Final Scientific Report

Cover Page

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Project Title: Rational design of insect control agent prototypes based on pyrokinin/PBAN neuropeptide antagonists

Investigators

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Institutions

ARO, Volcani Center

USDA, ARS, College Station

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

Sex pheromones, melanization, Lepidoptera, *Heliothis peltigera*, *Spodoptera littoralis*, Pyrokinin/PBAN-receptor, Diapause, aphicide, *Helicoverpa zea*, biostable, bioavailable, peptidomimetic, biophore, conformation, amphiphilic,

Abbreviations commonly used in the report, in alphabetical order:

Ab: antibody; Ac: acetylated; BBC: backbone cyclic; CAP2b: Cardioacceleratory activating peptide; CR: cross reactivity; DDW: double distilled water; DH: diapause hormone; DMSO: dimethyl sulfoxide; ECLs: extra-cellular loops; ELISA: enzyme linked immunosorbent assay; ICC: immunocytochemical; ICLs: intra-cellular loops; ETH: ecdysis-triggering hormone; HRP: horseradish peroxidase; HTS: high throughput screening; GPCR: G-protein coupled receptor; LPK: leukopyrokinin; MALDI: Matrix-assisted laser desorption/ionization; NMR: nuclear magnetic resonance; Np: neuropeptide, NPMs: non peptide molecules; ORF: open reading frame; PBAN: pheromone biosynthesis activating neuropeptide; PDB: protein data bank; PEG: polyethylene glycol; PK: pyrokinin; PT: pheromoneotropin; PVK: periviscerokinin; SAR: structure activity relationship; SMLs: small molecule libraries; TM: transmembrane; TOF: time of flight;

Final Scientific Report

Budget: IS: \$120,000

US: \$120,000

Total: \$240,000

Altstein

Signature
Principal Investigator

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Authorizing Official, Principal Institution

Final Scientific Report

Publication Summary

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	0	10	1	11
Submitted, in review, in preparation	3	4	1	8
Invited review papers	0	0	0	0
Book chapters	1	0	0	1
Books	0	0	0	0
Master theses	0	0	0	0
Ph.D. theses	0	0	1	1
Abstracts	0	0	0	0
Not refereed (proceedings, reports, etc.)	0	1	0	1

Postdoctoral Training: None

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	-	2	3
Longer Visits (Sabbaticals)	-	-	-	-

