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trans-Pro isosteres in the development of non-selective and selective mimetic agonists of insect pyrokinin neuropeptides; A review

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Abstract: The pyrokinin (PK) family plays a multifunctional role in an array of important physiological processes in a variety of insects. A PK active core analog containing an (*E*)-alkene, *trans*Pro isosteric component was evaluated in five disparate PK bioassays and/or in a recombinant PK receptor cell line, representing six different insect species. The assays included pheromone biosynthesis in the moth *Heliothis peltigera*, melanization in the larval *Spodoptera littoralis*, pupariation acceleration in the larval fly *Neobellieria bullata*, diapause termination in the moth *Heliothis zea*, and hindgut contraction in the cockroach *Leucophaea maderae*. This constrained analog demonstrated unselective agonist activity that approached, matched, or exceeded the activity of parent PK peptides of equal length in all six PK assays. The results provide strong evidence for the orientation of Pro and the core conformation adopted by PK neuropeptides during interaction with disparate PK receptors. A PK active core analog incorporating a second *trans*Pro motif, the dihydroimidazoline moiety, was found to demonstrate pure, selective agonism in the melanotropic bioassay, with no significant activity in three other PK bioassays. Both types of *trans*Pro isosteric analogs feature modification adjacent to the primary tissue-bound peptidase hydrolysis site that is expected to enhance biostability over natural PK peptides. The research further identifies two novel scaffolds with which to design either selective or non-selective mimetic PK analogs as potential leads in the development of environmentally favorable pest management agents capable of disrupting PK-regulated systems.

Keywords: pheromonotropic, pupariation, melanization, myotropic, peptidomimetic, neuropeptide, diapause break, PBAN

INTRODUCTION

The pyrokinin (PK) family of peptides plays a multifunctional role in the physiology of insects. In 1986 the first member of the family, leucopyrokinin (LPK), was isolated from the cockroach *Leucophaea maderae* [1] with over 30 members of this peptide class identified thereafter. All family members share the common *C*-terminal pentapeptide FXPRL-amide (X=S, T, G or V) and include subfamilies such as PKs, myotropins (MTs), pheromone biosynthesis activating neuropeptide (PBAN), diapause hormone (DH), melanization and reddish coloration hormone (MRCH), pheromonotropin (PT), as well as pheromonotropic β and γ peptides derived from the cDNA of moths [1]. The PK family has been shown to stimulate sex pheromone biosynthesis in moths [1-4], and mediate critical functions associated with feeding (gut contractions) [1, 5, 6] development (egg diapause, pupal diapause and pupariation) [1, 7] and defense (melanin biosynthesis) [1, 5, 8] in a variety of insects. The peptides do not exhibit species specificity and experiments have shown that all of the functions listed above can be stimulated by more than one peptide [1, 5, 8], and that the C-terminal pentapeptide common to the PK neuropeptide class retains activity in each of the disparate functions. Although neuropeptides of the PK class are potent regulators of physiological processes critical to insect survival, they hold little promise as pest management agents because they are subject to rapid degradation by peptidases in the hemolymph, tissues and gut of pest insects.

In previous work, a highly rigid cyclic PK analog *cyclo*[Asn^1]LPK (*cyclo*[NTSFTPTL]), featuring a *trans*Pro, type I β-turn, was determined to retain significant bioactivity in several PK/PBAN bioassays, including hindgut contractile (cockroach *Leucophaea maderae*) [6], oviduct contractile (cockroach *Leucophaea maderae*) [1, 5], pheromonotropic (silk worm *Bombyx mori*) [3], egg diapause induction (silk worm *Bombyx mori*) [1], pupariation (flesh fly *Neobelieria bullata*) [3-6], and diapause termination (tobacco budworm *Heliothis virescens*) [7] assay systems. These results are consistent with the suggestion that a *trans* oriented Pro and the type I β-turn structure holds broad significance for many physiological functions elicited by the PK neuropeptide family of peptides.

