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BARD Project Number: IS-3356-02

Date of Submission of the report: October 17 2006

Project Title: Rationally designed insect neuropeptide agonists and antagonists: application for the characterization of the pyrokinin/PBAN mechanism of action in insects.

Investigators

Principal Investigator (PI): Miriam Altstein

Co-Principal Investigator (Co-PI): Ronald Nachman

Collaborating Investigators:

Institutions

ARO, Volcani Center

USDA, ARS, College Station, TX

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Sex pheromones, melanization, Lepidoptera, *Heliothis peltigera*, *Spodoptera littoralis*, PBAN-receptor.

Abbreviations commonly used in the report, in alphabetical order:

BBC: Backbone cyclic; **LPK:** *Leucophaea maderae* pyrokinin; **MT:** myotropin; **PBAN:** pheromone biosynthesis activating neuropeptide; **PK:** pyrokinin; **PT:** pheromonotropin

Budget: IS: \$159,000

US: \$148,000

Total: \$307,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	-	9	3	12
Submitted, in review, in preparation	7	1	2	10
Invited review papers				
Book chapters				
Books				
Master theses			1	1
Ph.D. theses				
Abstracts		5		5
Not refereed (proceedings, reports, etc.)		1		1

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	-	2
Longer Visits (Sabbaticals)	-	-	-	-

Description of Cooperation:

The two research groups have been studying the PK/PBAN family of neuropeptides for many years addressing similar questions concerning the mode of action of these neuropeptides from different and complementary aspects. Prior to the onset of the project the Israeli group concentrated on two (out of five) known functions of the neuropeptides (pheromone biosynthesis and cuticular melanization), whereas the US group concentrated on myotropic activity and pupariation. Both groups joined the project with considerable developed knowledge and research tools (*in vivo* and *in vitro* bioassays, a selection of PK/PBAN peptides and their analogs and a few chemical libraries) which set a solid basis for a collaborative study of the above issues. Within the frame of this BARD project both groups integrated their experience and know-how in a manner that was synergistic and which facilitated rapid advances of the specific objectives that were set within the frame of the grant proposal.

Specifically, the study integrated the methods, materials and know-how that have been gained in both laboratories to study the role of the PK/PBAN in the physiology of insects. The study was performed in full collaboration where all peptides that were synthesized in the US laboratory were sent to analysis in Israel and *vice versa*. The Israeli group provided the US group with the BBC compounds for analysis and search for agonists and antagonists on myotropic activity in the cockroach *Leucophaea maderae* and pupariation in the flesh fly *Neobellieria bullata*, and the US group provided the Israeli group with PK/PBAN elicitors (LPK, and MT) as well as a long list of novel PK analogs with potentially enhanced bioavailability and bioactivity (see below) for analysis and search for agonists and antagonists for

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melanin formation in *Spodoptera littoralis* larvae and pheromone biosynthesis in *Heliothis peltigera* female moths. Throughout the study exchange of information and evaluation of results have been carried out on a regular basis and the results that were obtained in both laboratories were integrated and served as a basis for the design and synthesis of novel improved compounds. Exchange of information and detailed discussions also took place during the exchange trips (in the 2nd and the 3rd years of the project) which involved further evaluation of the results and discussions of the possibilities to explore future research avenues.

As summarized below, this study resulted in a discovery of an arsenal of highly potent selective and non selective agonists and antagonists of a variety of PK/PBAN mediated activities, revealed and provided helpful information on the structure activity relationship (SAR) of agonistic and antagonistic peptides, led to the identification of a scaffold with which biostable peptidomimetic agonists and antagonists can be obtained, provided a wide range of novel tools to study the mechanisms that underlay the activity of this family and provides a solid basis for the development of novel, environmentally-friendly insect control agents.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	-	-	-	-
Issued (allowed)	-	-	-	-
Licensed	-	-	-	-

