

Chimerism

A Strategy to Expand the Utility and Applications of Peptides

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Summary

The modular nature of peptides can be exploited in the synthesis of chimeric sequences that combine diverse motifs in a single molecule. A theoretical consideration of the classification of peptides further expounds the multigeneric nature of peptide chimeras. Strategies for chimeric peptide syntheses include the chemical cross-linking of monomers and tandem combination by conventional SPPS. Additional details of chimeric peptide synthesis are also provided elsewhere in this volume. This chapter also explores some of the more common applications of chimeric peptides with particular emphasis on the molecular pharmacology of sequences that include address motifs for G protein-coupled receptors. Specific details of the biological properties of chimeras containing mastoparan, an amphiphilic tetradecapeptide component of wasp venom, further illustrate the novel and often unpredictable biological actions of chimeric constructs. These and numerous additional studies confirm that chimerism is an established strategy for the synthesis of molecular probes and bioactive agents.

Key Words: Bradykinin; chimerism; galparan; G protein-coupled receptor; ligand binding; mastoparan; secretion; vasopressin.

1. Introduction

The continual and necessary refinement of both synthetic methodologies and materials has clearly established peptide synthesis as a common discipline at the interface of chemistry and biology. Predictably, the range of applications in which peptides have demonstrated utility has also rapidly increased in recent years. The Peptide Institute, Protein Research Foundation (PRF), Osaka (www.

prf.or.jp) currently lists 233,109 entries (83,164,416 residues) in a database of real and predicted peptide and protein sequences (SEQDB). A similar number of entries (298,109) are also to be found in the database of synthetic compounds that includes unnatural amino acids and chemically synthesized peptides (SYNDB). Though biologically active peptides usually comprise fewer than 50 residues, mostly coded or proteinogenic amino acids, the PRF revised its definition of the term “peptide” in 1998 to also include sequences of 50 to 100 residues.

The size differential between a large peptide of 100 residues and a small protein, or perhaps a protein domain, is a purely arbitrary distinction. However, both (1) the technology-driven acceleration in the rate of new peptide generation and (2) the increased size limit from 50 to 100 residues will vastly increase the number of sequences that need to be accommodated within the PRF databases. The problem is exacerbated by the fact that there is a temporal continuum to peptide production. Thus, it is impossible to know how many *bona fide* peptides are missing for the existing databases. Many of these sequences will have been erroneously produced and immediately discarded or simply never reported in the scientific literature. Some of these unaccounted-for peptides have no doubt been physically lost and now exist only in a virtual sense. Should peptide databases include ghosts? Unfortunately, such questions are relatively trivial when one considers future implications. The total number of all possible peptides must be finite, but what a number! Moreover, it is entirely reasonable to speculate that the sequences contained within the two current PRF databases are but a tiny fraction of this total.

Databases are most useful if they can be interrogated to extract information and provide answers to simple questions. These processes might themselves promote a logical subdivision of the database, a reactive classification of entries. Alternatively, some form of comparative analysis could be used to provide order to the database, a more proactive process of classification. The question arises as to how might all peptide sequences be arranged to provide some degree of logical order. One possibility would be to compare only primary sequences, a primitive though exhaustive form of parsimony analysis. Such an approach would assemble a few clusters of sequence-related peptides, such as opioid receptor ligands. The problem is, however, that sequence analysis would not identify most groups of peptides with similar biological roles. The diversity of sequences pertaining to peptide hormones and neuropeptides is testimony to this point. Thus, a meaningful subdivision of the database of all peptide sequences would require the use of a complex algorithm that compares sequence, structure, and biological activities.

The next question to arise from contemplation of the database of all peptide sequences is how best to represent relationships between different sequences.

