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Insect Neuropeptide Antagonists

Abstract: *The development of a new integrated approach to the generation of a novel type of insect neuropeptide (Np) antagonists and putative insect control agents based on backbone cyclic compounds is described. The approach, termed the backbone cyclic neuropeptide-based antagonist (BBC-NBA), was applied to the insect pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family as a model, and led to the discovery of a potent linear lead antagonist and several highly potent, metabolically stable BBC antagonists, devoid of agonistic activity, which inhibited PBAN-mediated activities in moths in vivo. This review briefly summarizes our knowledge of insect Nps, describes the PK/PBAN Np family, presents the basic concepts behind the BBC-NBA approach, and introduces the advantages of this method for generation of Np agonists, antagonists and insecticide prototype molecules.* © 2002 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 60: 460–473, 2001

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INTRODUCTION

Insect neuropeptides (Nps) regulate many physiological and behavioral processes during development, reproduction and senescence, and maintain growth, homeostasis, osmoregulation, water balance, metabolism, and visceral activities. In the past two decades, a large number of insect Nps have been identified, some of which are similar in structure to vertebrate Nps. Despite the intensive studies of insect Nps, the Nps that mediate these biological activities and the mechanisms by which they exert their action are far from being fully characterized or understood. Understanding the mode of action of key Np families in insects is of major scientific value and carries a high practical potential. The multiple functional capacities of insect Nps make them a prime scientific target in advancing the understanding of the physiology of insects, and a prime practical target in the development of novel insect-control strategies based on interference with the steps associated with their activity.

The possibilities of gaining a better insight into the mode of action of insect Nps, and of exploiting them as novel means of pest management, rely primarily on our understanding the cellular basis of their activity. One way of getting a better insight into the mode of activity and the functional diversity of insect Nps is by the use of receptor-selective agonists and antagonists. Despite the high scientific and practical potential of agonists and antagonists, their application has not been widely implemented so far in insects, mainly because of the lack of defined methods for obtaining antagonists on the basis of a known Np agonist, and because of the inability to predict which conformation

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will lead to a highly potent inhibitory or stimulatory receptor-selective activity.

In the past few years, we have developed, in cooperation with the laboratory of Gilon et al., of the Hebrew University of Jerusalem, Israel, a novel integrated approach termed backbone cyclic neuropeptide-based antagonist (BBC-NBA), which overcomes the above limitations. This approach was applied to the insect pyrokinin (PK)/pheromone biosynthesis activating neuropeptide (PBAN) family, and resulted in the discovery of highly potent, stable, and selective antagonists.

This review briefly summarizes our knowledge of insect Nps, describes the PK/PBAN Np family, presents the basic concepts behind the BBC-NBA approach, and introduces the advantages of this method for generation of Np agonists, antagonists and insecticide prototype molecules.

INSECT NPS

The development of chemical, biochemical, and genetic engineering technologies, as well as the growing awareness of the major role Nps play in the physiology of organisms, has stimulated active interest in insect Np studies, and has resulted in the discovery of nearly 150 insect Nps in the past decade, most of which have been isolated from cockroaches (e.g., *Leucophaea maderae, Periplaneta americana*, *Diploptera punctata*), locusts (e.g., *Locusta migratoria* and *Schistocerca gregaria*), moths (e.g., *Manduca sexta*, *Bombyx mori*, and various Heliothinae species), and the fruit fly *Drosophila melanogaster*. Most of the Nps are specific to insects, although recent immunocytochemical studies have indicated the presence of molecules that are comparable with vertebrate Nps. Their function in insects has not yet been discovered.

Many insect Nps have been characterized, their amino acid sequences have been determined, and their c-DNA and genes have been cloned from various insect species. These studies revealed that some Nps may occur in multiple forms (e.g., adipokinetic hormones; allatostatins, myotropic, and FMRF-related peptides), which is a well-known phenomenon among invertebrate Nps. The multiple peptide forms are often encoded by the same gene, and result from repeated internal gene duplication and subsequent diversification. Np diversity can also result from duplication of the whole gene and subsequent mutations. Many Nps elicit more than one biological response in a given insect species or in other species, and several biological activities in a particular insect species may be regulated by more than one peptide. A detailed, well-documented review on the structural, biochemical, and physiological characterization of insect Nps has been presented by Gäde.[1]

Despite the enormous amount of structural information on insect Nps that has been accumulated in recent years, our understanding of their mode of action remains very limited and rudimentary. It is still necessary to develop additional in vivo bioassays that involve whole organisms, and to create novel tools and technologies in order to unravel the complex coordination of the many Nps involved in the regulation of the physiological processes and behavioral patterns. In addition, there is a need to study the cellular and molecular factors that underlie their activity, and to discover their pathways of synthesis and release and their targets of action. These issues are currently being addressed by means of highly advanced molecular biology and genetic engineering techniques, immunocytochemical studies, and advanced chemical, immunochemical, biochemical, and molecular methods. Of great importance are the studies of Np receptors, and the development of receptor-selective agonists and antagonists. While the study of insect Np receptors is gaining a lot of attention (to date, a dozen different insect Np receptors have been characterized in various laboratories, and recently 44 genes that represent the vast majority, and perhaps all, of the G-protein coupled receptors (GPCRs) encoded by the *Drosophila* genome have been reported[2]), only a few insect Np antagonists have been discovered. Currently, the only ones that have been reported are proctolin antagonists[3] and the PK/PBAN antagonists that were developed by means of the BBC-NBA approach.[4–8]

A summarized description of the BBC-NBA approach for the generation of selective, highly potent antagonists and their exploitation as research tools and as a basis for the design of novel, environment-friendly insect control agents is presented below.

