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REVIEW

Peptoids as potential therapeutics

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Peptoids are oligomers of N-substituted glycine units. These molecules are almost perfectly suited for combinatorial approaches to drug discovery because large libraries can be synthesized easily from readily available primary amines. Moreover, major advances in screening methodology have allowed peptoid libraries of hundreds of thousands of compounds to be mined inexpensively and quickly for highly specific protein-binding molecules. These advances and the potential utility of peptoids as pharmacological agents are reviewed.

Keywords Bio-inspired polymer, combinatorial chemistry, drug development, HTS, peptoid, peptide, peptidomimetic, pharmacology, synthetic library

Introduction

An estimated 300 to 500 biomolecules have been successfully targeted with small-molecule agents [1,2]. This number is astoundingly small when compared with the number of human genes that have been identified, as well as those genes identified from bacterial or viral pathogens. Thus, developing new methods and molecules that allow the currently large collection of 'undruggable' proteins to be targeted pharmaceutically is an important task. In particular, the ability to manipulate protein-protein interactions would enable new therapeutic possibilities.

Improvements in the targeting of protein-protein interactions are possible, although perhaps not with compounds that satisfy Lipinski's class rules, as it can be challenging for a small molecule to cover sufficient protein surface area to disrupt protein-protein interactions [3]. Screening programs to identify agents that are capable of disrupting certain protein-protein interactions have led to the discovery of several molecules [4], although the eventual success of most of these compounds as drugs remains uncertain. Peptides that bind to protein-protein interaction surfaces often can be identified, and a popular strategy among chemical biologists interested in disrupting protein-protein interactions is producing modified peptides or miniature proteins that mimic one of the native binding partners of a protein complex and contain some type of conformational constraint that induces a stable secondary structure, such as a helix [5-12]. However, these molecules often have undesirable pharmacokinetic properties, although some interesting exceptions are emerging [9].

One potential solution to the conundrum of undesirable pharmacokinetic properties has been to develop peptide-like molecules that combine the favorable protein-binding characteristics of peptides with the cell permeability and *in vivo* stability of more drug-like molecules. Ideally, these peptide-like molecules would also be modular and simple to manufacture, thus facilitating the optimization of lead compounds. Peptoids, oligomers of N-substituted glycine units, constitute one such class of molecules [13]. The invention of peptoids in the context of a biotechnology company, their decreased popularity as possible drug candidates (mainly for non-scientific reasons) and their recent resurgence as a research focus in academic laboratories, particularly with respect to the discovery of ligands to defined molecular targets, are discussed in this review. In addition, advances in combinatorial library screening technology that, while not restricted to peptoids, take advantage of the many favorable properties of these molecules are also described.

Initial peptoid development at Protos/Chiron

In 1988, Chiron Corp, one of the original biotechnology companies and now part of Novartis AG [14], spun out the chemistry start-up company Protos Corp to develop small-molecule drugs using Chiron's biopharmaceutical resources. Researchers at Protos employed the approach of mimicking the natural molecular diversity in biopolymers – combining a small number of chemical building blocks into an oligomeric sequence to generate a large number of distinct compounds [13]. This modular, or combinatorial, approach is efficient because only a single chemistry

requires optimization, and the same coupling chemistry is used to link every building block together. Given the favorable protein binding characteristics but unfavorable pharmacokinetic properties of peptides, research efforts were focused on developing peptidomimetic oligomers that retained the diversity of side chains and a polar backbone, yet were engineered to remove sensitivity to proteases. In particular, peptoids were targeted. These molecules closely resemble peptides, except that the side chains extend from the main-chain nitrogen rather than the α-carbon [13] (Figure 1A). Peptoids are achiral, protease resistant and adopt different conformations than peptides, yet still retain the same density of functionality and backbone polarity of peptides.

The initial synthetic targets were libraries of peptoid trimers. Side chains similar to those occurring in the natural amino acids were employed in the synthesis of these libraries. The original synthesis method was in direct analogy to Fmoc (9-fluorenylmethyl chloroformate) solid-phase peptide synthesis, and the generation of samples of 25 grams for 10 to 12 representative Fmoc-protected peptoid monomers was required [13]. The preparation of a sufficient quantity of all the monomers needed required almost 1 year, highlighting the practical problems that are inherent in large-scale combinatorial chemistry.