In this manuscript, we review recent research undertaken to provide definitive evidence of the importance of a *trans* oriented Pro for a wide spectrum of PK bioactivities and to identify *trans*Pro mimetic motifs that can serve as novel scaffolds in the development of non-selective and/or selective mimetic PK analogs with greater biostability than natural neuropeptides.

RESULTS AND DISCUSSION

The *C*-terminal pentapeptide FXPRLa is highly conserved and thus, shared by PK family of neuropeptides. This pentapeptide has further been identified as the active core in pheromonotropic bioassays (X = S) [1, 3, 5, 6,] and in an expressed PBAN receptor assay from the moth *Heliothis virescens* [2] and *S. littoralis* [5, 8], although the *C*-terminal hexapeptide YFXPRLa (X= S) exhibits much greater potency. In the pheromonotropic assay of the heliothine insect *H. zea* the core PK *C*-terminal pentapeptide sequence exhibits similar potency whether the variable X position is occupied by an S or a T [1]. The *C*-terminal pentapeptide common to the PK class has also been found to retain significant activity in other bioassays, such as melanotropic, pupariation and hindgut myotropic preparations.

Nachman et al. conducted a conformational study of the rigid, cyclic PK/PBAN analog *cyclo*[NTSFTPRL] (*cyclo*[Asn^1]LPK) in aqueous solution containing no organic solvents using a combination of NMR spectroscopic and molecular dynamics calculations [3, 6]. The specific conformation of this constrained, cyclic analog in aqueous solution was shown to be extremely rigid, featuring a *trans*-oriented Pro in the second position of a type-I β-turn over residues Thr-Pro-Arg-Leu within the core region. A *trans*Pro is a defining characteristic of a type I β-turn [3, 6]. The very large (for Thr-2, Thr-5, and Leu-8) and very small (for Ser-3 and Arg-7) coupling constants found indicated that the backbone of *cyclo*[Asn^1]LPK was rigidly held in a single or a few closely related conformations, since conformational averaging would have given averaged, intermediate values [6].

Despite the conformational constraint imposed upon the cyclic PK analog *cyclo*[Asn^1]LPK, it was found to retain 10% of the pheromonotropic activity of the 33-residue Bom-PBAN-I in a pheromonotropic bioassay in the silkworm *B. mori* [6], the same percentage of activity retained by the linear *C*-terminal PBAN hexapeptide. The analog *cyclo*[Asn^1]LPK was also found to retain significant bioactivity in several other PK bioassays, including hindgut contractile (cockroach *Leucophaea maderae*), oviduct contractile (cockroach *Leucophaea maderae*), egg diapause induction (silk worm *Bombyx mori*), diapause termination in the moth *Helicoverpa zea*, and pupariation (flesh fly *Neobelieria bullata*) assay systems[3, 6, 7].

Nonetheless, two other turn conformations, in addition to the *trans*Pro type I turn, have also been proposed for the core pentapeptide region based on NMR experiments of a natural PK and/or core analogs in solution; specifically a *cis*Pro type I' [3] and a type II β-turn [4].

(*E*)-alkene, *trans*-Pro isostere: In order to provide more definitive evidence that a *trans*Pro, and a type I β-turn, represented the active conformation for the PK neuropeptide class, the PK analog **PK-Etz** (Ac-Tyr-Phe-SerΨ[(*E*)-**CH=C**]Pro-Arg-Leu-NH_2), incorporating a *trans*Pro isostere ('Etzkorn': SerΨ[(*E*)-**CH=C**] Pro), was evaluated in five diverse PK bioassay systems and on a recombinant PK GPCR receptor cell line. These bioassays were the pheromone biosynthesis assay in the moth *H. peltigera*, the melanization assay in the Egyptian cotton leaf worm *S. littoralis*, the pupariation assay in the fleshfly, *Neobellieria bullata*, the hindgut myotropic assay in the cockroach *Leucophaea maderae*, and the diapause termination assay in the moth H. zea [5]. The recombinant PK GPCR receptor cell line used was the HevPBANR receptor from the tobacco budworm *H. virescens* expressed in CHO-K1 cells [2]. In **PK-Etz**, the peptide bond of the Pro is replaced with a rigid double bond that locks in the *trans* orientation [2, 5] (Figure 1).

