Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 47–50

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Comparison of Library Screening Techniques Used in the Development of dsDNA Ligands

Patrick Chaltin, Filip Borgions, Arthur Van Aerschot and Piet Herdewijn*

Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

Received 22 January 2002; revised 13 September 2002; accepted 4 October 2002

Abstract—The gel retardation and FID (fluorescent intercalator displacement) techniques have been compared for the selection of dsDNA binding ligands out of library mixtures. The selection procedure involves the synthesis and screening of unnatural oligopeptide libraries based on an iterative deconvolution procedure. Both methods yield comparable selection results and binding constants for the selected compounds, meaning that they can be considered as complementary in the discovery process of new antigene compounds. Furthermore, a quinazolin-2,4-dione amino acid has been identified as possessing interesting properties for interaction with dsDNA.

Several established techniques for discovering drugs exist, that is the discovery by serendipity, by lead compound modification and by a process called structure-based drug design.[1] Development of new drugs can furthermore be accomplished by screening large numbers of molecules from natural or synthetic sources for activity. The advantage of screening for new active compounds is that this technique requires a minimum of knowledge about the disease process, the structure of the screened molecules or the biological target. Using this method, it is possible to discover totally new structure types with the desired biological activity, from natural products or chemical libraries. However, screening is a more-or-less random process, involving trial and error. As such, it is unpredictable in outcome and time consuming in labor.[1,2] Recently, it has become feasable to test large numbers of compounds against many targets in a short time in the so-called high throughput screening (HTS) systems, yielding lead structures in short periods of time.[3]

In the antigene field, screening is being applied in order to discover novel dsDNA interacting motifs. Thereby individual compounds and library mixtures have been analyzed on solid supports[4] or in solution. A variety of solution-screening methods have already been applied for this purpose, for example selection or analysis of nucleic acid ligands with affinity chromatography, melting temperature determination, gel shift, fluorescent intercalator displacement (FID), footprinting, competition dialysis, mass spectrometry, surface plasmon resonance with chip technology and several other methodologies.[5–7] Recently a new technique, based on measurements of DNA hybridization stability upon ligand binding, was added as screening methodology ('hybridization stabilization assay').[8] Most of these investigations use however individual compounds in their activity tests.

In order to increase the discovery rate of new dsDNA interacting compounds, screening of libraries is advantageous. Recently, solution screening of combinatorial library mixtures for dsDNA binding has been described by using FID[9] and DNase I footprinting.[10,11] Although a lot of information can be gathered by using footprinting assays to screen mixtures for dsDNA ligands, the technique possesses also several limitations. The method is slow, time-consuming, demands relatively high technical skills and the selection of active compounds is not straightforward due to sensitivity problems.[11]

Previously, we used gel retardation assays for the solution screening of library mixtures for their dsDNA interaction capacities.[12] This approach yielded several unnatural oligopeptides with affinities in the 10^{-4} M range for a 14-mer dsDNA target sequence, corresponding to the binding site of the IL-6 nuclear factor

*Corresponding author. Tel.: +32-16-337381; fax: +32-16-337340; e-mail: piet.herdewijn@rega.kuleuven.ac.be

0960-894X/03/$ - see front matter
PII: S0960-894X(02)00836-3

(NF-IL6) [5′-(AGATTGTGCAATGT)-3′:5′-(ACATTGCACAATCT)-3′]. To evaluate this strategy and to compare the gel retardation method with the recently described high-throughput FID method,[13] we repeated the selection procedure using the latter approach. An ethidium bromide displacement experiment was used to select dsDNA binding unnatural peptides from libraries and the results were compared with those obtained from the gel retardation assays. Gel shift screening of library mixtures has already been applied to select RNA interacting molecules.[14,15]