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A. Abstract

The general objective of this BARD project focused on rationally designed insect neuropeptide (NP) agonists and antagonists, their application for the characterization of the mechanisms of action of the pyrokinin/PBAN (PK-PBAN) family and the development of biostable, bioavailable versions that can provide the basis for development of novel, environmentally-friendly pest insect control agents. The specific objectives of the study, as originally proposed, were to: (i) Test stimulatory potencies of rationally designed backbone cyclic (BBC) peptides on pheromonotropic, melanotropic, myotropic and pupariation activities; (ii) Test the inhibitory potencies of the BBC compounds on the above activities evoked either by synthetic peptides (PBAN, LPK, myotropin and pheromonotropin) or by the natural endogenous mechanism; (iii) Determine the bioavailability of the most potent BBC compounds that will be found in (ii); (iv) Design, synthesize and examine novel PK/PBAN analogs with enhanced bioavailability and receptor binding; (v) Design and synthesize ‘magic bullet’ analogs and examine their ability to selectively kill cells expressing the PK/PBAN receptor. To achieve these goals the agonistic and antagonistic activities/properties of rationally designed linear and BBC neuropeptide (NP) were thoroughly studied and the information obtained was further used for the design and synthesis of improved compounds toward the design of an insecticide prototype. The study revealed important information on the structure activity relationship (SAR) of agonistic/antagonistic peptides, including definitive identification of the orientation of the Pro residue as *trans* for agonist activity in 4 PK/PBAN bioassays (pheromonotropic, pupariation, melanotropic, & hindgut contractile) and a PK-related CAP_{2b} bioassay (diuretic); indications that led to the identification of a novel scaffold to develop biostable, bioavailable peptidomimetic PK/PBAN agonists/antagonists. The work led to the development of an arsenal of PK/PBAN antagonists with a variety of selectivity profiles; whether between different PK bioassays, or within the same bioassay between different natural elicitors. Examples include selective and non-selective BBC and novel amphiphilic PK pheromonotropic and melanotropic antagonists some of which are capable of penetrating the moth cuticle in efficacious quantities. One of the latter analog group demonstrated unprecedented versatility in its ability to antagonize a broad spectrum of pheromonotropic elicitors. A novel, *trans*Pro mimetic motif was proposed & used to develop a strong, selective PK agonist of the melanotropic bioassay in moths. The first antagonist (pure) of PK-related CAP_{2b} diuresis in flies was developed using a *cis*Pro mimetic motif; an indication that while a *trans*Pro orientation is associated with receptor agonism, a *cis*Pro orientation is linked with an antagonist interaction. A novel, biostable PK analog, incorporating β -amino acids at key peptidase-susceptible sites, exhibited *in vivo* pheromonotropic activity that by far exceeded that of PBAN when applied topically. Direct analysis of neural tissue by state-of-the-art MALDI-TOF/TOF mass spectrometry was used to identify specific PK/PK-related peptides native to eight arthropod pest species [house (*M. domestica*), stable (*S. calcitrans*), horn (*H. irritans*) & flesh (*N. bullata*) flies; Southern cattle fever tick (*B. microplus*), European tick (*I. ricinus*), yellow fever mosquito (*A. aegypti*), & Southern Green Stink Bug (*N. viridula*)]; including the unprecedented identification of mass-identical Leu/Ile residues and the first identification of NPs from a tick or the CNS of Hemiptera. Evidence was obtained for the selection of Neb-PK-2 as the primary pupariation factor of the flesh fly (*N. bullata*) among native PK/PK-related candidates. The peptidomic techniques were also used to map the location of PK/PK-related NP in the nervous system of the model fly *D. melanogaster*. Knowledge of specific PK sequences can aid in the future design of species specific (or non-specific) NP agonists/antagonists. In addition, the study led to the first cloning of a PK/PBAN receptor from insect larvae (*S. littoralis*), providing the basis for SAR analysis for the future design of 2nd generation selective and/or nonselective agonists/antagonists. Development of a microplate ligand binding assay using the PK/PBAN pheromone gland receptor was also carried out. The assay will enable screening, including high throughput, of various libraries (chemical, molecular & natural product) for the discovery of receptor specific agonists/antagonists. In summary, the body of work achieves several key milestones and brings us significantly closer to the development of novel, environmentally friendly pest insect management agents based on insect PK/PBAN NPs capable of disrupting critical NP-regulated functions.