Two dimensions are clearly insufficient to describe all possible relationships among all possible sequences. One approach to this problem is to imagine all peptide sequences existing in a vast space through which it is possible to travel and explore their interrelationships; the hypothetical space of all possible peptide sequences (peptide space). A similar concept of a hypothetical space containing gene kits was eloquently developed as a model to explore genetic relationships and the origins of life (*I*), and the following description of peptide space owes much to this work. The borders around peptide space identify it as a subdivision of organic chemical space, a larger volume containing all possible carbon-based chemical entities. Within peptide space one might imagine each hypothetical peptide sequence to exist within a stackable box that contains a small-scale homogeneous reference sample and documentation providing contemporary synthetic instructions and appropriate analytical data (m.p., AA analysis, mass spectra, etc.). Moreover, the number of opened boxes colored green, representing those sequences that have been synthesized and might be found in the SEQDB and SYNDB databases, would be mere specks of color in a 3D (or 4D) spatial continuum of unopened red boxes. We might also assume that peptide space is organized by the complex algorithm required to take account of sequences, structure, and biological roles. By analogy to the even larger space of all possible molecules, in which we find clusters of opened boxes containing broadly similar polymeric structures (genes, proteins, lipids, plastics, rock crystals, etc), the distribution of green boxes in peptide space is certainly not random.

As we travel through peptide space we observe clusters of green boxes indicative of a higher order of organization. This spatial organization of peptides into representative groups, a kind of retro-molecular-cladism, is a physical map of the different functions and/or applications of mostly extant peptides. Depending on the bias of the sorting algorithm, we might logically expect to find clusters of green boxes that could include groups of peptides such as hormones, neuropeptides, enzyme inhibitors, and secondary structure mimetics. The spatial relationships between boxes will of course require constant modifications and fine-tuning as more data become available to influence the sorting algorithm. It is intriguing to consider how Merrifield's introduction of solid phase synthesis, and significant modifications including the Fmoc strategy (*see* Chapter 1), has accelerated the rate of box opening.

One additional important concept to consider is that of peptide classification. If each sequence in peptide space is considered an individual *species*, then our clusters of green boxes are representative of a higher order of classification, the *genus*. With this concept in mind, **Table 1** is an attempt to produce a *generic* classification of peptides. The number of individual species in each genus is, of course, variable and no doubt the future will see the *evolution* of new genera

Table 1
A Possible Classification Scheme of Peptides

Class. biologically active sequences

Order. endogenous mediators

- Hormone
- Neuropeptide
- Pheromone
- Toxin
- Protein fragment
- Natriuretic
- Cytokine
- Chemokine
- Growth factor

Order. antagonists and inhibitors

- Receptor antagonist
- Integrin antagonist
- Enzyme inhibitor
- Antimicrobial
- Antitumoral
- Antibiotic

Order. protein modulator

- Ion channel modulator
 - Enzyme modulator
 - Receptor modulator
 - Modulator of protein–protein interaction
 - Molecular switch
 - Secretagogue
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and higher orders recognizable as green clusters and their interrelationships in the red continuum. An algorithm that compared only primary sequences would produce an entirely different classification; some peptides have multiple biological actions allied to multiple genera.

One might also imagine the dramatic increase in volume of the space of all possible peptides required to accommodate the PRF’s redefinition and expansion of qualifying sequence length. On the contrary most, if not all, chimeric peptides studied to date already existed in the pre-1998 space, but we have only yet opened a very small fraction of the total number of dusty red boxes containing this intriguing class of biomolecule. I use the term “class” here in a strictly nonhierarchical, nonbiological sense as the majority of chimeric pep-

Table 1 (Continued)*Order. cellular delivery system or component*

- Cell-specific homing sequence
- Organelle targeting sequence
- Signal sequence
- Cell-penetrating vector
- Delivery vehicle

Order. immunological peptide

- Antigen
- Immune cell active sequence
- Chemotactic
- Immunosuppressant
- Antibody fragment

Class. nonbiologically active sequences*Order. carrier peptide*

- C-peptide
- Neurophysin
- Preprohormone
- Prohormone

Order. structural mimetic

- Secondary structure mimetic
- Protein domain mimetic
- Enzyme substrate
- Pore former