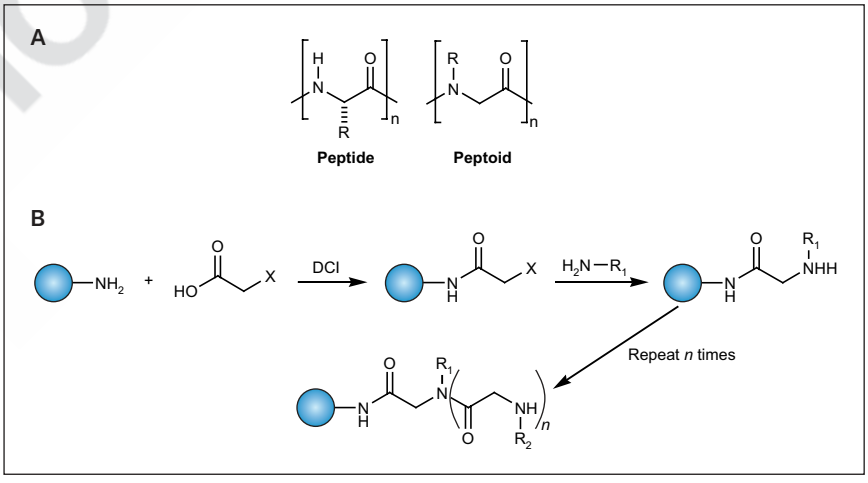
Researchers at Protos initially attempted to create soluble mixtures of compounds to feed automated HTS assays that had already been developed in-house. Libraries were created in this early period using a 'split and pool' approach (the solid-phase resin beads are separated into equal portions at the start of each coupling cycle, and then recombined), which was capable of generating compound mixtures of defined composition [15]. Automated synthesis equipment, built in-house, was employed to facilitate these efforts [16]. Equipped with this synthesis technology, combinatorial peptoid libraries could be created rapidly. The pace of synthesis resulted in Fmoc-protected peptoid monomers that required almost 1 year to be produced to be consumed in only a few days. Two bottlenecks in combinatorial peptoid drug discovery became apparent: (i) the rate of monomer consumption greatly outstripped the rate of monomer supply; and (ii) once activity was identified within a compound library, the identification of individual active compounds in the library (the process of deconvolution) would be laborious.

The submonomer method of peptoid synthesis

These bottlenecks stimulated research that led to a major breakthrough in 1991, when the solid-phase 'submonomer' method of peptoid synthesis was developed [17]. This approach considers the peptoid structure not as a homopolymer of N-substituted glycine units but, rather, as a copolymer alternating between acetate and amine units. The submonomer synthesis cycle consists of two chemical steps starting from a resin-bound amine: an acylation step using a diimide-activated haloacetic acid, followed by a displacement step using a primary amine (Figure 1B). For general use, bromoacetic acid is preferred. However, in cases in which there are side-chain functionalities that contain unprotected heteroatoms, particularly those occurring in many heterocycles (eg, imidazoles, pyridines, pyrazines and indoles [18]) the use of bromoacetic acid results in undesired side reactions from the alkylation of the heteroatoms. In these cases, choloroacetic acid can be used [19], thereby avoiding these unwanted alkylation reactions because of the reduced ability of the chloride to act as a leaving group.

The appeal of the submonomer approach is that side chains of interest require only a reactive primary amine in order to be incorporated into the peptoid structure. However,

Figure 1. Synthesis of peptoids using the submonomer method.



(**A**) General structures of peptides and peptoids. (**B**) The 'submonomer' solid-phase synthesis of peptoids. The blue sphere represents an amine-functionalized bead. R_1 and R_2 represent chemically diverse aliphatic, heterocyclic or aromatic side chain moieties.
X = Br or Cl, **DIC** *N,N'*-diisopropyl carbodiimide, **n** number of

not every amine can be incorporated in high yield; poor nucleophiles or hindered amines are incorporated slowly, as are amines with poor solubility. In addition, amines with competing pendant nucleophilic centers in the side chain must be protected. The displacement reactions are typically slower than the acylation step, requiring high concentrations of amine (~ 1 M) and reaction times of 20 min to 2 h at room temperature. Typically, acid-removable groups are used, so that resin cleavage and side-chain deprotection can occur simultaneously using trifluoroacetic acid. Nonetheless, hundreds of amines are commercially available in gram quantities that can be used directly and efficiently as submonomers [20].

The submonomer method of accessing synthetic oligomers of defined sequence is one of the most efficient techniques known in terms of coupling yields and monomer diversity/availability. The reactions are not air sensitive nor particularly moisture sensitive. Thus, the entire process can be readily automated and even adapted to almost any commercial peptide synthesizer, because peptide couplings also use a two-step monomer addition cycle [21]. Both the acylation and amination steps can be accelerated by microwave irradiation [22], which is particularly useful for otherwise slow couplings [23]. The longest peptoids reported in reasonable yield (that were produced in one continuous synthesis cycle) are approximately 50 monomers in length, suggesting that the yield for one coupling cycle is in excess of 99% [24]. The submonomer method is also unique in that no main chain-protecting groups are required, because reactive intermediates are attached to the resin. The mono-alkylation of an amine with an alkyl halide, for example, is difficult to achieve in solution, but on a solid phase can be readily achieved. The practical advantages of this synthetic approach are particularly well suited for the preparation of combinatorial libraries.

