H), 8.33 (s, 1H), 8.16 (s, 1H), 8.09 (d, 1H, J = 8.0 Hz), 8.04 (d, 2H, J = 8.8 Hz), 7.94 (d, 2H, J = 8.8 Hz), 7.93 (d, 1H, J = 8.8 Hz), 7.69 (m, 1H), 7.56–7.51 (m, 2 H), 3.88 (s, 3H), 1.51 (s, 9H).

BOCNH-8-CONH-1-CONH-6-OMe. (10.9 mg, 82%); ^1H NMR (DMSO, 400 MHz) δ 10.67 (s, 1H), 10.28 (s, 1H), 9.56 (s, 1H), 8.30 (s, 1H), 8.15 (s, 1H), 7.96 (d, 2H, J = 8.8 Hz), 7.94 (s, 1H), 7.90 (d, 2H, J = 8.8 Hz), 7.55–7.54 (m, 2H), 6.95 (s, 1H), 3.86 (s, 3H), 3.75 (s, 3H), 1.51 (s, 9H).

BOCNH-8-CONH-1-CONH-5-OEt. (4.8 mg, 37%); ^1H NMR (DMSO, 400 MHz) δ 10.67 (s, 1H), 9.56 (s, 1H), 8.29 (s, 1H), 8.15 (m, 1H), 8.08 (d, 2H, J = 8.8 Hz), 7.92 (d, 1H, J = 8.8 Hz), 7.87 (d, 2H, J = 8.8 Hz), 7.76 (s, 1H), 7.54 (d, 1H, J = 9.4 Hz), 4.29 (q, 2H, J = 7.1 Hz), 1.51 (s, 9H), 1.31 (t, 3H, J = 7.1 Hz).

General procedure for preparation of individual DMABA-trimers. Each of the individual samples of BOCNH-X-CONH-8-CONH-10-OEt trimers (X = **5**, **9**) (1.0 equiv) was dissolved in 4.0 N HCl/EtOAc (1 mL), and the mixtures were stirred at 25 °C for 2 h. The solvent was removed under a stream of N_2 and the residues were dried in vacuo for 4 h. Each sample was treated with 4-dimethylaminobutyric acid (2 equiv), EDCI (2 equiv), DMAP (2.5 equiv), and DMF (1 mL). The solutions were stirred for 16 h at 25 °C. The solvent was removed under a stream of N_2 and the residues were taken up in H_2O (10 mL) and extracted with EtOAc (4×10 mL). The combined organic layers were dried (Na_2SO_4), filtered, and concentrated in vacuo. The resulting solids were slurried in Et_2O (10 mL) and centrifuged. The pellets were again slurried in Et_2O (10 mL) and collected by filtration to afford the desired DMABA-trimers (51–80% yield).

$Me_2NCH_2CH_2CH_2CONH$-9-CONH-8-CONH-10-OEt (15). (18.9 mg, 80%); ^1H NMR (DMSO, 400 MHz) δ 10.72 (s, 1H), 10.45 (s, 1H), 10.07 (s, 1H), 8.52 (s, 1H), 8.35 (s, 1H), 8.22 (s, 1H), 8.14 (s, 1H), 8.05 (d, 1H, $J=8.9$ Hz), 7.88 (dd, 1H, $J=1.9$ Hz, $J=8.9$ Hz), 7.78 (s, 1H), 7.67 (d, 1H, $J=9.4$ Hz), 7.60–7.55 (m, 2H), 7.46 (d, 1H, $J=8.8$ Hz), 7.17 (s, 1H), 4.34 (q, 2H, $J=7.0$ Hz), 2.36 (t, 2H, $J=7.4$ Hz), 2.25 (t, 2H, $J=7.0$ Hz), 2.14 (s, 6H), 1.74 (pent, 2H, $J=7.4$ Hz), 1.35 (t, 3H, $J=7.0$ Hz).

$Me_2NCH_2CH_2CH_2CONH$-5-CONH-8-CONH-10-OEt (16). (2.2 mg, 51%); ^1H NMR (DMSO, 400 MHz) δ 10.70 (s, 1H), 9.95 (s, 1H), 8.52 (s, 1H), 8.35 (s, 1H), 8.22 (s, 1H), 8.14 (s, 1H), 8.05 (d, 1H, $J=8.9$ Hz), 7.78 (s, 1H), 7.60–7.55 (m, 1H), 7.54–7.40 (m, 2H), 7.17 (s, 2H), 4.33 (q, 2H, $J=7.0$ Hz), 2.34 (t, 2H, $J=7.4$ Hz), 2.24 (t, 2H, $J=7.0$ Hz), 2.13 (s, 6H), 1.75 (pent, 2H, $J=7.4$ Hz), 1.35 (t, 3H, $J=7.0$ Hz).

Ethidium bromide displacement assays

Hairpin oligonucleotides (0.887×10^{-5} M bp) were mixed with ethidium bromide (0.444×10^{-5} M) in a 2:1 ratio of base-pair:ethidium bromide in a 0.1 M Tris–HCl, 0.1 M NaCl, pH 8 buffer. The fluorescence measurements were conducted at 545 nm excitation and 595 nm emission. For the rapid screening of libraries, 96-well plates (Costar: black, 360 μL, flat-bottom) were loaded with the premixed ethidium bromide/DNA solution (100 μL) and single aliquots of each library (1 μL of 10 mM solutions in DMSO, 99 μM final concentration) were added. Each plate was incubated at 25 °C for 30 min before reading on a fluorescence plate reader (Molecular Devices SpectraMax Gemini) using 545 nm excitation and 595 nm emission.

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