Order. molecular support

- Linker
- Scaffold
- Regioselectively addressable functionalized template

This scheme is almost certainly incomplete and some genera, including receptor antagonists, can be further divided using a variety of classification criteria. There are also many other peptides generated by the enzymatic processing of larger precursors that might be included in the order of carrier peptides.

tides synthesized and studied to date are essentially hybrid species comprising recognizable sequences from two or more ancestors, often from different genera. A relatively simple algorithm that considers only sequences would have little organizational exasperation with chimeras. However, the complex algorithm that organized peptide space using sequence, structural, and functional data might have labored to accommodate single boxes containing chimeric peptides. As we shall see, chimeric peptides can display multiple biological actions

that may or may not reflect their ancestral components. Thus, peptide space might need to include multiple clones of some boxes to accommodate the multi-generic characteristics of their contents.

One purpose of this chapter is to highlight some key findings related to the properties and applications of chimeric peptides. No doubt the future will see a tangible change in the balance and distribution of green and red boxes in the subspace of all possible chimeric peptides.

1.1. Development of Chimeric Ligands for G Protein-Coupled Receptors

G Protein-coupled receptors (GPCRs) are the generic molecular target for approx 50% of all current drugs and a very common target for peptide mediators that include hormones and neuropeptides. Moreover, we can predict that more peptide mediators await discovery and that some of these will prove to be the ligands for the numerous orphan GPCR gene sequences identified by molecular cloning and *in silico* analyses of draft genome sequences. The differential expression of GPCRs in central and peripheral sites enables drug action to be selectively directed to appropriate cells and tissues.

The small nonapeptide hormones [Arg⁸]vasopressin (AVP; H-CYFQNCPRG-NH₂; intramolecular bond between Cys residues) and bradykinin (BK; H-RPP GFSPFR-OH) were among the first biologically active peptide hormones to be isolated some four decades ago, and are now known to collectively modulate most physiological and many pathological processes. Extensive characterization of numerous structural analogs of AVP and BK has since provided extensive knowledge of the SAR enabling both GPCR binding and activation (2,3). The molecular cloning of cDNAs encoding a variety of AVP and BK receptor subtypes confirmed that these proteins display the common 7-transmembrane architecture of rhodopsin-like (Type I) GPCRs (4,5).

The overwhelming majority of studies that defined, and continue to define, the SAR of peptidyl analogs of both AVP and BK have concentrated on the synthesis of individual nonapeptides or shorter deletion analogs (2,3). However, amino-terminal extended analogs of both hormones, including Val-Asp-AVP (6) and Lys⁰-BK (kallidin) (4), are also biologically active. A report in 1995 (7) also indicated that sequences derived from BK (residues 2–9 and 2–8) could be utilized in the design of galanin receptor antagonists as a carboxyl-terminal extension of galanin (1–13). (Indeed, my personal interest in chimeric GPCR ligands was fuelled by hearing Ülo Langel describe these and other studies at around the same time.) Intriguingly, the same galanin-derived sequence as an amino-terminal extension of mastoparan (MP), which has been labeled galparan, is a galanin receptor ligand and a potent secretagog (8). Observations

Table 2
Some Pharmacological Properties of Chimeric Ligands for GPCRS

Peptide Sequence	Comments
1. AVP-BK H-CYFQNCPRGRPPGFSPFR-OH	K_d B ₂ bradykinin receptor = 52 nM. 20% Antidiuretic activity of AVP; 20–30% activity of oxytocin in uterotonic tests; Hypotonic effect comparable to BK (35).
2. H22 [Phaa _D Tyr(Et) ² Arg ⁶]AVP(1-8)- εAhx- _D Arg ⁰ [Hyp ³ _D Phe ⁷ Leu ⁸] BK(1-9)	Combines a V _{1a} -selective vasopressin antagonist and a B _{2a} -selective antagonist joined by a flexible εAhx linker. High affinities for both V _{1a} (K_d = 0.3 nM) and B ₂ (K_d = 4.8 nM) receptors.
3. H28 [Phaa _D Tyr(Et) ² Arg ⁶]AVP(1-9)- DF-NH ₂	A V _{1a} -selective antagonist with a carboxyl-terminal extension providing the integrin anatgonist sequence <i>RGDF</i> . Retains high affinity (K_d = 0.4 nM) for the V _{1a} vasopressin receptor. Antagonizes both uterotonic (PA ₂ = 7.8) and pressor (PA ₂ = 7.2) activities of oxytocin and vasopressin respectively (35).