Peptoid drug discovery efforts at Chiron

Chiron acquired Protos in 1992 and transformed the company into its Small Molecule Drug Discovery division. To initiate the integrated drug discovery program, combinatorial compound mixtures were used because of the reduced upfront synthesis and screening effort required. The complexity of mixture was limited to a modest level (typically hundreds of compounds per pool). In 2002, macro-bead based methods were developed [25] that allowed the screening of smaller mixtures, or even individual compounds created via 'split and pool' synthesis to yield one bead, one compound (OBOC) libraries [26]. However, mixture screening only identifies active mixtures, and the identification of an active individual compound requires deconvolution. Library deconvolution was originally conducted by the iterative re-synthesis of successively smaller compound pools until a single active compound was identified [27].

Several chemically diverse peptoid libraries were synthesized at Chiron. Each library was constructed from a set of 15 to 20 monomers and was typically trimeric in order to limit the molecular mass. Sophisticated computational tools were also developed to help design the libraries [28]. By 1994, synthesis and screening efforts had yielded several potent (nanomolar) peptoid trimer ligands for G-protein coupled receptors [27] and the urokinase receptor [29]. The reports of an α-adrenergic receptor antagonist and a μ-opiate receptor antagonist were significant because these were the first reported demonstrations that a diverse combinatorial library of synthetic compounds could provide high-affinity ligands for pharmaceutically relevant receptors [27].

The critical question then became whether these peptoids possessed the appropriate pharmacokinetic properties to be considered as drug candidates. In 1996, an approximately one-nanomolar α_1-adrenergic receptor ligand was demonstrated to be soluble and metabolically stable *in vitro* and to have receptor antagonist activity in animals [30]. The intravenous administration of CHIR-2279, a peptoid trimer, to dogs antagonized the epinephrine-induced increase in intraurethal pressure (pA_2 = 6.86, compared with pA_2 = 7.71 for the standard anti-hypertensive drug prazosin). In both rats and guinea pigs, CHIR-2279 antagonized the epinephrine-induced increase in mean arterial blood pressure in a dose-dependent manner. These data suggested a good correlation between the *in vitro* and *in vivo* potencies of the compound. The rates of systemic clearance of CHIR-2279 following intravenous administration were 60 and 104 ml/min/kg in rats and guinea pigs, respectively.

More recently, another peptoid trimer, CHIR-5585, an inhibitor of the urokinase plasminogen activator receptor (uPAR) with activity in the sub-micromolar range *in vitro*, was also demonstrated to be an antagonist *in vivo*. The compound was administered to rats intranasally and observed to be delivered with high efficiency to the CNS [29]. Micromolar concentrations of the compound were detected in the olfactory bulbs, cortex, trigeminal nerve and deep cervical lymph nodes. These results suggest that intranasal delivery of peptoids is efficient and may deliver these compounds directly to the CNS, bypassing the blood-brain barrier.

A benzoylated peptoid trimer was evaluated by a group of researchers at Novartis Pharmaceuticals Corp [31]. The compound was administered to rats both orally and intravenously to study absorption and disposition properties. The extent of oral absorption was low (3 to 8%), which was consistent with the low absorptive clearance rate of the trimer (6.7×10^{-4} ml/min/cm) [31]. The peptoid was demonstrated to have good metabolic stability *in vivo* and was excreted intact in the feces. A quick clearance of the compound suggested rapid biliary excretion, which the investigators considered might be attributed to the overall high hydrophobicity of the compound [31].

These three animal studies of three different peptoid trimers do not provide sufficient data to make broad conclusions regarding the *in vivo* properties of peptoid drug candidates, particularly with regard to oral bioavailability, but do suggest some desirable attributes (eg, metabolic

stability) and some less-desirable attributes (eg, rapid clearance). Clearly, the choice of side chain must have a significant impact on the pharmacokinetic and pharmacodynamic properties of specific peptoids.

The peptoid program at Chiron had demonstrated that combinatorial library screening technologies could rapidly deliver hits to pharmaceutically relevant targets. Chiron decided not to pursue the development of peptoids as drugs, and instead chose to leverage its leadership position in the combinatorial technology arena. It is instructive to examine the apparent reasons behind this decision, as most were non-scientific.