BBC-NBA APPROACH

The BBC-NBA approach is based on backbone cyclization of peptides and the cycloscan concept (that were developed by Gilon et al.,[9] see below) and comprises the following steps (Figure 1):

1. Identification of the Np that controls the required function.
2. Elucidation of the shortest sequence of the Np that constitutes the active site.
3. Discovery of a peptidic linear lead antagonist on the basis of the sequence found in (2).

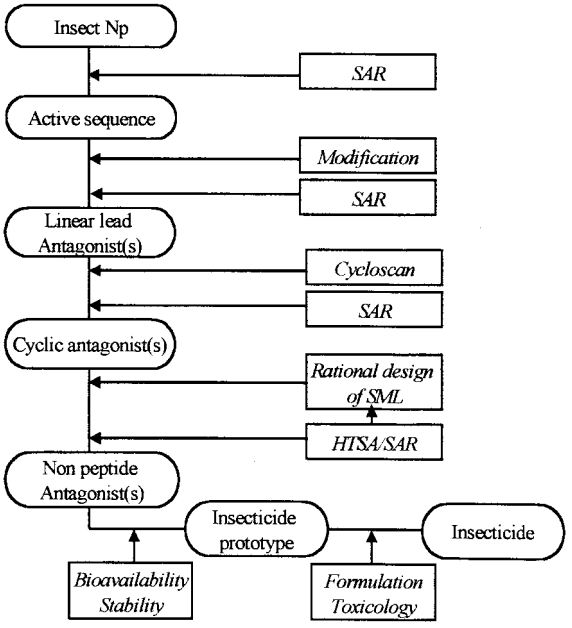

FIGURE 1 BBC-NBA approach for generation of antagonists and insecticide prototypes. HTSA: high throughput screening assay; Np: neuropeptide; SAR: structure–activity relationship; SML: small molecules.

4. Discovery of a potent BBC antagonist devoid of agonistic activity, based on (3).
5. Determination of the structural requirements for antagonistic activities, on the basis of (4).
6. Design and synthesis of small molecule combinatorial libraries of nonpeptidic compounds, based on the information obtained in (5).
7. Discovery of potent small-molecule nonpeptide antagonists out of the libraries in (6).

Further development of the antagonists obtained in (7) into insecticide prototypes involves the selection of the metabolically and environmentally stable, bioavailable, and cost-effective compounds for further formulation, toxicology, and field trials.

The BBC-NBA approach is dependent on the availability of simple and quantitative in vitro (e.g., binding assay) and/or in vivo bioassays for monitoring bioactivity; and on the availability of advanced chemical knowledge and techniques to design, synthesize, and determine the structure of the compounds of interest. The approach is general and can be applied to any Np.

The rationale behind the steps and the basic concepts that make up the BBC-NBA technology are described below.

Disclosure of the Active Sequence in the Np

There are two types of antagonists: the competitive antagonist, which binds to the same site as the agonist but does not elicit signal transduction; and the type that inhibits binding and/or signal transduction by means of allosteric effects. Antagonists of the first type are usually derived from agonists, but exhibit different structural and conformational features; those of the second type are usually nonpeptides, and are identified by screening of natural product libraries or combinatorial libraries. From this perspective it is clear that the prerequisite for the development of a competitive antagonist is the identification of the smallest active sequence in the Nps that binds and activates the receptor; this is the basis on which modifications can be made, that lead to the discovery of a lead antagonist.

Elucidation of the active site of a Np is achieved by means of structure–activity relationship (SAR) studies. In the case of large Nps (having more than 15 amino acid residues), peptide mapping is performed. This involves determination of the activities of peptide fragments containing up to 10 amino acid residues, which span the entire sequence of the Np. In cases of smaller Nps or when active peptides are discovered by peptide mapping, des-amino acid scanning is performed, namely, SAR studies of a library of peptides that lack one to five amino acids from either the C- or the N-terminus. It should be mentioned that in a Np family in which there is sequence homology among the various members, the homologous sequence is usually the active region. Once such a sequence has been revealed it is possible to continue to the next step: the discovery of a competitive lead antagonist.

Discovery of a Competitive Lead Antagonist

Lead antagonists are usually partial antagonists that bind to the receptor site but can only partially inhibit the transduction system. Discovery of a lead antagonist is a prerequisite for the further discovery of improved antagonists (that are highly potent, selective and devoid of agonistic activity), which can be achieved by SAR or by imposing conformational constraints on the lead antagonist molecules (see below).

Most of the lead competitive antagonists discovered up to now were based on vertebrate Np agonists. The empirical practices that emerged from these studies indicate that conversion of agonists to antagonists can be obtained when the one of the following occurs:

1. Naturally occurring L-amino acids are systematically replaced by their non-natural D- isomers or by amino acid residues with D hydrophobic amino acid residues, such as D-Phe or D-Trp.[10–23]
2. Amino acid residues are omitted from agonistic sequences, or functional side chains are omitted or replaced with other residues (as in [Sar^1]-angiotensin II (1–7)amide, where Asp^1 was replaced with Sar and Phe^8 was replaced with an amide group, or in [D-Phe^6,Des-Met^{14}]-bombesin (6–14)ethyl amide where the six N-terminal amino acids and Met^{14} were omitted).[24]
3. A C-terminal amide is replaced with a free acid (as in bombesin and gastrin).[25,26]
4. Peptide bonds are reduced (as in the case of bombesin).[27]
5. Conformational and/or topographical alterations are imposed on agonist molecules (for reviews, see Refs. 22 and 28–31).

Implementation of the above empirical practices for a given agonist necessitates detailed knowledge of the SAR and of any available information regarding the bioactive conformation.

To the best of our knowledge these approaches have been applied to a very limited number of insect Nps in order to discover antagonists. The D-Phe approach was applied to the insect Np proctolin and resulted in the discovery of a peptide with antagonistic activity,[3] and we recently discovered a lead antagonist for the insect PBAN by applying these empirical rules[5,6,8,32] (see below).

