Effect of insecticidal fusion proteins containing spider toxins targeting sodium and calcium ion channels on pyrethroid-resistant strains of peach-potato aphid (*Myzus persicae*)

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Abstract:

BACKGROUND: The recombinant fusion proteins Pl1a/GNA and Hv1a/GNA contain the spider venom peptides δ-amaurobitoxin-PI1a or ω-hexatoxin-Hv1a respectively, linked to snowdrop lectin (GNA). Pl1a targets receptor site 4 of insect voltage-gated sodium channels (NaCh) while Hv1a targets voltage-gated calcium channels. Insecticide-resistant strains of peach-potato aphid (*Myzus persicae*) contain mutations in NaCh. The pyrethroid-resistant "*kdr*" (794J) and "*super-kdr*" (UKO) strains contain mutations at residues L1014 and M918 in the channel α-subunit respectively, while the "*kdr*+*super-kdr*" strain (4824J), insensitive to pyrethroids, contains mutations at both L1014 and M918.

RESULTS: Pl1a/GNA and Hv1a/GNA fusion proteins have estimated LC_{50} values of 0.35 and 0.19 mg ml^{-1} when fed to wild-type *M. persicae*. For insecticide-resistant aphids, LC_{50} for the Pl1a/GNA fusion protein increased by 2- to 6-fold, correlating with pyrethroid resistance (wild-type < *kdr* < *super-kdr* < *kdr*+*super-kdr* strains). In contrast, LC_{50} for the Hv1a/GNA fusion protein showed limited correlation with pyrethroid resistance.

CONCLUSION: Mutations in the sodium channel in pyrethroid-resistant aphids also protect against a fusion protein containing a sodium channel-specific toxin, despite differences in ligand-channel interactions, but do not confer resistance to a fusion protein targeting calcium channels. The use of fusion proteins with differing targets could play a role in managing pesticide resistance.

Key words: biopesticide; insecticide resistance; Homoptera / Hemiptera; voltage-gated ion channels; fitness cost

1 **INTRODUCTION**

The peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is a serious worldwide insect pest of agricultural and horticultural crops, which, through its sap-sucking feeding habit, can transmit viral diseases.[1] Pyrethroids are a major class of insecticides used to control this pest, but populations of *M. persicae* can rapidly develop resistance to pyrethroids, leading to increased economic loss to agricultural producers.[2] Pyrethroids target the insect voltage-gated sodium channel, a large trans-membrane protein composed of a single 260kDa polypeptide (the alpha subunit), which contains four repeating and homologous domains (I–IV), with each domain being constituted by six hydrophobic transmembrane segments (S1–S6).[3] The insect sodium channel is similar in structure to the vertebrate sodium channel, containing different allosterically coupled receptor-binding sites for various neurotoxicants, but the two types of channel are distinguishable in the pharmacology. Therefore insecticides such as pyrethroids can be specific for insect sodium channels, showing no effect on mammals.[4,5]

Pyrethroids are hydrophobic compounds, and are thought to bind to the lipid-exposed interface formed by helices IIIS6, IIS5, linker helix IIS4-IIS5 and the IS4-IS5 linker,[6,7] affecting the functional properties of the sodium channel. By preventing closure of the sodium channel, pyrethroids cause paralysis in insects.[5] However, with the extensive use of pyrethroids, many insects have developed resistance to these insecticides, associated with mutations in the sodium channel. The pyrethroid resistance shown by *M. persicae* is typical of that seen in many species.[8-10] In aphids carrying the *kdr* mutation, there is a leucine to phenylalanine substitution (L1014F) within segment 6 of domain II (IIS6) of the channel protein,[11] which confers an intermediate level of resistance to pyrethroids. In aphids carrying the *super-kdr* site mutation, there is an additional methionine- to-threonine substitution (M918T) in the linker between segment 4 and segment 5 of domain II (IIS4-IIS5 linker) of the sodium channel protein,[8] which makes *M. persicae* highly resistant to pyrethroids. Data

presented by Eleftherianos et al.[1] shows that whereas the EC_{50} for a typical pyrethroid insecticide on wild-type *M. persicae* is in the range 0.5 - 2.8 ppm, a homozygous *kdr* mutation increases the EC_{50} by 20-75 fold, and a heterozygous *kdr*+*super-kdr* mutation increases resistance by 100-500 fold. The emergence of insecticide resistance is one factor driving a need for new specific environmentally benign pesticides, which could be used in strategies to manage resistance to chemicals like pyrethroids more effectively.

Spider toxin peptides have been suggested as environmentally friendly biopesticides. Toxins have been isolated from a range of arachnids, and most are small cysteine-rich proteins that principally target neuronal ion channels to cause paralysis of the spider's prey.[4,12] Toxins can be selected that are insect-specific, and have no effects on members of other taxons. This advantage would make them ideal candidates for use in pest control and crop protection, if a suitable delivery system which would get around the problem of toxicity being dependent on injection into the body fluid of the pest could be devised.[13] Recombinant fusion proteins, containing insecticidal peptides or proteins fused to a "carrier" protein are a method, which gives oral toxicity to neuroactive toxins.[14,15] The carrier protein transports the insecticidal peptide or protein across the insect gut epithelium into the haemolymph, from which it can access the central nervous system (CNS), which is the site of action. The mannose-specific lectin from snowdrop (*Galanthus nivalis* agglutinin: GNA), which has been shown to transport peptides into the insect haemolymph, is currently being used for making fusion proteins. Fusion proteins containing GNA as a carrier possess good stability towards proteolysis in the insect gut and high toxicity.[16]