The first issue was one of risk. Because almost all known successful small-molecule drugs have typically been conformationally constrained heterocyclic structures, there was no precedent for a compound resembling a peptoid oligomer that exhibited drug-like properties. The second issue was financial. The Small Molecule Drug Discovery division of Chiron was expected to be financially self-sufficient; gaining revenue from partnerships with other companies in the form of technology transfer deals was therefore necessary. Because there was a large demand from pharmaceutical companies to establish combinatorial synthesis efforts in-house, technology transfer deals were an obvious choice for Chiron to enable the company to become financially self-sufficient. The company provided a variety of solid-phase synthesis tools (synthesizers, devices and machines), protocols to make a wide variety of compound classes, computational tools to design compound libraries, analytical tools for identifying hit compounds, HTS strategies and, significantly, information on how these diverse efforts could be integrated into an efficient workflow. Several technology transfer deals were executed (eg, Jannsen-Cilag [32] and Ciba-Geigy Ltd [33]), which helped to not only disseminate the use of these techniques throughout the industry, but also to provide essential funding for Chiron's internal drug discovery program. This strategy was demonstrated to be successful, as many important small-molecule heterocyclic hit compounds were identified from Chiron's internal combinatorial drug discovery program. However, despite progression in the development of combinatorial library screening technologies, the development of peptoids remained limited.

Thus, in the early days of peptoid development, these compounds played a critical role in the development of combinatorial drug discovery techniques, primarily because of the efficiency demonstrated by submonomer chemistry. Peptoids are one of the fastest ways to 'prototype' new combinatorial approaches with synthetic molecules. However, the actual value of peptoids as therapeutic agents remains unclear and deserves further exploration; this is particularly true in the current pharmaceutical climate in which company pipelines are becoming smaller, and as important targets that function through protein-protein interactions are being identified.

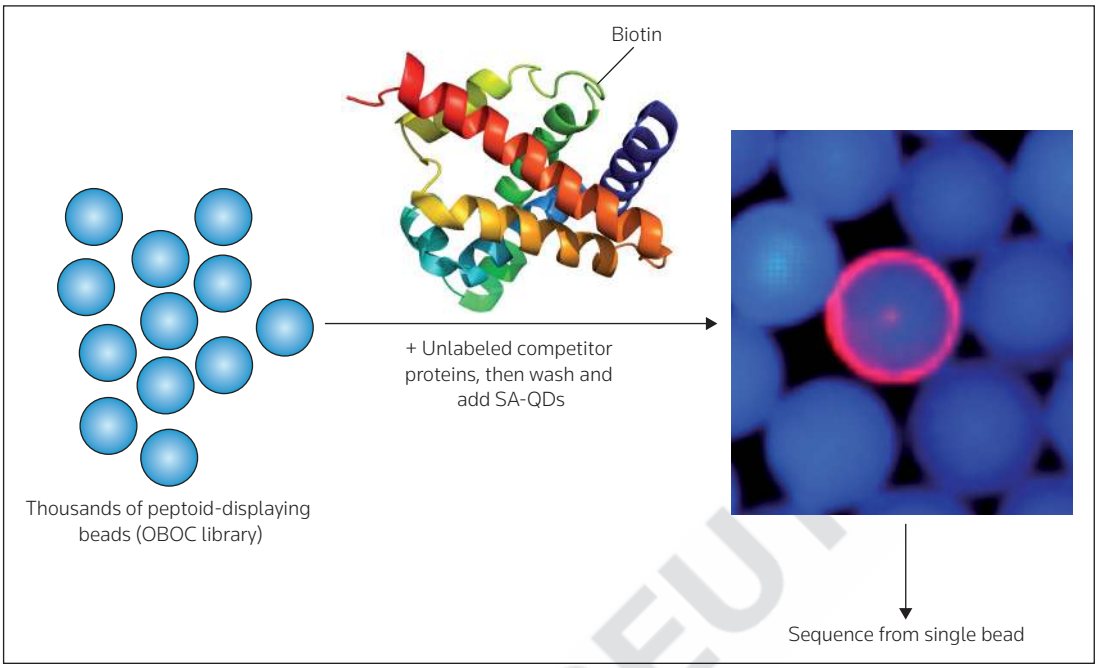
The second generation of research: Demonstrating the generality of peptoids

The pioneering studies at Chiron demonstrated that peptoids could be effective pharmacological agents with which to target cell surface receptors. Following these efforts, Montoliu *et al* created libraries of several thousand tripeptoids and pentapeptoids using the positional scanning format. Peptoid mixtures were screened successfully for several interesting activities. Among the molecules identified were peptoids that were capable of the prevention of neuronal excitoxicity [34], the attenuation of vanilloid receptor function [35], the inhibition of the proliferation of human neoplastic cells [36] and the blockade of multidrug resistance pumps [37,38]. The findings suggested that peptoid libraries were likely to be a general source of bioactive receptor ligands. The investigators also demonstrated that a highly diverse, but modestly sized, library (in most of these studies, 5000 to 10,000 compounds made from 22 different amines were screened) was sufficient to provide high-quality ligands. Most of the compounds isolated in these efforts were discovered to have potency in the low-micromolar range.