AVP-BK displays negligible affinity for neurohypophysial peptide receptors in binding assays (see **Subheading 3.2.**) but is an agonist at the B₂ receptor to which it binds with moderate affinity. However, bioassays indicate that AVP-BK possesses both antidiuretic and uterotonic activities. These observations might indicate some form of enzymatic processing to generate a smaller biologically active fragment. H22 and H28, high-affinity antagonists for the V_{1a} vasopressin receptor, present sequences of a B₂ receptor antagonist and a fibronectin-related integrin binding sequence respectively.

_DTyr(Et) is *O*-ethyl-D-tyrosine; εAhx is aminohexanoate; Phaa is phenylacetyl; Hyp is hydroxyproline.

such as these confirm the multigeneric character of chimeric peptides. Moreover, studies with similar peptides also lead to the discovery of the cell penetrant transportans (see Chapter 5).

A first report detailing some biological properties of chimeric constructs combining AVP and BK appeared in 1997 (9). The broad aim of these initial studies was to determine whether single peptides combining address sequences for AVP and BK receptors retained binding affinities. These and a variety of subsequent studies have clearly revealed that chimeric peptides can selectively bind GPCRs for both AVP and BK. **Table 2** reveals the structures and some biological properties of chimeric GPCR ligands that bind receptors for AVP and BK.

1.2. M375 and M391: Case Study to Illustrate Multigeneric Character of Chimeric Peptides

Additional details of these two peptides illustrate both the unique properties of the MP sequence and the fact that chimeric peptides interact with multiple protein targets.

M375 [Phaa_DTyr(Me)²Arg⁶Tyr⁹]AVP(1-9)-MP

M391 [Phaa_DTyr(Me)²Arg⁶Tyr⁹]AVP(1-9)-εAhx-MP

These are primary sequences of the chimeric peptides M375 and M391. _DTyr(Me) is *O*-methyl-D-tyrosine; Phaa is phenylacetyl; εAhx is aminohexanoate.

M375 and M391 were synthesized in Stockholm as part of a program to evaluate the biological properties of peptides combining sequences of GPCR receptor ligands with MP. [Phaa_DTyr(Me)²Arg⁶Tyr⁹]AVP, a linear antagonist of AVP, selectively binds the commonly expressed V_{1a} subtype of vasopressin receptor (10). M375 (K_d = 3.8 nM) and M391 (K_d = 9.0 nM) also display selective, high-affinity binding to the V_{1a} receptor (10). These data clearly indicate that substantial carboxyl-terminal extension is compatible with the binding of antagonists to the V_{1a} receptor protein. However, these peptides also interact with at least three other unrelated proteins. Both M375 and M391 are potent insulin secretagogues acting at a distal site in the secretory pathway distinct from that modulated by MP alone (11). These observations indicate that MP-containing chimeras translocate across the plasma membrane of cells to specifically interact with intracellular proteins that probably include heterotrimeric G proteins. This interesting property of MP chimeras has since been exploited in the development of transportan and its deletion analogs as a novel class of synthetic cell-penetrating peptide. M375 and M391 also interact with proteins involved in intracellular calcium homeostasis. Both peptides inhibit ER and SR Ca²⁺-ATPases and promote calcium release by activating the ryanodine receptor in vitro (12).