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B. Achievements

B.1. Significance of main scientific achievements or innovations

B.1.1. Joint US/Israel:

1. Definitive identification of the orientation of Pro as *trans* for agonist activity in 4 PK/PBAN bioassays (pheromonotropic, pupariation, melanotropic, & hindgut contractile); and identification of a novel scaffold to develop biostable, bioavailable peptidomimetic PK/PBAN agonists/antagonists.
2. Development of an arsenal of PK/PBAN novel agonists and antagonists with a variety of selective and non selective profiles; whether between different PK bioassays, or within the same bioassay between different natural elicitors. These provide a wide range of tools to neuroendocrinologists studying the mechanisms and/or role of PKs in arthropods; and new leads for the development of novel, environmentally-friendly pest arthropod management agents, some of which show a capability to penetrate the moth cuticle.
3. Developed novel, potentially biostable and highly bioavailable PK analogs containing β -amino acids at key core positions that demonstrate strong agonist and antagonist properties.
4. Proposed a novel, *trans*-peptide bond mimetic motif ('Jones'). A PK analog (Jones D) incorporating this motif demonstrated a strong, selective agonist response in the melanotropic bioassay in moths. The work identifies a new scaffold to develop biostable, bioavailable melanotropic agonists.
5. Developed a novel, cyclic PK analog that is a broad antagonist of all three natural elicitors in the melanotropic assay.

B.1.2. US:

1. First use of state-of-the-art MALDI-TOF/TOF mass spectrometry to distinguish between the isomeric Leu and Ile residues (indistinguishable by previous mass spectrometric methods) in invertebrate peptides.
2. Utilized state-of-the-art MALDI-TOF/TOF mass spectrometry directly on nerve tissue of single specimens to identify PK and PK-like peptides native to specific pest arthropods. Knowledge of specific PK sequences can aid in the future design of species specific (or non-specific) neuropeptide agonists/antagonists.
3. Utilized MALDI-TOF mass spectrometry to map the location of PK and PK-like peptides in the nervous system of the model insect, the adult fruit fly (*Drosophila melanogaster*).
4. Provided evidence for the selection of Neb-PK-2 as the primary pupariation factor of the flesh fly (*Neobellieria bullata*) among all native PK candidates.
5. Definitive identification of the orientation of Pro as *trans* for agonist diuretic activity of PK-like CAP2b/PVK peptides in the housefly; and identification of a novel scaffold for the development of biostable, bioavailable peptidomimetic agonists/antagonists of this important sub-family of PK

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peptides. In addition, development of a structure-activity relationship profile for this PK-like neuropeptide family.

6. Identification of the first antagonist of the PK-like CAP2b/PVK neuropeptides for diuretic activity in the housefly. It is a pure antagonist, with no agonist activity.

B.1.3. Israel:

1. Determination of the bioavailability of linear and cyclic peptides through the cuticle and finding that linear peptides are bioavailable and can penetrate through the cuticle even when applied in aqueous solutions.

2. Cloning of the PK/PBAN receptor from *S. littoralis* larvae which is the first receptor of this family cloned from larvae. The cloned receptor enables comparison of its structure with similar receptors cloned from the pheromone glands of various female moths and provides a basis for SAR analysis which is of major importance for the further design of improved selective and non-selective agonists and antagonists and potential antagonists-based insect control agents.

3. Development of polyclonal antibodies against the receptor which is a useful tool for its further characterization and for the development of a microplate binding assay.

4. Development of a microplate ligand binding assay using the PK/PBAN pheromone gland receptor which enable screening of various libraries (chemical, molecular and natural product) for the discovery of receptor specific agonists and antagonists and sets the basis for its development into a high throughput assay (HTA) for screening of the above libraries.