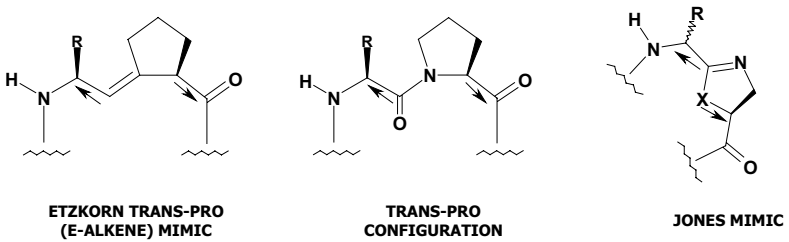

Figure 1. Comparison of a *trans*-Pro configuration (middle) and the (*E*)-alkene *trans*-Pro mimetic 'Etzkorn' (SerΨ[(*E*)-**CH=C**]Pro) motif (left). In this motif, the peptide bond that binds the amino group of the Pro is locked into a *trans* orientation by replacement with a double bond, which lacks the ability to rotate between *trans* and *cis* orientations as does a normal peptide bond[2, 5, 7]. In addition, comparison of a *trans*-Pro configuration (middle) and the 'Jones' dihydroimidazoline mimetic motif (right; X=N) [8].

Analog **PK-Etz** demonstrated activity essentially equivalent to parent PK analogs of equal length in four of the bioassay systems [5, 7] and on the recombinant HevPBANR receptor cell line [2]. In the melanization, pheromonotropic, pupariation and hindgut contractile assays, as well as with the expressed HevPBANR receptor, **PK-Etz** matched or approached the activity of natural PK isomorphs. Of particular note is the fact that **PK-Etz** exceeded the efficacy (maximal response) of the natural PBAN1-33 in the melanotropic bioassay by close to a statistically significant factor of 2 (at 1 nmol) [5]. In the

diapause termination bioassay in the moth *H. zea*, **PK-Etz** exceeded the potency of the native 24-residue diapause hormone DH by a factor of 13 [7]. The enhanced potency is likely a consequence of greater biostability towards peptidases that inactivate the native hormone, as **PK-Etz** incorporates the *trans*Pro isosteric motif adjacent to the primary hydrolysis site for tissue-bound peptidases. Furthermore, the N-terminal acetyl group blocks the N-terminus of **PK-Etz** from attack by aminopeptidases.

The relatively potent agonist activity of **PK-Etz** provides strong evidence that a *trans*Pro represents an important conformational aspect of the interaction of PK hormones with their receptors in the six disparate PK bioassay and/or recombinant receptor cell systems, each representing a different insect species.

Establishment of a *trans*Pro orientation provides valuable evidence for the identity of the active PK conformation. Three previous studies have led to the proposal of different β-turn types (type I, type II and type I') for the PK core region (as discussed above). Of these studies, only Nachman et al. [3-6] used both a conformationally rigid PK/PBAN analog along with aqueous solutions free of added organic solvents that artificially promote the formation of secondary structure. Of note is the fact that Wang et al. [4] admit that their observation of a type II β-turn could have been the result of conformational averaging of a type I β-turn (identified in the study by Nachman et al.) and an extended conformation in the flexible analog used. The type I β-turn proposed by Nachman et al. features a *trans*Pro that was clearly evident in the rigid, cyclic analog *cyclo*[Asn^{1}]LPK and has now been confirmed by the potent activity of **PK-Etz**, which locks in a *trans* orientation with an alkene bond that is unable to rotate. This finding is not consistent with a type I' β-turn proposed in the study by Clark and Prestwich [9] that used the highly flexible HezPBAN, as this turn type features a *cis*Pro rather than a *trans*Pro.