1.3. Other Chimeric Peptidyl Secretagogues

The observations that M375 and M391 are novel insulin secretagogues prompted more recent studies to engineer additional chimeric MP analogs that promote mast cell secretion (13). These studies identified differential peptidyl secretagogues (DPS) that selectively stimulate the exocytosis of 5-HT or the secretory granule marker β-hexosaminidase respectively. Moreover, many of these MP analogs are significantly more potent secretagogues than MP itself. Our recent contributions to this area have focused on chimeric peptides combining MP with either sequences related to small peptide hormones and neuropeptides or specific peptides known to modulate secretion. Of more than 70 peptides we have screened, MP S (INWKGIASMAibRQVL-NH₂), a synthetic MP analog con-

taining α -aminoisobutyric acid (Aib), a known helix promoter, is the most potent and selective activator of β -hexosaminidase secretion. In contrast, M436 (INL KALAALAKKILVTHRLAGLLSRVPTNVGSKAF-NH₂), a chimeric construct combining MP with sequences of human calcitonin gene-related peptide, is the most selective and potent activator of 5-HT secretion from RBL-2H3 (**13**). Intriguingly, M436 is also an antagonist of the CGRP₁-like receptor (**14**), further indicating that chimeric peptides can display both receptor- and non-receptor-mediated activities.

1.4. Additional Applications of Chimeric Peptides

Predictably, peptide chimeras have found utility for many additional applications other than those listed here. A detailed discussion of the growing literature pertaining to peptide chimeras, particularly GPCR ligands, is beyond the scope of both this chapter and this book. However, to partly address my own bias toward the study of peptide hormones and secretagogues, brief mention is made of a few notable examples of chimeric peptides using sequences derived from other peptide genera. The sequence and some properties of vasonatrin, a 27-AA chimera of atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP), were reported in 1993 (**15**). Intriguingly, in vivo studies indicated that vasonatrin possessed venodilatory actions (CNP), natriuretic actions (ANP), and a unique vasodilatory action in arteries characteristic of neither CNP nor ANP (**15**). A substance P-opioid chimeric peptide (YPFFGLM-NH₂) has been reported as a prototypic, ant-tolerance-forming analgesic (**16**). These unique properties of YPFFGLM-NH₂ appear to be the consequence of the co-activation of both μ -opioid receptors and substance P receptors in the spinal cord. Chimeric analogs of other neuropeptides that include opioids, deltorphins, Met-enkephalin, and FMRFa have also been developed as novel receptor probes (**17,18**).

There is no doubt that the range of peptide genera included in chimeric design will increase. One important development is the application of homing sequences to deliver peptides, drugs, or nanoparticles to tumor cells or neovasculature (**19–23**). Such sequences include (1) CGFECVRQCPERC, which binds lung endothelial cell membrane dipeptidase (**19**); (2) KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK, which binds cells and blood vessels in selected tumours (**20**); (3) CGNKRTRGC, which recognizes tumor cells in lymphatic vessels (**21**); and (4) SMSIARL, which binds to vasculature in the human prostate gland (**22**). These sequences, together with a range of GPCR ligands as reviewed in **ref. 24**, offer enormous potential for the selective delivery of cytotoxic agents to tumor cells and neovasculature.

One final development worthy of note here is the development of novel anti-cancer agents that are dimeric bradykinin analogs. There has been a long-term interest in cross-linked kinin analogs as both agonists and antagonists of GPCRs

(9,25). However, more recent studies have indicated that dimeric bradykinin antagonists such as CU210 specifically inhibit the growth of lung cancer lines (26). Such findings are particularly interesting since monomeric bradykinin agonists and antagonists lack this activity.

It is entirely reasonable to suggest that other chimeric peptides have intrinsic biological properties that await discovery through rational analyses. Increased understanding of these properties might provide generic guidelines for chimeric peptide construction. Moreover, as more data become available, peptide space will require constant reorganization to assimilate this new information. As the number of green boxes increases, their spatial organization may well indicate new peptide genera or, indeed, traces of some other hierarchical classification. However, it is very difficult to envisage when, where, and how such a process might end.