B.2. Details of cooperation:

This BARD investigation involved a synergistic collaboration between a physiologist/molecular biologist from Israel and a bioorganic chemist from the US that led to an impressive array of accomplishments that could not have been achieved alone. Their combined efforts also expanded the number of PK bioassays – the pheromonotropic and melanotropic bioassays in Israel and the pupariation acceleration and hindgut contractile bioassays in the US. Using knowledge of, and experience in, peptide and peptidomimetic analog structure, and the relationship of structure to activity, Dr. Nachman made key modifications to the linear lead PK/PBAN antagonist previously developed in Israel in order to enhance and/or modify the selectivity, biostability and/or bioavailability profile. This work led to the development of an arsenal of PK/PBAN antagonists with a variety of selectivity profiles; whether between different PK bioassays, or within the same bioassay between different natural elicitors. Among specific examples include the development of a novel, selective, amphiphilic PK antagonist (1605) of the pheromonotropic response in moths and that demonstrates a capability to penetrate the moth cuticle in biologically significant quantities. This structure of analog 1605 provides

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an excellent opportunity for future investigations to change the hydrophobic character in an incremental fashion via replacing the hexanoyl group with other fatty acids of varying chain length to probe the relationship between hydrophobic character and optimal penetration rates and time-release properties. Other novel amphiphilic analogs (1524, 1373, 1374) feature non selective antagonism of natural PK elicitors in both pheromonotropic and melanotropic assays. One of these analogs, 1373, demonstrates great versatility in its ability to antagonize a broad spectrum of elicitors in the pheromonotropic assay. Although not pure, the β -amino acid analog, 1461 has been shown to be a potent, selective antagonist in the melanotropic assay. Di- β -amino acid analog 1602 features protection to both primary and secondary sites of tissue-bound peptidase attack and greater bioactivity than the native peptide in *in vivo* topical application trials. A novel, cyclic analog has been shown as a broad and potent antagonist of all three natural elicitors in the

melanotropic assay. The collaborative effort between Drs. Nachman and Altstein led to definitive evidence that the PK agonists adopt a *trans*Pro configuration during interaction with the receptors of all four different PK bioassays utilized in this project. This effort led to the identification of a scaffold with which to develop biostable peptidomimetic agonist and antagonist analogs. During the course of the work, Dr. Nachman proposed a novel *trans*-peptide bond (*trans*Pro) mimetic motif and incorporated it into the PK sequence. Dr. Altstein subsequently found that the resulting analog proved to be a potent, selective agonist of the melanotropic assay in moths. This now identifies a new scaffold to develop biostable, bioavailable melanotropic agonists. Together all of these new analogs provide a wide range of tools to neuroendocrinologists studying the mechanisms and/or role of PKs in arthropods; and new leads for the development of novel, environmentally-friendly pest arthropod management agents.

In addition, expertise in molecular biology of the PK receptors on the Israeli side and state-of-the-art mass spectrometric peptidomic techniques on the US side have combined to add further information and potential tools to the accomplishments of this BARD project and for further studies by our groups or others. Mass spectrometric techniques have been used for the first time to distinguish between the isomeric residues Leu and Ile in neural tissue samples. The complete sequences of seventeen novel PK and PK-like peptides have been identified in specific arthropod pests of man, animals and crops – including the identification of the first neuropeptides from ticks and from the central nervous system (CNS) of a member of the important insect order Hemiptera. Mass spectrometric techniques were utilized to map the location of PK and PK-like peptides in the nervous system of the adult fruit fly. The work has provided compelling evidence for Neb-PK-2 as the primary pupariation factor in the flesh fly among all known native PK and PK-like candidates. The work further identified that PK-like

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‘CAP2b/PVK’ peptides adopt a *trans*Pro configuration during interaction with the receptor in a housefly diuretic assay, and the active core for full activity requires the C-terminal heptapeptide. The work also led to the development of the first antagonist analog (pure) of this sub-family of peptides. In the process, novel scaffolds have been identified for the development of biostable, peptidomimetic agonist/antagonist analogs of the PK-like CAP2b/PVK peptides. In the Israeli laboratory, cloning of the PBAN receptor has provided a basis for induction of mutations and further SAR analysis, which is of major importance for the further design of improved selective and non-selective agonists and antagonists and potential antagonists-based insect control agents; and the microplate binding assay, which can be turned into a HTS binding or functional assay, can serve for screening of combinatorial libraries that will be designed in the future by us or by others. For further details of the collaboration please refer to the cover page above.