Patent Summary

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0

Final Scientific Report

Abstract

The general objective of this study was to develop rationally designed mimetic antagonists (and agonists) of the PK/PBAN Np class with enhanced bio-stability and bioavailability as prototypes for effective and environmentally friendly pest insect management agents. The PK/PBAN family is a multifunctional group of Nps that mediates key functions in insects (sex pheromone biosynthesis, cuticular melanization, myotropic activity, diapause and pupal development) and is, therefore, of high scientific and applied interest. The objectives of the current study were: (i) to identify an antagonist biophore (ii) to develop an arsenal of amphiphilic topically active PK/PBAN antagonists with an array of different time-release profiles based on the previously developed prototype analog; (iii) to develop rationally designed non-peptide SMLs based on the antagonist biophore determined in (i) and evaluate them in cloned receptor microplate binding assays and by pheromonotropic, melanotropic and pupariation *in vivo* assays. (iv) to clone PK/PBAN receptors (PK/PBAN-Rs) for further understanding of receptor-ligand interactions; (v) to develop microplate binding assays for screening the above SMLs. In the course of the granting period A series of amphiphilic PK/PBAN analogs based on a linear lead antagonist from the previous BARD grant was synthesized that incorporated a diverse array of hydrophobic groups (**HR-Suc-A[dF]PRLa**). Others were synthesized via the attachment of polyethylene glycol (PEG) polymers. A hydrophobic, biostable PK/PBAN/DH analog DH-2Abf-K prevented the onset of the protective state of diapause in *H. zea* pupae [$EC_{50}=7$ pmol/larva] following injection into the preceding larval stage. It effectively induces the crop pest to commit a form of 'ecological suicide'. Evaluation of a set of amphiphilic PK analogs with a diverse array of hydrophobic groups of the formula **HR-Suc-FTPRLa** led to the identification of analog T-63 (**HR=Decyl**) that increased the extent of diapause termination by a factor of 70% when applied topically to newly emerged pupae. Another biostable PK analog PK-Oic-1 featured anti-feedant and aphicidal properties that matched the potency of some commercial aphicides. Native PK showed no significant activity. The aphicidal effects were blocked by a new PEGylated PK antagonist analog PK-dF-PEG₄, suggesting that the activity is mediated by a PK/PBAN receptor and therefore indicative of a novel and selective mode-of-action. Using a novel *transPro* mimetic motif (dihydroimidazole; 'Jones') developed in previous BARD-sponsored work, the first antagonist for the diapause hormone (DH), DH-Jo, was developed and shown to block over 50% of *H. zea* pupal diapause termination activity of native DH. This novel antagonist development strategy may be applicable to other invertebrate and vertebrate hormones that feature a *transPro* in the active core. The research identifies a critical component of the *antagonist* biophore for this PK/PBAN receptor subtype, i.e. a *trans*-oriented Pro. Additional work led to the molecular cloning and functional characterization of the DH receptor from *H. zea*, allowing for the discovery of three other DH antagonist analogs: *Drosophila* ETH, a β -AA analog, and a dF analog. The receptor experiments identified an agonist (DH-2Abf-dA) with a maximal response greater than native DH. 'Deconvolution' of a rationally-designed nonpeptide heterocyclic combinatorial library with a cyclic bis-guanidino (BG) scaffold led to discovery of several members that elicited activity in a pupariation acceleration assay, and one that also showed activity in an *H. zea* diapause termination assay, eliciting a maximal response of 90%. Molecular cloning and functional characterization of a CAP2b antidiuretic receptor from the kissing bug (*R. prolixus*) as well as the first CAP2b and PK receptors from a tick was also achieved. Notably, the PK/PBAN-like receptor from the cattle fever tick is unique among known PK/PBAN and CAP2b receptors in that it can interact with both ligand types, providing further evidence for an evolutionary relationship between these two NP families. In the course of the granting period we also managed to clone the PK/PBAN-R of *H. peltigera*, to express it and the *S. littoralis*-R Sf-9 cells and to evaluate their interaction with a variety of PK/PBAN ligands. In addition, three functional microplate assays in a HTS format have been developed: a cell-membrane competitive ligand binding assay; a Ca flux assay and a whole cell cAMP ELISA. The Ca flux assay has been used for receptor characterization due to its extremely high sensitivity. Computer homology studies were carried out to predict both receptor's SAR and based on this analysis 8 mutants have been generated. The bioavailability of small linear antagonistic peptides has been evaluated and was found to be highly effective as sex pheromone biosynthesis inhibitors. The activity of 11 new amphiphilic analogs has also been evaluated. Unfortunately, due to a problem with the *Heliothis* moth colony we were unable to select those with pheromonotropic antagonistic activity and further check their bioavailability. Six peptides exhibited some melanotropic antagonistic activity but due to the low inhibitory effect the peptides were not further tested for bioavailability in *S. littoralis* larvae. Despite the fact that no new antagonistic peptides were discovered in the course of this granting period the results contribute to a better understanding of the interaction of the PK/PBAN family of Nps with their receptors, provided several HT assays for screening of libraries of various origin for presence of PK/PBAN-R agonists and antagonists and provided important practical information for the further design of new, peptide-based insecticide prototypes aimed at the disruption of key neuroendocrine physiological functions in pest insects.