The library used consisted of oligopeptides with the general structure Ac-Arg-Ual-Sar-X_1-X_2-X_3-Arg-$CONH_2$, with X_n representing each of 12 amino acid building blocks depicted in Figure 1, except arginine (Arg). An iterative deconvolution strategy was used to optimize the oligopeptide structure, in which in a first step X_1 was fixed and optimized, followed by X_2 and finally X_3. The peptide libraries and individual compounds were synthesized on solid support by using the Fmoc-based strategy, the mix and split technique and standard coupling reagents used in protein chemistry.[12] Following assembly, the peptides were cleaved from the solid support and used in screening assays after extraction and in the case of individual peptides an additional purification by reversed phase HPLC was applied.[12]

During the gel retardation screening of the peptides with the general structure Ac-Arg-Ual-Sar-X_1-X_2-X_3-Arg-$CONH_2$ the strongest binding peptides were selected by measuring the residual amount of free dsDNA. The first screening round of twelve libraries with fixed position X_1, each consisting of a mixture of 144 peptides, yielded three amino acids leading to a 20% or higher decrease of unbound dsDNA at a 2 mM sublibrary concentration. The library with Chi on position X_1 yielded a residual amount of free DNA of 54%, while for Hpr and Tal values around 80% were obtained. Consequently, the oligopeptide library with the general structure Ac-Arg-Ual-Sar-**Chi**-X_2-X_3-Arg-$CONH_2$ seemed to possess the highest interaction capacities with the dsDNA target and was therefore selected for further deconvolution, together with the Ac-Arg-Ual-Sar-**Tal**-X_2-X_3-Arg-$CONH_2$ peptides.

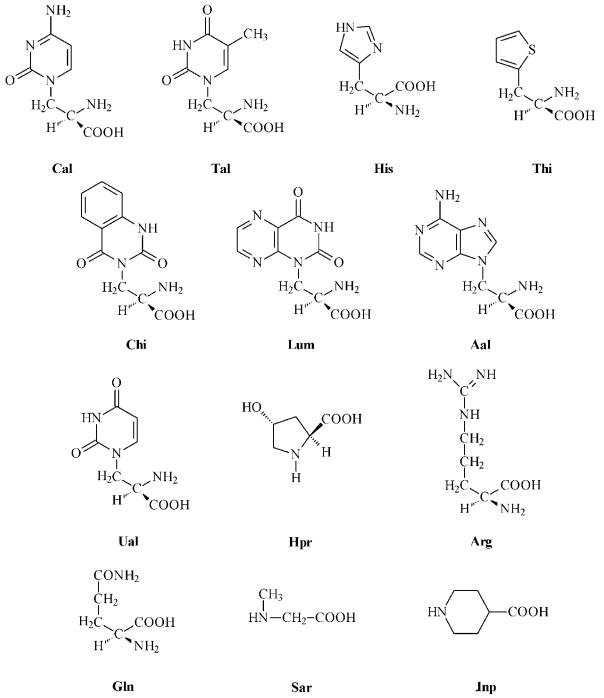

Figure 1. Amino acid building blocks used for synthesis of the libraries: β-(cytosin-1-yl)-α-D-alanine (Cal), β-(thymin-1-yl)-α-D-alanine (Tal), L-histidine (His), β-thienyl-D-alanine (Thi), β-(1*H*-quinazolin-2,4-dion-3-yl)-α-D-alanine (Chi), β-(lumazin-1-yl)-α-D-alanine (Lum), β-(adenin-9-yl)-α-D-alanine (Aal), β-(uracil-1-yl)-α-D-alanine (Ual), *trans*-4-hydroxy-L-proline (Hpr), L-arginine (Arg), L-glutamine (Gln), sarcosine (Sar) and isonipecotic acid (Inp).

In the following cycle of screening, two times twelve samples each containing 12 different synthetic peptides needed to be screened. The screening experiments of these peptide mixtures (1 mM) showed that for both series the quinazoline derivative (Chi) possessed high interaction capacities on position X_2. For the case where X_1 was already fixed as Chi, likewise strong binding peptides could be observed with either His, Cal, Aal or Lum on position X_2. For Chi on position X_2 residual amounts of dsDNA were 45%, while this ranged around 53% for Aal and Lum, 57% for Cal and 61% for His. Further gel based screening experiments, also including selectivity tests with modified dsDNA targets, led to the selection of Chi on position X_2 for both libraries: Ac-Arg-Ual-Sar-**Chi-Chi**-X_3-Arg-$CONH_2$ and Ac-Arg-Ual-Sar-**Tal-Chi**-X_3-Arg-$CONH_2$.