Improvement of the Antagonistic Activity by Conformational Constraint

Application of the above-mentioned approaches [(1)–(4) above] to various vertebrate Nps resulted in the discovery of linear peptide lead antagonists. Linear peptides are characterized by many limitations and are far from being ideal research tools, let alone insecticide prototypes, because of their high susceptibility to proteolytic degradation, their low bioavailability, and their lack of selectivity (which results from high conformational flexibility). An effective approach to overcome these limitations is the introduction of conformational constraint into the linear lead peptides, which leads to slower equilibrium rate, thus reducing the flexibility of the molecule.

Conformational constraint can be imposed by various methods (for reviews, see Refs. 28–30). Cyclization of peptides is one of the most common and most attractive methods to introduce conformational constraint into peptides, and thus to restrict their conformational space.[33] The conformational constraint confers the following attributes on the peptides: (a) Selectivity—the cyclic structure may restrict the conformational space to a conformation which mediates one function of the peptide and excludes those which mediate other functions. (b) Enhanced metabolic stability—the cyclic structure may exclude the conformation which is recognized by degrading enzymes from the conformational space, thus preventing enzymatic degradation. (c) Increased biological activity—the rigidified structure will be more potent than the linear one, since it spends more time in the bioactive conformation because of the much slower equilibrium between the conformations. However, this is only true when the conformational space of the cyclic peptide overlaps with the bioactive conformation. In most cases cyclization will yield an inactive peptide because of mismatching.[4] (d) Improved bioavailability—due to reduction of polarity reduction of polarity.

Nature has also chosen the cyclization route for restricting the conformation of peptides, and many natural cyclic peptides are known today, some of which are in therapeutic use (e.g., insulin, oxytocin, and cyclosporin). There are four modes of cyclization in peptides formed by (a) a covalent bond between two side chains (e.g., an amide bond between Asp and Lys, or a disulfide bond between two Cys residues); (b) covalent linking between the amino and carboxy termini of the peptide; (c) a bond between an Asp or Glu side chain and the amino terminus; and (d) a bond between a Lys or ornithine side chain and the carboxy terminus (for review, see Ref. 9).

The four natural modes of cyclization cannot easily be applied to most of the peptides, for two reasons: (a) not every peptide contains functional amino acid residues, which can be covalently interconnected; and more importantly (b) even when there are such residues, in most cases they are crucial for the biological activity of the peptide, and using them for ring closure causes a loss of or marked reduction in the bioactivity. The same holds true for the amino and carboxy termini, which might be important for the activity of naturally occurring peptides. In addition, the natural modes of cyclization result in only a small number of conformational combinations, which are too few to effectively screen the conformational space available for a linear peptide with a given sequence. In order to overcome the above-mentioned problems, the concept of backbone cyclization was developed by Gilon et al.[9]

Backbone Cyclization: A Tool for Imposing Conformational Constraint on Peptides

Backbone cyclization is a mode of cyclization that imposes conformational constraints on peptides without hindering their biological activity. Backbone cyclization involves covalent interconnection of the peptide backbone atoms (N^{α} and/or C^{α}) to each other, to side chains, or to amino or carboxy termini[9]—thus retaining the functionality and the activity of the side chains, and offering a great variability in the spatial orientation of the constituent residues. The backbone cyclization approach introduces many advantages to cyclic peptides over linear peptides (such as enhanced stability against proteolytic degradation, high selectivity, higher potency; and improved bioavailability[4,34–37]). The advantages introduced by the BBC peptides make them excellent research tools (especially for studying multipeptide families that exhibit functional diversity) and highly potent leads for the development of insecticide prototypes. In addition, BBC peptides have a constraint conformation that facilitates easy determination of their bioactive conformation by NMR[37–39] and x-ray analysis,[40] provided they are active as the endogenous (parent) peptide. This information is most important for further design of nonpeptide small molecules, which are the basis for the further design of insecticide prototypes (see below).

Cycloscan: Conformationally Constrained BBC Peptide Libraries

Another major problem that hampers the ability to obtain efficient Np antagonists arises from the lack of means to predict, a priori, the structure of an antagonist or to determine which conformations will lead to a highly potent inhibitory activity, even when leads are available. Lead antagonists have been obtained, so far, by two main strategies: (a) by serendipity from natural products, chemical libraries or phage display libraries, using multireceptor screening; and (b) by systematic SAR studies of the endogenous agonist and its analogs. As indicated previously, we have chosen, within the general framework of the BBC-NBA approach, to apply the second method, which leads to the discovery of a lead compound.

Once a linear lead compound has been discovered, its antagonistic activity can be improved (for selectivity and metabolic stability) by manipulations of its conformational constraints in combination with SAR studies. Improved antagonists can thus be obtained by application of the backbone cyclization method to the sequence of the lead antagonist. However, in order to obtain the optimal BBC peptide based on a given sequence, namely a compound that will best match the bioactive conformation (and thus exhibit the highest antagonistic activity), it is necessary to synthesize a large number of BBC peptides in order to screen the conformational space of the peptide in a systematic way. Cycloscan is the methodology that was developed by Gilon et al. for that purpose.[41]

Cycloscan is defined as a selection method based on conformationally constraint BBC peptide libraries with the aim of efficiently screening the conformational space and thus quickly achieving identification of a BBC peptide lead compound that overlaps the bioactive conformation. Cycloscan is performed by designing and synthesizing libraries of BBC peptides and screening them with the appropriate bioassay. The peptides in each library differ from each other in distinct parameters which affect their conformation and hence their bioactivity. This is achieved by the gradual introduction of discrete modifications that ensure efficient screening of the conformational space of the parent peptide. The majority of the peptides from such libraries should be inactive, because they do not overlap the bioactive conformation. However, the peptide(s) that do fit into the bioactive conformation should be very potent and should offer all the advantages mentioned above.