Final Scientific Report

Achievements

New pest control strategies based on PK/PBAN Np methodology can be achieved via topical application of amphiphilic analogs that feature surfactant-like properties that allow them to both penetrate the hydrophobic insect cuticle and dissolve in the aqueous hemolymph to reach the internal target receptor. In addition, optimal control will be facilitated by an analog mixture featuring diverse surfactant properties that simultaneously allow both rapid cuticle penetration for an immediate physiological response along with a slower time-release component that can elicit a prolonged effect. Thus, a series of amphiphilic PK/PBAN analogs based on a linear lead antagonist from the previous BARD grant were synthesized that incorporated a diverse array of hydrophobic groups according to the following formula: **HR-Suc-A[dF]PRLa**. Other amphiphilic analogs were created by attaching polyethylene glycol (PEG) polymers to the PK/PBAN core. Evaluations of these amphiphilic series of analogs in melanotropic and pheromonotropic assays led to the following results revealed that small linear peptides are available through the cuticle and can inhibit pheromone biosynthesis to a similar extent than that obtained by metabolically stable cyclic peptides. The hydrophobic, biostable PK/PBAN/DH analog DH-2Abf-K (2Abf-Suc-FKPRLa), inspired by a lead agonist analog from the preceding BARD grant, was evaluated in another PK/PBAN assay, diapause termination/prevention in *H. zea*. The analog prevented the onset of the protective state of diapause in *H. zea* pupae [$EC_{50}=7$ pmol/larva] following injection into the preceding larval stage. It thus induces the crop pest to commit a form of ‘ecological suicide’. In order to pursue topically active analogs, a set of 11 amphiphilic PK analogs containing a diverse array of hydrophobic groups with the formula **HR-Suc-FTPRLa** were injected into pupae. Three of the most active were tested via topical application of 1-10 nmoles onto newly-emerged pupae; and one, T-63 (**Decyl-Suc-FTPRLa**), increased the extent of diapause termination by 70%. Several biostable PK analogs, inspired by previous BARD work, and two PEGylated PK analogs (see above) were evaluated in an aphid feeding assay. Two of the bioavailable analogs, PK-Oic-1 (FT[**Oic**]RLa: $EC_{50}=0.04$ nmol/ μ L/LT₅₀=1.0 day) and PK-dF-PEG₈ (**P₈**-YF[**dF**]PRLa: $EC_{50}=0.13$ nmol/ μ L/LT₅₀=1.3 day), featured anti-feedant and aphicidal properties that matched the potency of some commercial aphicides. Native PK showed no significant activity. The aphicidal effects of PK-Oic-1 could be blocked by a new PEGylated PK antagonist analog PK-dF-PEG₄ (**P₄**-YF[**dF**]PRLa), suggesting that the activity is mediated by a PK/PBAN receptor and therefore indicative of a novel and selective mode-of-action. Biostable PK analogs represent genuine leads for a new class of aphicides. Using the dihydroimidazole (‘Jones’) scaffold that we developed as a novel *trans*Pro mimetic motif in previous BARD-sponsored work, the first antagonist for the diapause hormone (DH), DH-Jo (Ac-GLWA[**Jo**]RLa), was developed, blocking over 50% of the *H. zea* pupal diapause termination activity of native DH. This novel antagonist development strategy may be applicable to

Final Scientific Report

other invertebrate and vertebrate hormones that feature a *trans*Pro in the active core. The research identifies a critical component of the *antagonist* biophore for this PK/PBAN receptor subtype, i.e. a *trans*-oriented Pro. Homology between the diapause termination and other PK/PBAN receptor subtypes indicates that it is highly likely that antagonist biophores for all of the subtypes also feature the *trans*Pro conformation. Additional work led to the molecular cloning and functional characterization of the DH receptor from *H. zea*, which allowed for the discovery of three other analogs with pure DH antagonist properties. The antagonists were *Drosophila* ETH (FFLKITKNVPRLa), β -AA analog Ac-W[β^3 F]G[β^3 P]RLa, and dF analog **2Abf-Suc-F[dF]PRAa**. The receptor experiments also identified an agonist (DH-2Abf-dA: **2Abf-Suc-F[dA]PRLa**) featuring a maximal response (144%) greater than that of native DH. In previous BARD work, a *trans*Pro configuration was shown to be a critical component of the agonist response for PK/PBAN NP. PK analogs containing the 'Etzkorn', (*E*)-alkene *trans*Pro isosteric motif demonstrated activity equipotent with native peptides in several PK bioassay/receptor systems. This motif was incorporated into a rationally designed nonpeptide mimetic PK core analog that also featured a reduced peptide bond and the removal of an N-terminal amino group. The resulting nonpeptide (**Hca-Etz-R(r)La**; **Hca**=hydrocinnamyl-, (**r**)=reduced peptide bond) contains no α -amino acids or peptidase-susceptible native peptide bonds. It proved to be a selective PK agonist, with significant activity (threshold: 1 nM) in a hindgut contractile assay, whereas no significant activity was observed in pupariation acceleration or melanization assays. Additionally, a rationally-designed nonpeptide cyclic bis-guanidino (BG) combinatorial library was synthesized and evaluated in a PK *N. bullata* pupariation acceleration assay. The BG scaffold contains the guanidinium group, representative of the sidechain of Arg, the most critical residue for virtually all PK/PBAN/DH activity. Five members of this BG library elicited significant activity in the pupariation acceleration assay, with the most active (BG7-6-9) exhibiting significant activity at a threshold of 1 pmol. Another (BG7-6-2) also showed activity in the *H. zea* diapause termination assay, eliciting a maximal response of 90% at 50 pmol/pupa. Molecular cloning and functional characterization of a CAP2b antidiuretic receptor from the kissing bug (*R. prolixus*) as well as the first CAP2b and PK/PBAN receptors from a tick (*R. microplus*) were completed. Notably, the PK/PBAN-like receptor from the cattle fever tick is unique among known PK/PBAN and CAP2b receptors in that it interacts with both ligand types, providing further evidence for an evolutionary relationship between these two Np families. In the course of the project we also managed to clone a sex pheromone gland receptor of the PK/PBAN family from *Heliothis peltigera* (*Hep-R*) female moth and to stably express this receptor together with the previously cloned *Spodoptera littoralis* larval receptor (*Spl-R*) in an insect cell line. The structural models, electrostatic potentials and cellular functional properties of both receptors were evaluated. Homology modeling indicated highly conserved amino-acid residues in appropriate structural positions as experimentally shown for class A G-protein coupled receptors. Structural differences could be proposed and electrostatic potentials of the two receptor models revealed net charge