To compare the gel shift method with FID for mixture screening, the libraries with general structures Ac-Arg-Ual-Sar-X_1-X_2-X_3-Arg-$CONH_2$, Ac-Arg-Ual-Sar-Chi-X_2-X_3-Arg-$CONH_2$ and Ac-Arg-Ual-Sar-Tal-X_2-X_3-Arg-$CONH_2$ were tested for their dsDNA interaction capacities in an ethidium bromide displacement experiment using the 96-well format.[13] The same 14-mer dsDNA fragment (not radiolabeled) used in the gel retardation experiments was applied as target and the assay was performed as described by Boger et al.[13] In a 100 μL assay volume, 1 μM dsDNA (14 μM in bp) and 7 μM ethidium bromide were used, establishing the optimal 1:2 EtBr/bp ratio. A 10 mM Tris, 10 mM NaCl buffer pH 7.4 was used. Repeated measurements with several oligopeptide concentrations were performed.[16]

As for gel mobility-shift screening, FID experiments with a 1 mM concentration of the 12 libraries with fixed positions X_1, led to the selection of Chi for X_1, with a relative fluorescence of 51%. Aal and His on this position yielded a comparable strength of binding with a fluorescence of respectively 52% and 54%. The Tal amino acid, which was selected in the gel shift screening, displayed a fluorescence of 62%, together with Cal and Lum (Table 1).

In the case of screening of the libraries Ac-Arg-Ual-Sar-**Chi**-X_2-X_3-Arg-$CONH_2$ and Ac-Arg-Ual-Sar-**Tal**-X_2-X_3-Arg-$CONH_2$, the highest amount of ethidium bromide was displaced by Chi-Aal-X_3 peptides. In this group of libraries with Chi on position X_1, also mixtures

with Chi, Cal, Thi, Lum and Inp showed high interaction capacities for position X_2. In the case where X_1 was fixed as Tal, the amino acids Tal and Cal possessed the highest interaction capacities at X_2, closely followed by Chi. Comparison of these results with the selection by gel shift experiments shows that for libraries with Chi fixed on X_1, the same compounds are selected except that Inp was not included after gel shift experiments. In the group of Tal-X_2-X_3, Chi was selected by gel shift testing. During FID, Cal and Tal showed the strongest binding (relative fluorescence of 38%), while Chi led to a lower EtBr displacement with a relative fluorescence of 47%.

The last step in the gel based screening approach was the synthesis and screening of 24 individual oligopeptides with Ac-Arg-Ual-Sar-**Chi-Chi**-X_3-Arg-$CONH_2$ or Ac-Arg-Ual-Sar-**Tal-Chi**-X_3-Arg-$CONH_2$ as general structure. Nine oligopeptides showing the highest affinity could be selected: seven in the group with a Chi–Chi (X_1–X_2) structure and two in the Tal–Chi category. For the peptides with the Tal–Chi (X_1–X_2) sequence, the compounds with Aal and Chi on X_3 displayed the strongest binding. More versatility of binding peptides was found in the Chi–Chi (X_1–X_2) series, with Ual, Tal, Chi, Cal, Aal, Lum and Thi emerging as strong interacting amino acids at position X_3.

From seven of the nine selected oligopeptides during gel based screening, dissociation constants were determined with gel retardation assays, in order to compare their affinity with each other and with known dsDNA interacting compounds. The selected peptides are shown in Table 2 with their respective apparent dissociation constants. Ranking the oligopeptides based on their apparent K_d delivers the following result: Chi-Chi-Chi > Tal-Chi-Aal ≈ Chi-Chi-Thi > Chi-Chi-Aal > Chi-Chi-Tal > Chi-Chi-Cal ≈ Chi-Chi-Ual, with the compound Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-$CONH_2$ possessing the highest interaction capacities with a K_d of 9×10^{-5} M.