The third generation of research: Advances in screening technology

In 1991, a group at UT Southwestern Medical Center embarked on an effort that was initially focused on the discovery of large numbers of peptoid ligands for proteins, with the intention of using these ligands for the construction of protein-detecting microarrays [39,40]. The approach was modeled on the powerful OBOC screening methods that had been invented for synthetic peptide libraries [26], and differed in several ways from the strategies used previously [24,27,34-38]. First, and most importantly, screening was conducted by binding a fluorescently-labeled target protein to the bead-displayed peptoid library. After trial and error to optimize the system, including the type of beads used and the nature of the dye used to label the protein, a reasonably effective screening protocol was developed that involved exposing a Texas Red-labeled protein to a library of tens to hundreds of thousands of peptoids displayed on TentaGel beads [41]. Peptoids that bound the Texas Red-labeled target protein were identified by a visual examination of the beads under a fluorescent microscope, and were isolated manually using a micropipette. The visual contrast between 'hits' and 'non-hits' (the beads themselves emit an inconvenient level of autofluorescence) was later improved by employing a biotinylated protein and detecting its binding to the bead by subsequent hybridization with a streptavidin-coated red quantum dot (QD). When observed under a fluorescence (non-confocal) microscope, emission from the surface-bound QD is visible as an intense red halo around the blue-green autofluorescence of the bead, enabling straightforward visual identification (Figure 2). The second distinguishing feature of this screening protocol was the sequencing of peptoids by Edman degradation [41]. Thus, a protocol was developed in which the bound proteins were stripped from the bead with 1% SDS and the bead was

Figure 2. Screen of a one bead, one compound (OBOC) peptoid library against a biotinylated protein doped into a complex mixture of unlabeled proteins.



The binding of a biotin-labeled protein to a 'hit' bead is visualized by subsequent incubation with streptavidin-coated quantum dots (**SA-QDs**). A fluorescence micrograph of an actual screening result is shown. The sequence of the peptoid on the bead is then determined by Edman degradation or mass spectrometry.