We know nothing of the practical or conceptual limitations of chimeric peptide design and two potential caveats are apparent.

First, how should the algorithm that organizes peptide space deal with failures? The chimeric peptide vasokinin, BK(1)-AVP(2-5)-BK(6-9) (H-RYFQN SPFR-OH), combines motifs from the amino and carboxyl termini of BK with a middle section from AVP. The peptide was conceived as a putative GPCR ligand but lacks affinity for receptors that bind BK or AVP (27). However, vasokinin is a competitive inhibitor of angiotensin-converting enzyme and could well display other biological activities that remain to be discovered. Where might vasokinin be located in the subspace of chimeric peptides, and should there be multiple boxes?

Second, it is most likely that the peptide classification scheme presented here will soon be outdated. Indeed, any such scheme is largely subjective and I apologize here for any notable omissions or inconsistencies. Future developments will no doubt require an increasingly complex classification system. Imagine the futility of attempting a rational classification of an animal kingdom that contained organisms with every possible body plan ranging from unicellular to multicellular, from diploblastic to triploblastic, and beyond. A multitude of reproductive strategies would ensure that our all-encompassing animal kingdom would collectively occupy every conceivable niche the home planet could offer. Thus, in a sense it is the missing animals that enable a logical and hierarchical classification of this planet's fauna. Evolutionary pressures will no doubt influence the opening of boxes in peptide space with successful sequences and new discoveries serving as templates for further modification and improvement. Certain clusters of green boxes will, therefore, rapidly expand in all dimensions at the expense of other domains that remain largely unexplored. However, we may reach a time when the sheer abundance of green boxes obscures any obvious spatial organization either of peptide space or its subspace of chimeric

peptides. Perhaps when the system becomes this complex it would be better to abandon any organizational algorithm using functional data and classify all peptides using primary sequence only. At this point in time the concept of chimerism would essentially become redundant and a single green box containing vaso-kinin would be located near other sequence-related nonameric peptides with quite different biological properties.

2. Materials

A generic list of materials pertinent to the detailed evaluation of chimeric peptide is provided.

1. Chimeric peptide(s).
2. Appropriate assay system components.

Clearly, a detailed list of all materials necessary to determine the many activities of chimeric peptides is not possible here. As this article has focused on chimeras that combine GPCR ligands with MP it is appropriate to include details of two assay systems that can be used to determine both receptor binding affinity and secretory efficacy. The details pertaining to receptor binding analyses relate specifically to studies with the vasopressin V_{1a} receptor (**5**), though they are broadly applicable to all other GPCRs that bind peptide ligands. The rat basophilic line RBL-2H3 is a convenient mast cell model for studying peptide-induced secretion of β -hexoseaminidase (**13**).

2.1. V_{1a} Receptor Binding Analysis

1. Rodent liver membranes (*see Note 1*).
2. Binding assay buffer: 20 mM HEPES, 10 mM $Mg(CH_3COO)_2$, 1 mM EGTA, 1 mg/mL BSA, pH 7.4.
3. Tritiated tracer ligand: $[^3H]AVP$ (agonist) or $[^3H][d(CH_2)_5[Tyr(Me)^2]AVP]$.
4. (V_{1a} -selective antagonist [**5**]) (*see Note 2*).
5. $[d(CH_2)_5[Tyr(Me)^2]AVP]$ (*see Note 2*).
6. Chimeric peptide(s).
7. Circulating water bath.
8. High-speed bench centrifuge.
9. Tissue solubilizer (Soluene 350).
10. Scintillation fluid.
11. Liquid scintillation counter.

2.2. β -Hexoseaminidase Secretion From RBL-2H3

1. RBL-2H3 cells in Dulbecco's modified Eagle's medium (DMEM).
2. Chimeric peptides.
3. Balanced salt solution (BSS).
4. 0.1% v/v Triton X-100.
5. 96-Well plates.

6. 1 mM *p*-nitrophenyl *N*-acetyl- β -D-glucosamide in 0.1 M sodium citrate pH 4.5.
7. 0.1 M Na₂CO₃/NaHCO₃, pH 10.5.
8. Microplate reader at 405 nm.