The main difference between a cycloscan library and normal combinatorial peptide libraries (either chemical or phage display) is that in the latter every peptide in the library has a different sequence, whereas in the former all the peptides in the library have the same sequence and they differ from each other only in their conformations. It is, therefore, possible to generate a large BBC library for each biologically active lead peptide discovered by peptide mapping or combinatorial libraries.

Cycloscan can be performed in either of two general ways: (a) sequence-biased cycloscan, in which all the peptides have the same primary sequence but differ in the size, chemistry, and location of the bridge; and (b) combinatorial cycloscan in which cycloscan may be further diversified by replacement of sequential amino acids. In cases where sequence-biased cycloscan is applied, the amino acid sequence of the linear lead antagonist is used as a basis for library construction. The diversity of sequence-biased cycloscan is not sequential but conformational, and includes the following modes: (a) the modes of backbone cyclization; (b) the position of the backbone bridge along the peptide sequence; (c) the size of the bridge; (d) the chemistry of the bridge. Each of these diversity parameters has been shown to affect the

Table I Amino Acid Sequence of PBAN and Peptides of the PK/PBAN Family[a]

Code Name	Species	Amino Acid Sequence
Hez-PBAN	*Helicoverpa zea*	LSDDMPATPADQEMYRQDPEQIDSRTKY**F**S**PRL**-NH_2
Bom-PBAN-I/MRCH	*Bombyx mori*	LSEDMPATPADQEMYQPDPEEMESRTRY**F**S**PRL**-NH_2
Bom-PBAN-II	*Bombyx mori*	RLSEDMPATPADQEMYQPDPEEMESRTRY**F**S**PRL**-NH_2
Lyd-PBAN	*Lymantria dispar*	LADDMPATMADQEVYRPEPEQIDSRNKY**F**S**PRL**-NH_2
Haz-PBAN	*Helicoverpa assulta*	LSDDMPATPADQEMYRQDPEQIDSRTKY**F**S**PRL**-NH_2
Agi-PBAN	*Agrotis ipsilon*	LADDTPATPADQEMYRPDPEQIDSRTKY**F**S**PRL**-NH_2
Mab-PBAN	*Mamestra brassicae*	LADDMPATPADQEMYRPDPEQIDSRTKY**F**S**PRL**-NH_2
Bom-DH	*Bombyx mori*	TDMKDESDRGAHSERGALC**F**G**PRL**-NH_2
Hez-DH	*Helicoverpa zea*	NDVKDGAASGAHSDRLGLW**F**G**PRL**-NH_2
Has-DH	*Helicoverpa assulta*	NDVKDGAASGAHSDRLGLW**F**G**PRL**-NH_2
Pss-PT	*Pseudaletia separata*	KLSYDDKVFENVE**F**T**PRL**-NH_2
Lom-PK-I	*Locusta migratoria*	pQDSGDGWPQQP**F**V**PRL**-NH_2
Lom-PK-II	*Locusta migratoria*	pQSVPT**F**T**PRL**-NH_2
Lem-PK	*Leucophaea maderae*	pQTS**F**T**PRL**-NH_2
Lom-MT-I	*Locusta migratoria*	GAVPAAQ**F**S**PRL**-NH_2
Lom-MT-II	*Locusta migratoria*	EGD**F**T**PRL**-NH_2
Lom-MT-III	*Locusta migratoria*	RQQP**F**V**PRL**-NH_2
Lom-MT-VI	*Locusta migratoria*	RLHQNGMP**F**S**PRL**-NH_2
Hez-MT-I	*Helicoverpa zea*	ME**F**T**PRL**-NH_2
Hez-MT-II	*Helicoverpa zea*	TMN**F**S**PRL**-NH_2
Bom-MT-III	*Bombyx mori*	TMS**F**S**PRL**-NH_2

[a] Bold letters indicate conserved amino acid sequences. DH: diapause hormone; MRCH: melanization and reddish coloration hormone; PBAN: pheromone biosynthesis activating neuropeptide; PT: pheromonotropin; PK: pyrokinin; MT: myotropin.

conformation and, hence, the biological activity.[4,34–36]

The concepts of backbone cyclization and cycloscan were initially used for obtaining conformationally constraint analogs of naturally occurring vertebrate Nps. The model peptide chosen for the development of these techniques was substance P (SP).[9] A variety of building units were prepared and incorporated into sequence-biased libraries of BBC SP analogs, and it was found that the cycloscan parameters such as ring size and ring chemistry had large effects on the activity of the various analogs.[34–36] Overall, the extensive research on BBC SP analogs has clearly proved the feasibility and effectiveness of the concepts of backbone cyclization and cycloscan, and has enabled these techniques to be applied to somatostatin[37] and recently to the insect family of PK/PBAN Nps[4,5,8] (see below). Several reason motivated us to chose the PK/PBAN family as a model for application of the BBC-NBA approach: (a) knowledge of the amino acid sequence and the large body of information derived from SAR studies; (b) the availability of biological assays which enable agonistic and antagonistic activities to be determined quantitatively; (c) the functional diversity of the family (which can be better understood with the aid of selective antagonists); and (4) the major role the PK/PBAN family plays in the physiology of moths and other insects. The last issue is of particular importance for the practical application of Np antagonists, as we anticipated that the availability of antagonists for this family may not only prove the feasibility of the BBC-NBA approach but may also result in the development of an important insect control agent. A brief description of the PK/PBAN family and its functional diversity is presented below.