Final Scientific Report

differences. A calcium mobilization assay has been developed and used to characterize the interaction of various PK/PBAN ligands with both the *Heliothis* and *Spodoptera* cloned receptors. The assay demonstrated that both receptors were fully functional and could initiate extracellular calcium influx to start PK/PBAN signal transduction. Evaluation of the signaling response of both receptors to PBAN and diapause hormone (DH) revealed a highly sensitive, though a differential response. Both receptors responded to PBAN whereas only *Spl*-PK/PBAN-R exhibited a high response toward DH. The structural, electrostatic and cellular functional differences indicate that different PK/PBAN *in vivo* functions may be mediated by different PK/PBAN receptors and elicited by a different peptide(s). Based on the structural analysis and the differences in both receptors we have generated 8 mutations (in regions predicted on the basis of the structural analysis to be important for ligand binding). The genes have already been synthesized, will be expressed in the near future and the interaction of the mutants with the above ligands will be evaluated and compared with that of the native receptors using the highly sensitive Ca flux assay in combination with the cAMP ELISA which has also been developed in the course of this granting period. This will enable further evaluation of the PK/PBAN receptors and their interaction with their ligands as well as exploration of the intra-cellular events associated with such interactions toward a better understanding the mode of action of this family of physiologically important peptides. The results advance our understanding of the mode of action of the PK/PBAN family, and might help in exploring novel high-affinity receptor-specific antagonists that can serve as a basis for the development of new families of insect-control agents. The Ca flux assay that has been developed will enable high throughput screening of NPMs and SMLs of various sources for selection of highly potent agonists and more important antagonists that can serve as potent insect control agents. The overall results of the present study represent a significant addition to the approaches used in moth neuroendocrinology studies and to the strategies that should be considered in development of environmentally friendly and biodegradable, rationally designed control agents, based on Np agonists or antagonists. Use of the noninvasive application route in such studies eliminates the stress and injury caused to the insect by injection which could significantly affect the *in vivo* measurement of any tested physiological parameter.

Final Scientific Report

Details of cooperation

The US sent a series of eleven antagonist PK/PBAN analogs of the general formula **HR**-Suc- A[dF]PRLa (**HR** = hydrophobic amino group) designed to create different time-release profiles to the Israeli partner for evaluation of bioavailability parameters for pheromonotropic and melanotropic activity. Some of the data are presented in the appendix the rest of the data collection is underway. The US partner have also sent a set of PEGylated antagonist PK/PBAN compounds to enhance topical bioavailability in PK/PBAN peptides for evaluation by the Israeli. Collection of data will be completed in the coming months. In addition, the US partner have sent a complete synthetic rationally designed BG (cyclic bis-guanidinium) combinatorial library to Israel for evaluation in pheromonotropic and melanotropic bioassays/expressed PBAN receptor cell preparations to identify non-peptide agonists and antagonists; another major goal of our BARD grant. This data collection is underway. Several individual members of this nonpeptide BG library that were deconvoluted using a *Neobellieria* pupariation assay and found to demonstrate activity in either the *Neobellieria* pupariation acceleration and/or the *Helicoverpa* diapause termination assays will be sent to Israel in the next couple of months for evaluation in *Spodoptera* melanization and *Heliothis* pheromonotropic assays when new synthetic batches have been purified. A rationally-designed, non-peptide PK/PBAN SML developed by the USDA group has also been to collaborating partners in Israel in the coming months for evaluation in melanotropic bioassay system. It was found to be a selective PK/PBAN agonist; active in a *Leucophaea* hindgut contractile assay, but inactive in *Neobellieria* pupariation acceleration and *Spodoptera* melanization assays.