The individually synthesized oligopeptides with the general structure Ac-Arg-Ual-Sar-Chi-Chi-X-Arg-NH_2 or Ac-Arg-Ual-Sar-Tal-Chi-X-Arg-NH_2, were also compared for their dsDNA affinity in the FID experiment, using the same procedure as for the mixtures. Evaluation of the results allowed selection of the strongest dsDNA interacting oligopeptides depending on the percentage of fluorescence decrease. In the group of peptides with the Tal–Chi (X_1–X_2) structure, Chi and Aal on the third position seemed to possess the highest and comparable affinities, followed by Ual and His. For peptides with the sequence Chi–Chi (X_1–X_2), the amino acids Aal, Cal, Chi, Lum, Tal, Thi and Ual showed the highest interaction capacities (Table 3).

Comparison with the selection of oligopeptides by gel shift assay, shows that exactly the same structures were selected in both screening procedures. Furthermore, based on the percentage of ethidium displacement, a ranking order of dsDNA affinity could be established: Chi-Chi-Chi > Chi-Chi-Thi > Chi-Chi-Aal ≈ Chi-Chi-Lum > Chi-Chi-Tal ≈ Chi-Chi-Cal > Chi-Chi-Ual > Tal-Chi-Chi ≈ Tal-Chi-Aal. The only important difference with the results obtained with the gel retardation approach is the ranking order of the peptide Ac-Arg-Ual-Sar-Tal-Chi-Aal-Arg-$CONH_2$, which was selected as the second strongest interacting molecule of seven peptides in the gel retardation assays.

Dissociation constant determination with the EtBr-displacement test was also performed for the peptide Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-$CONH_2$, determined as the strongest binding compound. The concentration of the compound leading to a 50% decrease in ethidium

Table 1. Fluorescence readings in an EtBr-displacement experiment with the oligopeptide library mixtures-X_1-X_2-X_3-,-Chi-X_2-X_3 and-Tal-X_2-X_3-Arg-NH_2[a]

X_1 or X_2	First selection (%)	Second selection (%)	
	X_1-X_2-X_3	Tal-**X_2**-X_3	Chi-**X_2**-X_3[b]
His	**54**	51	46
Gln	67	58	59
Hpr	75	74	64
Sar	75	77	66
Inp	75	73	**39**
Tal	**62**	**39**	52
Cal	62	**38**	**33**
Ual	69	73	68
Lum	62	71	**39**
Aal	**52**	64	**21**
Chi	**51**	**47**	**26**
Thi	68	71	**36**

[a]The results are reported as % fluorescence relative to control wells. In the first selection round a 1 mM concentration of oligopeptide mixtures was used, while in the second selection cycle 750 μM was applied.

Table 2. Seven selected peptides with their apparent K_d[a]

Selected peptide sequences	Apparent K_d (M)
Ac-Arg-Ual-Sar-Chi-Chi-Cal-Arg-$CONH_2$	$6.3\times10^{-4}\pm0.7$
Ac-Arg-Ual-Sar-Chi-Chi-Ual-Arg-$CONH_2$	$6.1\times10^{-4}\pm0.8$
Ac-Arg-Ual-Sar-Chi-Chi-Tal-Arg-$CONH_2$	$4.9\times10^{-4}\pm0.6$
Ac-Arg-Ual-Sar-Chi-Chi-Aal-Arg-$CONH_2$	$3.9\times10^{-4}\pm0.5$
Ac-Arg-Ual-Sar-Chi-Chi-Thi-Arg-$CONH_2$	$2.3\times10^{-4}\pm0.3$
Ac-Arg-Ual-Sar-Tal-Chi-Aal-Arg-$CONH_2$	$2.2\times10^{-4}\pm0.3$
Ac-Arg-Ual-Sar-Chi-Chi-Chi-Arg-$CONH_2$	$9.1\times10^{-5}\pm0.1$

[a]The K_d values are averaged from three to four gel mobility-shift experiments.