PK/PBAN FAMILY

The PK/PBAN family of peptides (Table I) is a multifunctional family that plays a major role in the physiology of insects. PBAN, one of the first members of the family, was first reported by Raina and Klun[42] as the Np that regulates sex pheromone production in female moths (*Helicoverpa zea)*. Its amino acid sequence was determined in 1989 by Raina et al.,[43] and the peptide was termed Hez-PBAN (nomen-

clature according to Raina and Gäde[44]). Since then seven PBAN molecules have been isolated from six additional moth species, their entire primary structures have been determined,[45–50] and the c-DNA and genes have been cloned.[48–54] PBAN molecules were found to be C-terminally amidated Nps consisting of 33–34 amino acids, and their primary structure revealed that they share a high degree of homology and an identical pentapeptide C-terminal sequence (FSPRL-amide), which also constitutes the active core.[55–63] Since 1984, the presence of PBAN-like activity has been demonstrated in a variety of moths and in other non-Lepidopteran species, and its mode of action has been studied extensively (for reviews, see Refs. 1, 64, and 65).

Further studies on the regulation of sex pheromone biosynthesis in moths have revealed that this function can be elicited by additional Nps isolated from various insects, all of which share the common C-terminal pentapeptide FXPRL-amide (X = S, T, G, V).[66–70] Among these peptides are the PKs (Lem-PK, Lom-PK-I, and Lom-PK-II)[71,72] and the myotropins (Lom-MT-I to IV) (myotropic peptides isolated from the cockroach *Leucophaea maderae* and the migratory locust, *Locusta migratoria*),[73] Hez-MT-I and Hez-MT-II isolated from *H. zea*,[51] and Bom-MT-III isolated from the silk moth *Bombyx mori*.[52] For details on the amino acid sequences and the characteristics of these peptides, see Table I and Ref. 1] Additional members of this family containing the "signature" C-terminal sequence are pheromonotropin (Pss-PT), an 18-amino acid peptide isolated from *Pseudaletia (Mythimna) separata*,[74] and diapause hormone (Bom-DH), isolated from the silk worm, *Bombyx mori*.[75] These peptides have been designated the PK/PBAN family. In addition to their ability to stimulate sex pheromone biosynthesis in moths, members of this family have been found to control a variety of additional physiological and behavioral functions, such as melanization and reddish coloration in moth larvae,[58,76] contraction of the locust oviduct,[71] myotropic activity of the cockroach and locust hindgut,[71,77] egg diapause in the silkworm,[75,78] and acceleration of pupariation in the flesh-fly, *Sarcophaga bullata* larvae.[79] Studies performed in several laboratories including ours have shown that the peptides do not exhibit species specificity.[1]

The involvement of PK/PBAN Nps in the above functions was demonstrated by means of a variety of bioassays (pheromonotropic, melanotropic, pupariation, and myotropic) that have been developed and optimized in several laboratories.[42,58,71,76,79–83] All of the above assays are quantitative and enable determination of agonistic activities (by monitoring the intensity of the response of any tested compound and comparing it with that elicited under similar conditions by a standard stimulator, e.g., PBAN1-33NH_2 and/or LPK) or of antagonistic activities (by monitoring the ability of a given compound to inhibit the response elicited by a given stimulator).

Despite the intensive studies on the bioactivity of this family, very little is known about the endogenous mechanism, and much remains to be determined concerning the structural, chemical, and cellular basis of their activity. Most of the studies were performed with synthetic peptides and involved exogenous application (mainly by injection) of the tested compounds, which does not necessarily reflect the endogenous natural mechanisms. Furthermore, most studies were performed with linear peptides, which are highly flexible and have a large conformational space, which decreases their receptor selectivity. Currently, it is still not known which endogenous peptide(s) mediate(s) each of the in vivo functions, whether each function is mediated by a different peptide, and whether each peptide mediates one or several functions. It is also not clear whether these functions are mediated by the same receptor or by different receptors, or whether the receptors of the various PK/PBAN peptides share functional homologies.

The major role the PK/PBAN Nps play in the physiology of insects, and their functional diversity motivated us to obtain a better insight into the mode of action of this family, by the use of selective, conformationally constraint agonists and antagonists.

IMPLEMENTATION OF THE BBC-NBA STRATEGY FOR THE DISCOVERY OF RECEPTOR-SELECTIVE ANTAGONISTS FOR THE INSECT PK/PBAN FAMILY

The first stage involved the optimization of two in vivo biological assays (pheromonotropic and melanotropic) for evaluation of agonistic and/or antagonistic activities of linear and BBC peptides.[58,80] Once the assays were available, we synthesized a variety of linear peptides derived from the sequence of Hez-PBAN1-33NH_2, and identified (by SAR studies) the minimal active sequence of PBAN that constitutes the active core of the PK/PBAN molecule.[56–59] The sequence comprises the six C-terminal amino acids of Hez-PBAN1-33NH_2 (YFSPRL-amide), five of which constitute the "signature" common sequence of the PK/PBAN family. Based on the hexapeptide active sequence, a ''biased library'' of linear peptides was synthesized, in which each amino acid was sequentially substituted with the amino acid D-Phe. The

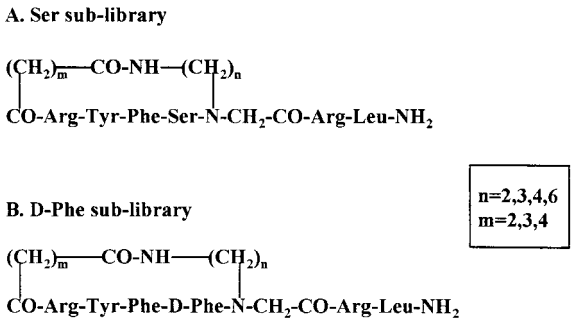

FIGURE 2 General structure of the BBC Ser (A) and the D-Phe (B) sublibraries of peptides.

peptides in the library were tested for their agonistic and antagonistic pheromonotropic and melanotropic biological activity, and a highly potent antagonist (RYFdFPRL-amide), capable of inhibiting sex pheromone biosynthesis by 80% (at 100 pmol)[5,6,8] and cuticular melanization by 46% at (1 nmol) (Altstein et al., unpublished) was discovered.