Dihydroimidazoline ('Jones') motif: The dihydroimidazoline moiety has been previously introduced by Jones and coworkers as a peptide bond isostere (with an amidine as an amide bond replacement) [10]. Until recently [8], it had not been previously proposed as a mimic of a *trans* peptide bond. Nachman et al. argued that the dihydroimidazoline moiety can function as a mimic or surrogate of the *trans* peptide bond, and in particular, a *trans*Pro, locking a *trans* orientation within the constrained five-membered dihydroimidazole ring (Figure 1). However, whereas the molecular modeling suggests that the dihydroimidazoline moiety can function as a mimic of a *trans*Pro, it is clear that it is not an exact mimic; and furthermore, is not as close a mimic as is the (*E*)-alkene moiety mentioned above [2, 5,8]. Therefore, analogs containing the

dihydroimidazoline moiety provide an opportunity for selective interaction with closely related receptors, as some receptors may display more tolerance to small deviations from the *trans*Pro structure of natural peptides than others.

Incorporation of the *trans*Pro surrogate, dihydroimidazoline ('Jones') moiety into a PK/PBAN C-terminal hexapeptide sequence led to analog **PPK-Jo** (Ac-YF[Jo]RLa) [8]. The analog is acetylated at the N-terminus to provide protection from aminopeptidase degradation. **PPK-Jo** demonstrated strong activity in the *in vivo S. littoralis* melanotropic assay, reaching a 115 and 96% activity at doses of 100 pmol and 1 nmol, respectively compared with that of 5 pmol PBAN and matching the efficacy of 5 pmol LPK. The parent PK hexapeptide analog YFTPRLa also elicited strong activity in this assay. However, YFTPRLa also demonstrated inhibition of the melanotropic activity of 5 pmol PBAN or 5 pmol PT at doses of 100 pmol and 1 nmol. Analog **PPK-Jo** shows no inhibition even up to a dose of 1 nmol. Unlike the parent PK/PBAN hexapeptide YFTPRLa, **PPK-Jo** is a pure melanotropic agonist in the *S. littoralis* assay. **PPK-Jo** failed to elicit significant agonist (or antagonist) activity in three other PK bioassays; i.e., an *in vivo H. peltigera* pheromonotropic assay, an *in vivo N. bullata* pupariation assay, and in an *in vitro* cockroach *L. maderae* hindgut myotropic assay [8]. Therefore, **PPK-Jo** is a pure, selective agonist for the melanotropic assay. It is apparent that the receptor associated with the melanotropic assay in *S. littoralis* is more promiscuous than those of the other PK assays, demonstrating more tolerance to deviations from the natural *trans*Pro structure. The development of a selective PK agonist can lead to a better understanding of the endogenous mechanisms of this important peptide class and can serve as a probe to study the plasticity of PK-regulated systems in insects and the receptors associated with them.

This work has demonstrated that the dihydroimidazoline motif can function as a mimic or surrogate of a *trans*Pro in certain circumstances, and has been identified as a novel scaffold with which to construct mimetic pseudopeptide of the PK, and other, peptide families that feature enhanced selectivity. Notably, the dihydroimidazoline motif in **PPK-Jo** introduces a major structural modification adjacent to the peptide bond connected to the amino group of Arg in the core pentapeptide, which has been identified as the primary tissue-bound peptidase susceptible site of the PK core [1, 3, 5-8]. Therefore, it seems plausible that incorporation of the motif could enhance the biostability of PK analogs. The work demonstrates that analogs containing the dihydroimidazoline moiety provide an opportunity for selective interaction with closely related receptors, as some receptors may display more tolerance to small deviations from the natural *trans*Pro structure of native peptides.

SUMMARY

The research with *trans*Pro isosteres reviewed herein not only provides evidence for the orientation of Pro and core conformation for the interaction of PK neuropeptides with receptors associated with a broad range of PK regulated processes, but also identifies two novel scaffolds with which to design mimetic analogs, both non-selective and selective, of this peptide class. Such analogs may provide leads in the development of novel insect-specific, environmentally favorable pest management agents capable of disrupting PK-regulated physiological systems.

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