3. Methods

The provided methods describe (1) the design and synthesis of peptide chimeras, (2) the analysis of receptor binding affinity, and (3) β -hexoseaminidase secretion from RBL-2H3. These protocols have proven particularly useful for the development of both GPCR ligands (9) and differential secretagogues (13).

3.1. Synthesis of Chimeric Peptides

Chimeras synthesized by tandem linkage (*see Subheading 3.1.1.*) is the quantitatively dominant form of construct studied to date. The relative ease of synthesis using conventional methodologies (*see Chapter 1*) is one explanation for the relative abundance of tandem constructs. A brief discussion of homo- and hetero-dimeric peptides constructed by the covalent crosslinking of monomers is also provided, in **Subheading 3.1.2.**

3.1.1. Tandem Chimeric Constructs

M375 and M391 are examples of tandem constructs synthesized according to the following generic template:



This is a simple generic template for chimeric peptide construction. For the design of chimeric GPCR ligands, both address and message motifs have been used as sequences **A** and **B**. The physical linkage (**C**) joining sequences **A** and **B** is usually one of three chemical entities: (1) a simple peptide bond (e.g., M375); (2) a flexible aminohexanoic acid spacer (e.g., M391) designed to release the constraint of a relatively rigid peptide backbone; or (3) another amino acid or sequence of amino acids. Examples of the latter strategy include Gly-Gly, used to join the cytotoxic sequence *D*(KLAKLAK)₂ to a prostate homing sequence (22); and lysine, which provides a primary amine for the attachment of other moieties. A variety of common modifications at the amino terminal (**D**) and the carboxyl terminal (**E**) are feasible with conventional SPPS. Thus, it is clear that even the simple template indicated here offers enormous potential for generating a vast array of structural chimeras.

3.1.2. Cross-Linked Dimeric Peptides

The covalent linkage of peptide monomers is an alternative strategy for the synthesis of peptide chimeras. The formation of cystine via the reduction of paired Cys residues is perhaps the most commonly employed methodology for

achieving this strategy. Cystine formation can be achieved by simply mixing free thiol-containing peptides in weakly alkaline conditions. However, such a strategy is compatible mostly with the formation of homodimers, as multiple dimers are produced when mixing different thiol-containing monomers. The 3-nitro-2-pyridinesulfonyl (Npys) protecting group, first introduced by Bernatowicz et al. (31), is a particularly useful side-chain protecting group for Cys that provides an active leaving group to facilitate the formation of unsymmetrical disulfide bonds by Npys-thiol exchange. Such a strategy can be used to conjugate thiol-containing cargoes to transportans and other cell penetrating peptides. Further details of the use Cys (Npys) are provided in Chapter 5. The vast array of other strategies employed for the synthesis of chimeras from peptide monomers is beyond the scope of this chapter. Bifunctional cross-linkers can be used to join unprotected peptides together, while Tam's group (32) has developed a numerous chemoselective methodologies. The latter approaches, based largely on thiol and carbonyl chemistries (32), offer enormous potential for the covalent coupling of peptide monomers and are perhaps not as commonly employed as they deserve to be.

3.2. V_{1a} Receptor Binding Analysis

Competitive displacement of a radiolabeled tracer ligand (*see Note 2*) is the usual method to determine the receptor binding affinity of a GPCR ligand. Additional details of these methods are provided elsewhere (28).

1. Thaw rat liver membranes on ice and dilute in binding assay buffer to a final protein concentration of 0.208 mg/mL.
2. Add 10 μ L tracer ligand and 10 μ L competing peptide to membranes to give a final volume of 500 μ L, containing 100 μ g of membrane protein. Using this system a concentration of tracer ligand from $0.2\text{--}1.0 \times 10^{-9}$ M provides a good specific:nonspecific binding ratio. Unlabeled peptides are usually added at a range of concentrations from 10^{-11} M to 10^{-6} M.
3. Incubate samples in 1.5-mL Eppendorf tubes at 30°C in a circulating water bath. Equilibrium binding is achieved after 90 min incubation.
4. To separate bound and free radioligand, sediment membranes by centrifugation (5 min, 13,000g) and wash pellets twice.
5. Dry Eppendorf tubes and solubilize membranes with 50 μ L tissue solubilizer.
6. Add scintillation fluid and determine radioactivity by liquid scintillation spectroscopy.