The sequences of the parent agonist and of the lead antagonist were used for the design and synthesis of two BBC peptide sublibraries (Figure 2). The first sublibrary (Ser sublibrary) was based on a slight modification of the C-terminal hexapeptide sequence (RYFSPRL-amide) of PBAN1-33NH_2, which was found to comprise the PBAN active core. The second sublibrary (D-Phe sublibrary) was based on the sequence of the lead antagonist: RYFdFPRL-amide. In both sublibraries, the Pro residue was replaced with the building units Fmoc-GlyN2, Fmoc-GlyN3, Fmoc-GlyN4, and Fmoc-GlyN6 (Fmoc: 9-flourenylmethyoxycarbonyl) (Figure 2). All the cyclic peptides in each sublibrary had the same primary sequence and the same location of the ring. The members of each sublibrary differed in their bridge sizes and in the position of the amide bond along the bridge. Screening of the two sublibraries for pheromonotropic antagonists resulted in the discovery of four compounds that fully inhibited sex pheromone biosynthesis at 1 nmol and were devoid of agonistic activity ($n + m$= 2 + 3; 3 + 2; 4 + 2; 6 + 2, see Figure 2). All antagonistic peptides originated from the D-Phe sublibrary. Substitution of the D-Phe amino acid with a Ser resulted in a loss of antagonistic activity.[4,5,7,8,32] Four precyclic peptides based on two of the BBC antagonists were also synthesized; their activity revealed that a negative charge at the N-terminus of the peptide abolished the antagonistic activity.[7] Assessment of the metabolic stability of the BBC peptides indicated that the peptides were very stable compared with their linear parent molecules (Figure 3).

The availability of the BBC libraries and the availability of a variety of bioassays for the PK/PBAN peptides facilitate further examination of the agonistic and antagonistic properties of these compounds with relation to additional functions (e.g., melanization, myotropic activity, and pupariation) elicited by PBAN1-33NH_2 and other peptides of this family (PBAN28-33NH_2; PT, PKs, and MTs). Along these lines we have recently tested the ability of the BBC peptides to inhibit sex pheromone biosynthesis elicited by PBAN28-33NH_2 (which constitutes the active core of PBAN). The data revealed that none of the BBC peptides (at 1 nmol) inhibited sex pheromone biosynthesis elicited by this peptide, hinting at the possibility that sex pheromone biosynthesis is mediated by at least two different receptors. We have also evaluated the ability of the BBC peptides from both sublibraries to inhibit melanotropic activity elicited by PBAN1-33NH_2. The results revealed five compounds that inhibited melanin formation (at 1 nmol) by 70–98%, and were devoid of agonistic activity ($n + m$ = 2 + 3; 3 + 2; 3 + 3; 4 + 2; 6 + 2, see Figure 2). As in the case of the pheromonotropic antagonists, all of the inhibitory compounds originated from the D-Phe sublibrary. A close examination of the data revealed that four out of the five are the same compounds that inhibited pheromone biosynthesis, whereas one BBC compound ($n + m$ = 3 + 3) is a selective melanotropic inhibitor. The discovery of a

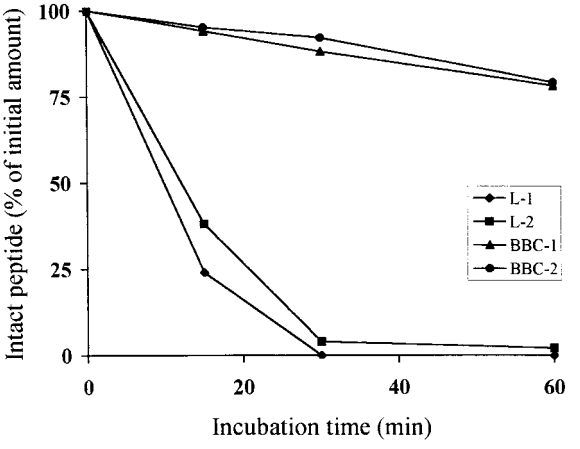

FIGURE 3 Time course of trypsin digestion of BBC and linear peptides. BBC (D-Phe BBC-1: $n + m$ = 6 +2; D-Phe BBC-2: $n + m$ = 4 + 2) and linear PBAN derived peptides (L-1: RYFSPRL-amide; L-2: YFSPRL-amide) were incubated at a concentration of 1 mg/mL with 8 μg/mL trypsin in 20 m*M* Tris-HCl buffer containing 20 m*M* $CaCl_2$, pH 8.0. Enzymatic reactions were terminated by the addition of 16 m*M* HCl at the indicated times and the amount of intact peptide was determined by analytical HPLC as previously described.[6]

selective inhibitor suggests that sex pheromone biosynthesis and cuticular melanization may be controlled via different mechanisms that involve different receptors. Further studies on the presence of selective and nonselective agonists and antagonists and attempts to solve the functional diversity of the PK/PBAN Nps as well as their role in the regulation of the physiology of insects are in progress.

INSECT NP ANTAGONISTS AS INSECT CONTROL AGENTS

Although Np-based antagonists carry a high potential for insect management, their application in pest control has not so far been implemented because of two major limitations. The first is the linear peptidic nature of Nps, which renders them nonselective, highly susceptible to proteolytic degradation, and unable to penetrate through biological tissues. The second is the lack of an approach to the design of antagonists, because of lack of knowledge of the three-dimensional (3D) structure of the receptor–agonist complex, and of the mechanism of receptor activation. As indicated above, the BBC-NBA approach overcomes the above limitations and provides a basis for the development of highly potent, stable and selective antagonist.