Table 3. Fluorescence readings in an EtBr-displacement experiment with the individual oligopeptides[a]

X_3	Tal-Chi-X_3 (%)	Chi-Chi-X_3 (%)
His	87	81
Gln	103	80
Hpr	92	91
Sar	99	80
Inp	99	81
Tal	90	60
Cal	90	60
Ual	83	66
Lum	95	**51**
Aal	**77**	**51**
Chi	**77**	**35**
Thi	100	**41**

[a]The results are reported as % fluorescence relative to control wells. In these experiments 750 μM oligopeptides was used.

bromide fluorescence was around 2×10^{-4} M, differing with a factor two with the apparent dissociation constant as determined in the gel based assay. This higher value is in agreement with the results described by Boger et al.[13]

The results of this study show that both screening methods, gel shift and FID, deliver quantitatively and qualitatively comparable selections in the optimization process of the dsDNA binding oligopeptide library. As such, the usefulness of the solution phase gel mobility-shift assay for selection of dsDNA binding molecules out of mixtures and individual compounds has been demonstrated. Based on these results, it can be concluded that gel shift and ethidium bromide displacement screening are equally suited for selection of dsDNA binding molecules.

The FID method is technically easy, requires little preparation and with the 96-well format a high throughput can be achieved. The technique is however not generally applicable to all dsDNA interacting molecules. Due to the presence of fluorophores in the ligands or to fluorophore quenching, investigation of binding properties of some dsDNA interacting molecules (f.e. combilexins) with FID would be troublesome. The gel shift assay requires certainly more technical expertise and is more time consuming, but there is no restriction as to the kind of molecules that can be assayed for their dsDNA interaction capacities. Furthermore, since for gel shift assays imaging is based on the highly sensitive detection of radioactivity, lower amounts of both the dsDNA and ligands can be used. Therefore, both methods can be considered as complementary for the discovery of new DNA ligands.

Secondly, results of the deconvolution procedure with the oligopeptide libraries reveal that β-(1*H*-quinazolin-2,4-dion-3-yl)-α-D-alanine possesses properties making it especially suited for interaction with dsDNA. Stacking of the heterocyclic ring systems inside or outside the duplex will certainly contribute to the binding, next to possible hydrogen bonding interactions. Stacking between the quinazolinedione rings of the amino acid can furthermore confer a certain pre-organization reducing the entropy cost of binding to double stranded DNA. In this respect, it is however surprising that not the lumazine side chain displays the strongest interaction capacities, as it could be expected that the lumazine heterocycle would be endowed with similar or stronger stacking capacities compared to quinazoline-dione. Perhaps the different orientation of the two ring systems, lumazine being coupled via N^1 and quinazolinedione via N^3, can account for it. In further experiments the quinazoline-dione side-chains were removed. Systematic replacement of every unnatural amino acid by glycine revealed that the side chains of sarcosine (Sar) and β-(uracil-1-yl)-α-D-alanine (Ual) contribute to a lesser extent to the affinity of the investigated peptides for the dsDNA target than the quinazoline-dione side chains, once more highlighting the importance of this heterocyclic system in our dsDNA binding peptides. Further optimization of the structure of these peptides and investigation of their binding mode are the subject of present research.

Acknowledgements

The authors are grateful to Geconcerteerde onderzoeksactie (GOA) of the K.U. Leuven and to the Flemish Fund for Scientific Research for financial support (G.0269.98 and G.0089.02). We thank Prof. DeWitte for the use of the FL600 Microplate Fluorescence reader and C. Biernaux for editorial help.