K_d values of unlabeled peptides can be calculated from experimentally derived IC_{50} values by correcting for ligand occupancy of the tracer ligand according to the equation:

$$K_d = IC_{50} \times [K_d^*/(L^* + K_d^*)]$$

where K_d^* is the dissociation binding constant of the tracer ligand and L^* is the free tracer ligand concentration. L^* can be accurately determined by counting a 100- μ L aliquot of the supernatant after membrane sedimentation. A 1000-fold excess of a suitable high affinity antagonist (*see Note 2*) is usually included in the assay to define the nonspecific binding component of the tracer ligand.

AVP and its analogs are relatively stable when incubated with rat liver membranes; there is little or no degradation of peptides during the 90 min incubation required to ensure binding has reached equilibrium. However, other peptide ligands including BK are more labile in the presence of biological membranes and may require the inclusion of a cocktail of protease inhibitors to prevent ligand, and possibly receptor, degradation (**29**). It is also noteworthy that some chimeric GPCR ligands, including galanin analogs (**33**), bind with Hill coefficients significantly <1 indicating complex receptor interactions with more than one recognition site.

3.3. β -Hexoseaminidase Secretion From RBL-2H3

RBL-2H3 is a widely used model of mucosal mast cells (**34**) employed to study both constitutive and regulated secretory pathways. Significantly, RBL-2H3 does not respond to a range of polybasic peptides that promote secretion from other mast cells but is sensitive to MP and its analogs (**13**). The secretion of β -hexoseaminidase, a secretory lysosomal marker, is a convenient assay of the efficacy of peptidyl secretagogues that correlates with the activation of phospholipase D, enzyme intimately involved in regulated secretion (**13**). Secretion assays are generally performed in a 24-well plate format using subconfluent cell monolayers.

1. Add peptidyl secretagogues to cells for a period of 15 min at 37°C in a final volume of 0.25 mL.
2. Collect medium and remove cell debris by centrifugation.
3. Secreted β -hexoseaminidase is assayed in samples of cell medium. Cellular β -hexoseaminidase activity is determined by washing cells in BSS and adding 100 μ L of 0.1% v/v Triton X-100 to extract residual β -hexoseaminidase from cell monolayers.
4. Transfer 5- μ L samples of medium or cellular extract to 96-well plates and incubate with 20 μ L of 1 mM *p*-nitrophenyl *N*-acetyl- β -D-glucosamide in 0.1 M sodium citrate pH 4.5, for 1 h at 37°C.
5. Add 200 μ L of 0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.5, and determine β -hexoseaminidase activity by colorimetric analysis on a microtiter plate reader at 405 nm.

4. Notes

1. Rat liver is the most convenient source of tissue for the preparation of crude plasma membranes that contain the V_{1a} subtype of vasopressin receptor. Methods for the

production of rat liver membranes that can be conveniently stored at -20°C are presented elsewhere (28). Detailed protocols for the production of kidney medulla membranes of rodent and bovine origin are also available and such preparations are suitable for studying binding to V_2 vasopressin and B_{2a} bradykinin receptors (28,29).

- Both $[^3\text{H}]\text{AVP}$ and $[^3\text{H}][\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$, a V_{1a} -selective antagonist, are suitable radiolabeled tracer ligands for the V_{1a} receptor. A high concentration of $[\text{d}(\text{CH}_2)_5[\text{Tyr}(\text{Me})^2]\text{AVP}$ or another analog can be used to define the nonspecific binding of the radioligand.

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