However, the design of Np antagonists for insecticidal applications requires, in addition to the antagonistic properties, the development of novel strategies for the generation of compounds that will conform with the common practice of the insecticide industry—namely, they should be compounds of low molecular weight, able to penetrate through the insect cuticle and gut, environmentally stable and cost effective in production. Small, nonpeptide antagonists fulfill the above requirements. The structural data derived from active and nonactive BBC, conformationally constrained agonists and antagonists (such as those described above), enable proceeding toward the discovery of such molecules.

The general strategy for the development of a Np antagonist-based insecticide prototype involves (Figure 1): (a) discovery of a conformationally constrained antagonist; (b) determination of the structural requirements for antagonistic activity; (c) design and synthesis, on the basis of (a) and (b), of small nonpeptide molecule libraries by means of chemical combinatorial approaches; (d) screening the libraries created in (c) for molecules that bind to the receptor (by means of a high throughput screening assay, HTSA); (e) evaluation of the in vivo potency, bioavailability, and selectivity of the molecules found in (d); and (f) selection, on the basis of (e), of an insecticide prototype (highly potent, small, metabolically stable, bioavailable, and cost effective) for formulation, toxicological experiments and field trials.

Although this strategic approach has been used to develop some vertebrate Np agonists and antagonist-based drugs (for review, see Refs. 84 and85) its application in pest control has not so far been implemented and still needs to be developed.

A brief description of the BBC-NBA-based approach for designing small molecule combinatorial libraries and their screening by high throughput assays is presented below.

Small Molecule Combinatorial Libraries

Recent advances in combinatorial and/or high throughput synthesis provide a large variety of methods for the generation of small molecule combinatorial libraries.[86] Although these methods provide a large source of chemical diversity for lead generation, the optimization and selection of the compounds in question, the huge number of compounds that have to be screened, the time required for selection of the desired compound, and the high cost of the entire process in many cases render the effort not commercially worth while, and it is definitely beyond the resources of an ordinary research laboratory in academia.

Design of the small-molecule combinatorial libraries within the framework of the BBC-NBA approach is based on a convergent method that combines rational design and combinatorial chemistry. The method facilitates the rapid generation of optimized compounds having desirable characteristics with a significant reduction in the overall time required for their development. This constitutes a major advantage in screening for the desired compounds, as the overall number of molecules in such libraries is much smaller than that in a randomly synthesized combinatorial library, thus enabling screening of individual compounds rather than their mixtures.

The approach involves two steps: (a) identification of the biophores essential for the antagonistic activity (of the most active antagonist found in the BBC libraries); and (b) incorporation of these essential biophores in novel scaffolded small molecule libraries.

The first step can be done by means of two complementary methods: (a) Ala-cycloscan (in which the most potent BBC antagonist serves as a parent peptide); and (b) BBC omission libraries (where BBC penta-, tetra-, tri-, and dipeptide sublibraries are prepared and screened for bioactivity). Once the bio-

phores have been identified, they can be incorporated into the novel libraries of scaffolded small molecules. This approach enables a given set of biophores to be screened within structurally related, homologous scaffolds, and facilitates the selection of small molecules that have the appropriate induced match to the receptor.

Many small molecules having therapeutic potential, which inhibit protein–protein or peptide–protein interactions, are scaffolded molecules having 2–4 biophore diversity, constrained into the appropriate 3D structure. The active molecules were discovered in each case by screening combinatorial libraries of the molecules in which the various R groups were different functional groups or had a positional diversity (for a detailed review on the above methods, see Ref. 86). An alternative approach, offered by the backbone cyclization strategy, involves transformation of BBC peptides into novel types of cyclic and bicyclic small molecules having 3–5 biophores by means of novel types of converged combinatorial-conformational libraries.

High Throughput Screening Assays

Generation of chemical combinatorial libraries (even those based on partial on rational design) requires the availability of an HTSA for fast screening of the libraries and selection of the biologically active compounds.

In the past decade, HTSAs have become common tools for discovering new drugs and pesticides. Recently, there have been tremendous advances in the efficiency of HTSAs made possible through a combination of molecular biology, assay technology, instruments, and robotics whose development has been driven by the demand for constantly increasing throughput. The approaches to meeting this demand have been as varied as the types of targets being studied. The types of biological assays in current use employ immunoassays, receptor binding assays, enzyme inhibition reactions, protein–protein binding, protein–DNA (RNA) binding, cell-based functional assays, and reporter genes. The properties covered by analytical measurements include radioactivity, luminescence, fluorescence, color or pH changes, light diffraction, and evanescence; more recently, they have involved the use of microchips. The assay technologies employed include filtration, extraction, in-plate binding, and biacore. Most assays are carried out in standard 96- or 384-microplate formats, although other formats also exist. A detailed overview of HTSAs and the most important considerations in their development are described by Devlin.[84]

One major goal in developing efficient screening methods is the achievement of simplicity, sensitivity, selectivity, and reproducibility of the assay. The assay must be able to screen each compound individually, in several concentrations, and must be highly sensitive, specific, and selective for the compound(s) of choice, with a high signal-to-noise ratio. Desirable additional characteristics include the use of small amounts of material and the need for a minimal number of separation steps. In many cases fulfillment of all of the above requirements is impossible and there is a need to compromise by using alternative targets and/or nonselective assays as well as screening mixtures rather than single compounds.