B.3. List of publications

B.3.1. List of published manuscripts (Enclosed)

1. Predel R, Russell WK, Tichy SE, Russell DH and Nachman RJ. (2003). Mass spectrometric analysis of putative capa-gene products in *Musca domestica* and *Neobellieria bullata*. *Peptides* 24, 1487-1491.
2. Altstein, M. (2004). Role of neuropeptides in sex pheromone production in moths. *Peptides*, 25, 1491-1501
3. Predel R, Wegener C, Russell, WK, Tichy SE, Russell DH and Nachman RJ. (2004) Peptidomics of CNS-associated neurohemal systems of adult *Drosophila melanogaster*: A mass spectrometric survey of peptides from individual flies. *J. Comp. Neurol.*, 474, 379-92.
4. Ben-Aziz, O., Zeltser, I. and Altstein, M. (2005). PBAN selective antagonists: Inhibition of PBAN induced cuticular melanization and sex pheromone biosynthesis in moths. *J. Insect Physiol.* 51, 305-314.
5. Nachman RJ, Russell WK, Coast GM, Russell DH and Predel R. (2005). Mass spectrometric assignment of Leu/Ile in neuropeptides from single neurohemal organ preparations in insects. *Peptides* 26, 2151-2156.
6. Neupert S, Predel R, Russell WK, Davies R, Pietrantonio PV and Nachman RJ. (2005). Identification of tick periviscerokinin, the first neurohormone of Ixodidae: Single cell analysis by means of MALDI-TOF/TOF mass spectrometry. *Biochem. Biophys. Res. Commun.* 338, 1860-1864.

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7. Nachman, R.J., Russell WK, Russell DH and Predel R. (2005). MALDI-TOF/TOF mass spectrometric assignment of Leu/Ile in PVK/CAP2b neuropeptides from single neurohemal organ preparations of four flies. *Pestcydy*, 3-4, 49-53.
8. Nachman RJ, Russell WK, Coast GM, Russell DH, Miller JA and Predel R. (2006) Identification of PVK/CAP2b neuropeptides from single neurohemal organs of the stable fly and horn fly via MALDI-TOF/TOF tandem mass spectrometry. *Peptides*, 27, 521-6.
9. Nachman RJ, Strey A, Zubrzak P and Zdarek J. (2006). A comparison of the pupariation acceleration activity of pyrokinin-like peptides native to the flesh fly: Which peptide represents the primary pupariation factor? *Peptides*, 27, 527-33.
10. Ben-Aziz, O., Zeltser, I. , Bhargava, K, Davidovitch, M. and Altstein, M. (2006). Backbone cyclic pheromone biosynthesis activating neuropeptide (PBAN) antagonists: Inhibition of melanization in the moth *Spodoptera littoralis* (Insecta: Lepidoptera). *Peptides*, 27, 2147-2156.

B.3.2. Manuscripts "in press", 'accepted' or 'submitted' (Enclosed)

1. Predel R, Russell WK, Neupert S, Russell DH, Esquivel JF, and Nachman RJ. (2006). Identification of the first neuropeptides from the CNS of Hemiptera: Capa peptides of the Southern Green stinkbug *Nezara viridula* (L.). *Peptides*, In Press.
2. Nachman RJ and Coast GM. Structure-activity relationships for in vitro diuretic activity of CAP2b in the housefly. *Peptides*, In Press.
3. Zheng, L., Njauw, C.N., Lytle, C., Altstein, M. and Martins-Green, M. (2006). Cloning and Characterization of the Pheromone Biosynthesis Activating Neuropeptide Receptor Gene in *Spodoptera littoralis*. *Gene*., Submitted.
4. Altstein, M., Ben-Aziz, O., Zeltser, I. , Bhargava, K, Davidovitch, M. Strey, A., Pryor, N., and Nachman, R.J. (2006). Inhibition of PK/PBAN mediated functions in insects: Discovery of selective and non-selective inhibitors. *Peptides*, Submitted.