References and Notes

1. Perun, T. J.; Propst, C. L. In *Nucleic Acid Targeted Drug Design*; Propst, C. L., Perun, T. J., Eds.; Marcel Dekker: New York, 1992; p 1.
2. Kaul, P. N. *Prog. Drug Res.* **1998**, *50*, 9.
3. Oldenburg, K. R. *Ann. Rep. Med. Chem.* **1998**, *33*, 301.
4. Lescrinier, T.; Hendrix, C.; Kerremans, L.; Rozenski, J.; Link, A.; Samyn, B.; Van Aerschot, A.; Lescrinier, E.; Eritja, R.; Van Beeumen, J.; Herdewijn, P. *Chem. Eur. J.* **1998**, *4*, 425.
5. Ren, J.; Chaires, J. B. *Biochemistry* **1999**, *38*, 16067.
6. Bischoff, G.; Bischoff, R.; Birch-Hirschfeld, E.; Gromann, U.; Lindau, S.; Meister, W.-V.; Bambirra, S.; Bohley, C.; Hoffmann, S. *J. Biomol. Struct. Dyn.* **1998**, *16*, 187.
7. Harrison, J. G.; Balasubramanian, S. *Nucleic Acids Res.* **1998**, *26*, 3136.
8. Gonzalez, C.; Moore, M.; Ribeiro, S.; Schmitz, U.; Schroth, G. P.; Turin, L.; Bruice, T. W. *Nucleic Acids Res.* **2001**, *29*, 85e.
9. Boger, D. L.; Dechantsreiter, M. A.; Takahiro, I.; Fink, B.; Hedrick, M. P. *Bioorg. Med. Chem.* **2000**, *8*, 2049.
10. Guelev, V. M.; Harting, M. T.; Lkokey, R. S.; Iverson, B. L. *Chem. Biol.* **1999**, *7*, 1.
11. Hamy, F.; Albrecht, G.; Flörsheimer, A.; Bailly, C. *Bioch. Bioph. Res. Commun.* **2000**, *270*, 393.
12. Chaltin, P.; Lescrinier, E.; Lescrinier, T.; Rozenski, J.; Hendrix, C.; Rosemeyer, H.; Busson, R.; Van Aerschot, A.; Herdewijn, P. *Helv. Chim. Acta* **2002**, *85*, 2258.
13. Boger, D. L.; Fink, B. E.; Brunette, S. R.; Winston, C. T.; Hedrick, M. P. *J. Am. Chem. Soc.* **2001**, *123*, 5878.
14. Hamy, F.; Felder, E. R.; Heizmann, G.; Lazdins, J.; Aboul-Ela, F.; Varani, G.; Karn, J.; Klimkait, T. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 3548.
15. Cho, B.; Taylor, D. C.; Nicholas, H. B., Jr.; Schmidt, F. J. *Bioorg. Med. Chem.* **1997**, *5*, 1107.
16. For the ethidium bromide experiments, the same target sequence was used as originally applied in the gel shift screening experiments [5′-(AGATTGTGCAATGT)-3′:5′-(ACATTGCACAATCT)-3′]. Wells of Costar black 96-well plates were loaded with 2 μL of a 50 μM dsDNA solution, 2 μL of a 0.35 mM EtBr solution and a varying volume of oligopeptides (individual or mixtures) to obtain the necessary concentrations. The appropriate volume of a Tris/NaCl buffer (10 mM Tris–10 mM NaCl pH, 7.4) was added to obtain a total volume of 100 μL per well. Before adding the DNA to the wells, it was rendered double-stranded by placing equal amounts of the two complementary strands for 3 min at 80 °C, at room temperature for 5 min and at 4 °C for 20 min. After incubation at room temperature for 30 min, each well was read on a FL600 Microplate Fluorescence reader, with 530/25 nm as excitation wavelength and 590/35 nm as the emission detection wavelength. Two control wells (no agent = 100% fluorescence, no DNA = 0% fluorescence) were used per 12 samples. Fluorescence readings are reported as % fluorescence relative to the control wells. For dissociation constant determinations, several oligopeptide concentrations are used. Generally two to three sets of measurements were performed to calculate average values.