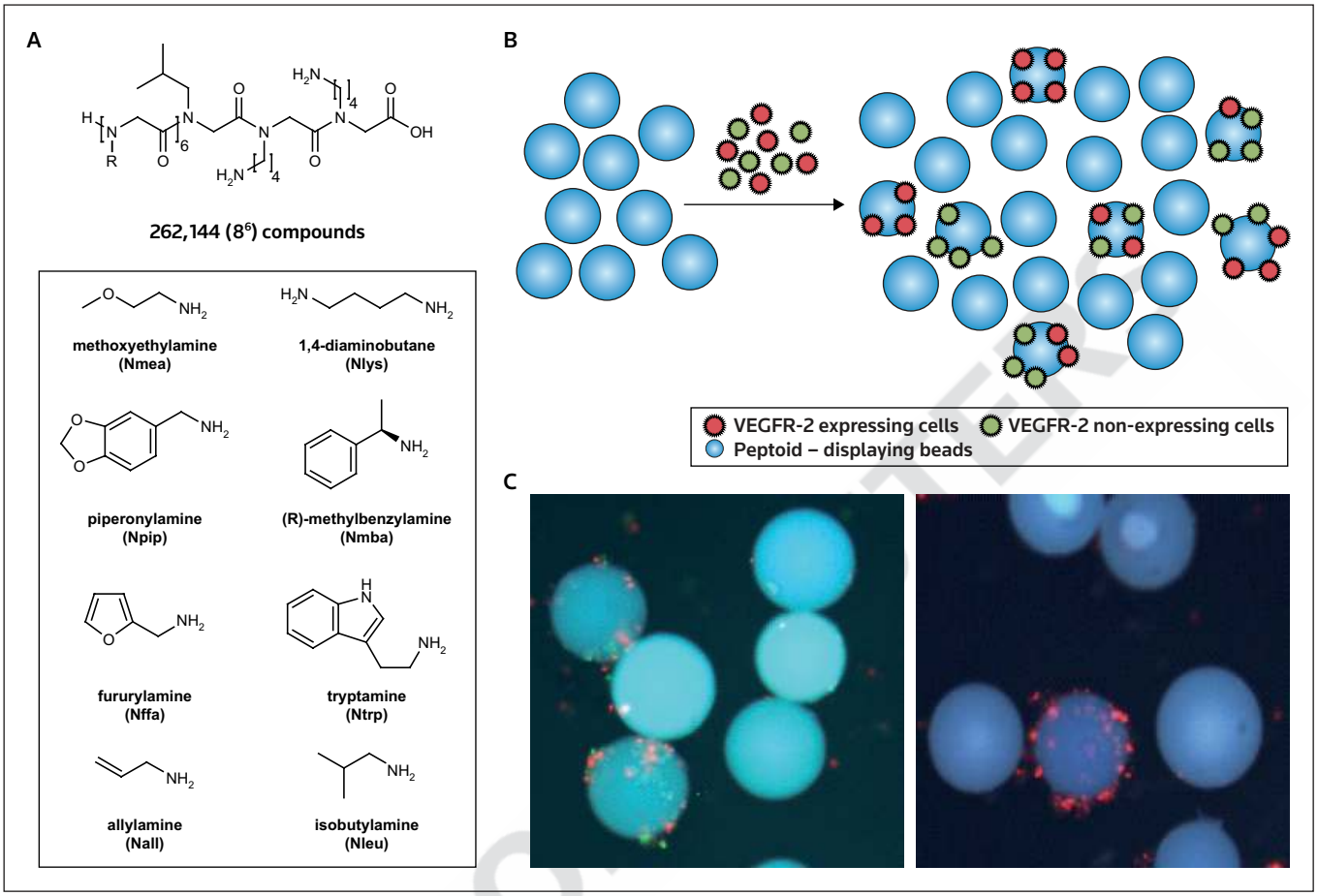
placed in the cavity of an automated Edman sequencer and characterized. Alternatively, if the peptoid was released from the bead, the sequence could be deduced by tandem mass spectrometry (MS) [42] or by partial Edman degradation/MS [43].

This on-bead screening method obviates the need for the tedious deconvolution step that had been used in earlier research, as well as the need for library encoding [44] that is necessary for libraries of compounds that cannot be characterized directly from a single bead [45,46]. These bead-based screens are also cheaper than high-throughput functional screens. However, hits from a binding screen may not be agonists or antagonists; they could simply be innocent ligands. The nature of the interaction can be determined easily and cheaply by functional testing of the few hits that arise from a binding screen. In addition, the conditions of a binding screen can be altered in almost any way desired, the most important consequence of which is that heavy demands can be placed on binding specificity for a compound to score as a hit. Indeed, the inclusion of a large excess of a highly heterogeneous mixture of unlabeled competitor proteins (eg, *Escherichia coli* cell lysate) increased the specificity of the ligands isolated in these screens, presumably by suppressing the tendency of relatively 'greasy', non-specific molecules from sequestering the target protein [41]. Such greasy compounds are a major challenge of common functional screening assays and consume substantial research time and effort in subsequent validation efforts; however, crude extracts cannot be added to most enzyme assays.

Using this on-bead screening approach, peptoid ligands for several soluble proteins, some never before targeted with a synthetic molecule, were isolated [41,47-52]. In general, all of the molecules isolated were ligands with excellent specificity for their target protein (ie, in the micromolar range). Using more rational methods, other researchers have also designed protein-binding peptoids or peptoid-containing compounds [53-55]. Some of these peptoids have been validated as being active in cell-based assays, demonstrating the cell permeability of peptoids [56], but their activities *in vivo* and pharmacokinetic properties have not been studied.

An advance in the bead screening technology was reported recently that may represent a general method for the isolation of a peptoid ligand for any cell surface receptor [57]. Integral membrane receptors are difficult to handle biochemically (because of their poor solubility) and thus could not be easily employed in the standard assay used for soluble proteins, but previous research had demonstrated the feasibility of conducting bead binding assays with living cells carrying the target receptor [58-60]. The novel feature of this new screen was the use of a two-color method that required the peptoids isolated as hits to display extremely high specificity for the target receptor, in this case VEGFR-2. Cells lacking VEGFR-2 were labeled with a green QD, and cells that carried VEGFR-2, but were otherwise identical, were labeled with a red QD. The QDs were taken up by endocytosis and therefore did not contaminate the cell surface. The cells were then suspended, mixed in an approximately 1:1 ratio, and incubated with

Figure 3. A two-color method to screen a one bead, one compound (OBOC) peptoid library against an integral membrane receptor for highly specific ligands.