Final Scientific Report

List of Publications (only reviewed and acknowledging BARD)

1. Coast, G.M., TeBrugge, V.A., Nachman, R.J., Lopez, J., Aldrich, J.R., Lange, A., and Orchard, I. (2010) Neurohormones implicated in the control of Malpighian tubule secretion in plant sucking heteropterans: The stink bugs *Acrosternum hilare* and *Nezara viridula*. *Peptides* 31: 468-473.
2. Predel, R., Neupert, S., Garczynski, S., Crim, J., Brown, M., Russell, W., Kahnt, J., Russell, D., Nachman, R.J. (2010) Neuropeptidomics of the mosquito *Aedes aegypti*. *J. Proteome Res.*, 9:2006-2015.
3. Paluzzi, J., Y. Park, R.J. Nachman and I. Orchard (2010). Isolation, expression analysis and functional characterization of the first anti-diuretic hormone receptor in insects. *Proc. Natl. Acad. Sci. USA* 107:10290-10295.
4. Coast, G. M., Nachman, R. J. and Lopez, J. (2011) The control of Malpighian tubule secretion in a predaceous hemipteran insect, the spined soldier bug *Podisus maculiventris* (Heteroptera, Pentatomidae). *Peptides* 32:493-499.
5. Zhang, Q., Nachman, R.J., Kaczmarek, K., Zabrocki, J., Denlinger, D. L. (2011) Disruption of diapause using agonists and an antagonist of diapause hormone. *Proc. Natl. Acad. Sci. USA* 108:16922-16926.
6. Predel R, Russell WK, Russell DH, Suh, CP-C. Nachman R J. (2012) Neuropeptides of the cotton fleahopper, *Psuedatomoscelis seriatus* (Reuter). *Peptides* 34: 39-43.
7. Nachman RJ, Hamshou M, Kaczmarek K, Zabrocki J, Smagghe G. (2012) Biostable and PEG-polymer conjugated insect pyrokinin neuropeptides demonstrate antifeedant activity and induce high mortality in the pea aphid *Acyrtosiphon pisum* (Hemiptera: Aphidae). *Peptides* 34: 266-273.

Final Scientific Report

8. Nachman RJ, Wang XJ, Etzkorn FA, Kaczmarek K, Zabrocki J, Lopez JD, Coast GM. (2013) Evaluation of insect CAP2b analogs with either an (*E*)-alkene, trans- or a (*Z*)-alkene, cis-Pro Isostere identifies the Pro orientation for antidiuretic activity in the stink bug. *Peptides* 41: 101-106.
9. Predel R, Neupert S, Russell WK, Hauser F, Russell DH, Li AY, Nachman RJ. (2013) CAPA gene products in the haematophagous sandfly *Plebotomus papatasi* (Scopoli) – Vector for leishmaniasis disease. *Peptides* 41: 2-7.
10. Hariton-Shalev, A., Shalev, M., Adir, N., Belausov, E. and Altstein, A. (2013). Structural and functional differences between pheromonotropic and melanotropic PK/PBAN receptors. *Biochim. Biophys. Acta.* 1830, 5036-5048.
11. Yang Y, Bajracharya P, Bravo Castillo P, Nachman RJ, Pietrantonio P. Molecular and functional characterization of the first tick CAP2b/periviscerokinin receptor from *Rhipicephalus (Boophilus) microplus*. *Gen. Comp. Endocrin.*, accepted.
12. Nachman, RJ. A new strategy for the development of antagonists and/or selective agonists of the insect pyrokinin (FXPRLamide) superfamily using a novel conformational-mimetic motif. *EUPa Open Proteomics*, submitted.