A natural HTSA target for the discovery of Np antagonists is the receptor, where the tested compounds from the combinatorial library are examined for their ability to compete with the ligand in binding to the receptor. The assay may involve highly purified components or it may utilize partially purified or even crude membrane preparations as a source of the receptor. Recombinant receptors or cell lines that express the receptor offer particular advantages because they ensure the steady supply of material during screening. Both membrane and recombinant receptors can be prepared in bulk and frozen for long-term storage and subsequent use. Receptor binding assays are usually carried out in 96- or 384-microplates, which are compatible with commercially available automated equipment. The assay can be performed in either of two formats. In the first, the receptor is added to a tagged (i.e., radiolabeled fluorescent or biotinilated) ligand in an appropriate buffer, in the presence of the tested compound, and separation of the bound and free ligand is performed by filtration. In the second format the receptor is adsorbed on the microplate, the tested compound and ligand are added to the microplate wells, and separation of free form bound ligand is performed by excessive washing.

Pending the development of a receptor-based HTSA, it is necessary to generate an appropriate ligand, to optimize the assay and fully validate it. Optimization usually involves determination of conditions for receptor preparation and establishment of optimal conditions for receptor–ligand interaction with low nonspecific binding; determination of the dependence of binding on receptor and ligand concentrations, and characterization of the time course of association and dissociation of the ligand with the receptor and the saturation isotherm.

It is important to note that the binding assay cannot distinguish between an agonist and an antagonist. Consequently, there is a need for further screening of the selected molecules with a bioassay, to determine

their antagonistic potency in vivo. Once bioactive small nonpeptide molecules are discovered they have to be further screened, by means of in vivo bioassays, for selectivity, metabolic stability, and bioavailability. Compounds that fulfill the required characteristics can then serve as the desired insecticide prototypes, which subject to appropriate formulation and preliminary toxicology evaluations, can be tested in preliminary field trials.

Recently, we have completed the development of a Radio-Receptor-Assay (RRA) for the PK/PBAN family as part of the effort to develop an HTSA based on the PK/PBAN receptor for screening nonpeptide small molecule combinatorial libraries that will be synthesized on the basis of the BBC antagonists. *Heliothis peltigera* pheromone gland membranes were used as the source tissue and the C-terminal hexapeptide sequence of Hez-PBAN1-33NH_2 (PBAN 28-33 NH_2) (which constitutes the active core of PBAN and contains the "signature" sequence shared by all members of the family) was used as a radioligand. In the course of the study we have:

1. determined the conditions for receptor preparation;
2. determined the optimal binding conditions in terms of specific vs nonspecific binding, buffers, reaction time, receptor and ligand concentration;
3. characterized some of its pharmacological properties;
4. determined the affinity toward various peptides derived from the PK/PBAN family and validated the RRA with the in vivo bioactivity of these peptides;
5. evaluated the dependence of its expression on moth age and species.

A detailed summary of these results is presented in Refs. 87 and 88.

The RRA enables us now to proceed toward the development of a microplate-based HTS assay for screening small molecule combinatorial libraries and selection of compounds that can interact with the PK/PBAN family of receptors, which should lead to the discovery of an insecticide prototype.

Achievement of this goal will complete the development of the overall BBC-NBA technology, which is aimed at the generation of Np antagonist-based nonpeptide insecticides. The generic nature of the technology will enable the design of additional Np antagonists that will have the potential to interfere with other Np-mediated functions in moths and other insects. The nonpeptidic nature of the compounds will enable production on the multiton scale at relatively low cost.

Beyond the development of the overall BBC-NBA technology, the immediate application of the approach lies in the disclosure of an anti-PK/PBAN insecticide prototype, which carries the potential to inhibit and/or impair sex pheromone production in female moths as well as all the other functions regulated by PBAN (melanization, diapause, pupariation, and muscle contraction) in moths and other insects. Since PBAN is active as a pheromonotropic factor common to many moth species, it is anticipated that the Anti-PK/PBAN compounds will inhibit pheromone biosynthesis in most of the key moth pests. PBAN is also found in male moths and the Anti-PK/PBAN compounds may have an antagonistic effect on male moths also, and thereby increase the effectiveness of their application.

The outcome of the BBC-NBA approach will result, most likely, in a number of nonpeptide compounds, some of which will exhibit antagonistic properties. The availability of several compounds that can inhibit a number of functions in a variety of insects will offer a major advantage, especially in integrated pest management (IPM) and integrated resistance management (IRM) strategies, and it will provide an arsenal of compounds that can be alternated and thus, help to minimize the development of resistance. Moreover, it is anticipated that the compounds that will emerge from the BBC-NBA approach will not include free radicals or compounds that generate them in their course of action, therefore they will not be toxic to living organisms. In addition, these compounds metabolized to digestable or nonharmful materials that leave no residues in the environment and can thus be considered environment friendly.

CONCLUDING REMARKS

The article presents a novel general approach (termed BBC-NBA) that combines rational design and a selection method for the generation of agonistic and/or antagonistic cyclic peptides based on the sequence of an insect Np. This approach, applied to PBAN, has led to the discovery of several agonists and antagonists, which exhibited pheromonotropic activity and effectively inhibited sex pheromone biosynthesis in female *H. peltigera* moths and formation of cuticular melanin in *Spodoptera littoralis* larvae. To the best of our knowledge, this is the first reported use of backbone cyclization for the design of insect Np antagonists. Beyond the immediate benefits introduced by the use of cyclic peptides as selective antagonists, the

information on the bioactive conformations of the antagonists gained in the course of this study may serve as a basis for the design of improved (small, cost effective, metabolically stable, and bioavailable) nonpeptide agonists and antagonists. Such compounds would be potential candidates for agrochemical applications, and could serve, after formulation and preliminary field experiments, as prototypes for the development of a novel group of highly effective, insect-specific, and environment friendly insecticides and/or insect-control agents.

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