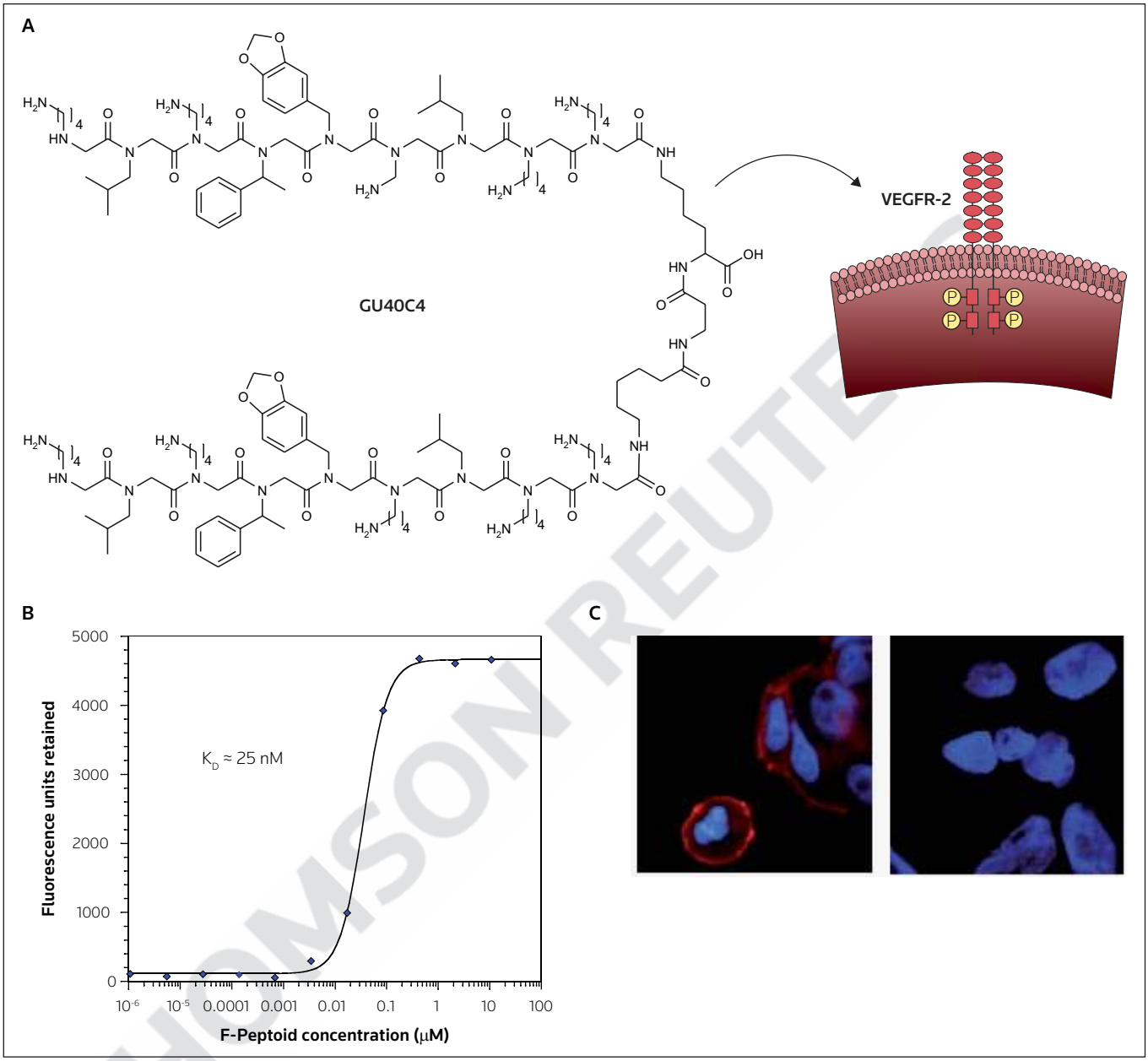
(**A**) The library used in a screen against VEGFR-2 and the amines employed in the construction of the library (shown in box). The diverse portion of the library consists of 8 monomers randomized at each of 6 positions, resulting in 8^6 or 262,144 possible compounds. (**B**) A schematic diagram of the screening protocol in which peptoid-displaying beads are exposed to cells that do or do not contain the target receptor. The green quantum dots (QDs) label cells lacking VEGFR-2 and the red QDs label cells carrying VEGFR-2. Beads that bind only the receptor-containing cells are collected. (**C**) Fluorescence micrographs of some of the beads after incubation with green-stained cells lacking VEGFR-2 and red-stained cells containing VEGFR-2. The left micrograph shows a field containing two beads that bound both cells lacking and containing VEGFR-2. The right fluorescence micrograph (one of the five hits obtained in this screen) shows beads that bound cells containing VEGFR-2.
(*Adapted from from the American Chemical Society and Udugamasooriya DG, Dineen SP, Brekken RA, Kodadek T:* ***A peptoid 'antibody surrogate' that antagonizes VEGF receptor 2 activity.*** *J Am Chem Soc (2008) 130(17):5744-5752. © 2008 American Chemical Society*)

approximately 300,000 beads displaying nonameric peptoids in which the six N-terminal residues had been randomized using eight different amines (Figure 3). After incubation under carefully controlled conditions to minimize non-specific adhesion of cells to the beads, the collection of beads was washed and inspected under a fluorescent microscope (Figure 3). Hundreds of beads that bound both red and green cells were observed. These beads presumably displayed a peptoid that recognized a molecule other than VEGFR-2. Five beads bound only red cells, suggesting that these beads were specific VEGFR-2 ligands. Subsequent *in vitro* binding studies confirmed that all five beads were ligands for the VEGFR-2 extracellular domain (ECD) at concentrations in the low-micromolar range [57] and that all bound a common site of the ECD that was distinct from the hormone-binding pocket [61].

One of the VEGFR-2-binding peptoids, GU40C4, was elaborated into a high-affinity ligand (K_d~ 25 nM) by dimerization, taking advantage of the knowledge that VEGFR-2 acts as a dimer (Figure 4) [57]. As anticipated, from the extreme stringency of the screen, subsequent staining experiments revealed that the dimeric peptoid only recognized cells that expressed VEGFR-2 (Figure 4C) [57].

This high-affinity dimer was demonstrated to be an antagonist of VEGFR-2-mediated angiogenesis in cell culture assays as well as in a mouse tumor model. In the animal experiment, the peptoid was delivered slowly over 20 days via an implanted Alzet pump [57]. A detailed pharmacokinetic evaluation of the study was in progress at the time of publication; according to the functional data available, the peptoid inhibited tumor growth for several days following

Figure 4. A high-affinity VEGFR-2-binding peptoid, GU40C4.



(**A**) Structure of GU40C4, a peptoid dimer that binds the dimeric VEGFR-2 with high affinity. (**B**) Data obtained from an *in vitro* binding assay that monitored binding of fluorescently labeled GU40C4 to immobilized VEGFR-2 extracellular domain with an approximate dissociation constant (K_d) value of 25 nM [52]. (**C**) Fluorescence micrographs of cells incubated with biotinylated GU40C4 and streptavidin-coated red quantum dots. The cells in the left panel are MCF-7 breast cancer cells that are known to express VEGFR-2. The cells in the right panel are HeLa cells, which do not express VEGFR-2. The nuclei of the cells were stained blue with 4',6-diamidino-2-phenylindole (DAPI). **F-peptoid** fluorescently labeled GU40C4

the end of the 20-day administration period. Similar to the early research from Chiron, this study suggests the potential pharmacological utility of receptor-targeted peptoids.

Conclusion: Prospects for the next generation of research

The currently available technology for creating and screening peptoid libraries is powerful. The question remains, however, as to whether peptoids will eventually be developed into useful pharmaceutical compounds or whether these molecules can, at least, be employed as potent 'tool compounds' with which to explore biological mechanisms [62]. Both possibilities appear to be feasible, but considerable further research remains to be completed.

Molecules that arise from primary screening efforts are usually ligands with activities in the low-micromolar range; peptoids are no exception. Thus, a key goal is the

development of a rapid and efficient method with which to develop these hits into more potent lead compounds, by first identifying the critical residues that are important for protein binding [63,64], and then optimizing these leads. Peptoids appear to be ideally suited to becoming lead compounds, given their regular structure and the availability of structurally diverse amine building blocks; however, a clear demonstration that the development of potent leads can be performed quickly and effectively has not yet been reported in the literature. Another outstanding question will be whether peptoids have appropriate pharmacokinetic properties to be useful therapeutics. As described above, only a small amount of investigation has been completed at Chiron and in the academic arena. The next 5 years should be instructive regarding the demonstration of peptoids as pharmacological agents.

Disclosure

Ronald N Zuckermann was a Research Scientist at Protos and from 1989 to 1992. After the Protos/Chiron merger Dr Zuckermann continued research in the Small Molecule Drug Discovery group at Chiron, and ultimately became a Chiron Research Fellow in 2003 in the BioPharmaceticals Division. Dr Zuckermann left the company in 2005 just prior to the Chiron/Novartis merger.

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• of special interest

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