δ-Amaurobitoxins, or δ-palutoxins, from the spider *Pireneitega luctuosus*, are a family of four similar 36-37 residue peptides containing 8 cysteine residues which are disulphide-linked to form a cysteine knot motif. Pl1a is specific for insect sodium channels, causing paralysis, and has no adverse effects when injected into mice.[17] The toxin acts by binding to receptor site 4 in the sodium channel protein, which involves

the extracellular loops of S1-S2, S3-S4 of domain II.[18] It affects the functional properties of the sodium channel α subunit by shifting the voltage dependence of activation, resulting in paralysis; the effect is similar to that produced by pyrethroids.[5] A PI1a/GNA fusion protein has been shown to be an effective oral insecticide towards insects of different orders, including aphids.[19]

Hv1a is a family member of insecticidal neurotoxins, which possess 36–37 residues, from the Australian funnel web spider *Hadronyche versuta*.[20] Hv1a arrests insect voltage-gated calcium channels and has no negative effects on mammals.[21-23] Hv1a contains three disulfide bonds which shape an inhibitor cystine knot motif, which confers chemical and thermal stablility and resistance to proteases.[24,25] The highly conserved C-terminal β hairpin of Hv1a contains the key residues for insecticidal activity.[20] An Hv1a/GNA fusion protein has been described previously, and its oral toxicity towards insects has been demonstrated.[16]

The present paper compares the toxicity of PI1a/GNA and Hv1a/GNA fusion proteins towards wild-type and pyrethroid-resistant strains of *M. persicae*, and shows that although the toxicity of PI1a/GNA is reduced by the *kdr* and *super-kdr* mutations in the sodium channel, it retains some activity. However, the mutations confer no resistance to Hv1a/GNA targeting calcium channels. This residual high insecticidal activity makes Hv1a/GNA a potential biopesticide for controlling pyrethroid-resistant aphids.

2 MATERIALS AND METHODS

2.1 Materials

Chemicals and reagents were of analytical grade and were supplied by Sigma or BDH Chemical Company otherwise unless stated. Restriction enzymes and other molecular biology reagents were supplied by Fermentas. A double stranded DNA incorporating a sequence encoding the mature PI1a toxin (P83256), with codons optimised for

expression in *Pichia pastoris*, was designed by the authors, synthesized and supplied by ShineGene Molecular Biotech, Inc. (Shanghai 201109, China; http://www.synthesisgene.com/). Other oligonucleotides required for cloning were supplied by Sigma Chemical Co. Recombinant snowdrop lectin was produced by the authors by expression in *Pichia pastoris*, as described by Baumgartner et al. (2004).[26]

The mutant strains of peach-potato aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) were kindly provided by Prof. Linda M. Field (Department of Biological Chemistry and Crop Protection, Rothamsted Research, UK). Strain 4106A has no mutation ("*wild type*"). Strain 794J is homozygous for the mutation L1014F (*kdr*), and is resistant to pyrethroids. Strain UKO is homozygous for the mutation M918L (*super-kdr*), and shows enhanced resistance to pyrethroids. 4824J is homozygous for L1014F (*kdr*) and M918T (*super-kdr*), and shows immunity to pyrethroids.[1] Aphids were cultured on fresh Chinese Leaf under conditions of 12h light, 12h dark, 18°C, 70% relative humidity.

2.2 Production of Pl1a/GNA and Hv1a/GNA fusion proteins

Assembly of expression constructs encoding Pl1a, PI1a/GNA and GNA and expression of the recombinant proteins in the yeast *Pichia pastoris* have been described elsewhere.[19] The fusion proteins, which contained C-terminal $(His)_6$ tags, were purified by metal affinity chromatography, dialysed and lyophilised as previously described.[14-16] Expression constructs for Hv1a and Hv1a/GNA and production of recombinant proteins have also been described previously;[16] the constructs used to express Hv1a and Hv1a/GNA for this paper were modified by inclusion of a predicted pro-region for the toxin.[27] Other recombinant proteins were produced as previously described.[15] Purified proteins were analysed by SDS-PAGE for quantitation by comparison to standards run on the same gel; proteins were also quantitated by using the BCA assay, and by absorbance.

2.3 Bioassays on peach-potato aphid

Bioassay of aphids using liquid artificial diet was carried out as described by Prosser and Douglas[28]. Adult aphids were transferred to control liquid diet, acclimatised for 24h, and then neonate nymphs produced over the following 24h were transferred to experimental diets, and allowed to develop to adult stage (8-9 days). 20 individuals per treatment were used to perform the bioassays. Each assay was repeated 3 times. Mortality was observed daily, and assays were continued until control aphids started to produce nymphs. Nymphs were not counted but the presence or absence of progeny was recorded. Effects of treatments on aphid growth were assessed by using Image J Software to measure insect length.

2.4 Statistical analysis

Mortality data were analysed using survival curves, with a Kaplan-Meier test to evaluate significance of differences (Origin 8.5 software). ANOVA analysis (with Bonferroni-Dunn post-hoc tests) was carried out to determine any significant differences between treatments in size parameters measured. Differences between treatments were considered significant at a probability level $p < 0.05$. LC_{50} values for different treatments were estimated by taking survival data for diets containing different concentrations of fusion proteins (over a range of 0.125 - 2.0 mg ml^{-1}) and fitting data points to a sigmoidal dose-response curve by non-linear regression (Prism v. 5 software).

3 RESULTS

3.1 Toxicity of separate components of fusion proteins

Effects of toxins and GNA components of insecticidal fusion proteins on the strains of peach-potato aphids (794J, UKO, 4824J and 4106A) were determined by bioassays in which components were fed separately in liquid diet from neonate nymphs. Concentrations were chosen to be equivalent to 1 mg/ml fusion protein. Results are

shown in Fig. 1. None of the treatments caused more than 30% mortality over a 7-day period of development against a background of no mortality in aphids on control diet; survival analysis showed that most differences to control were not significant (effect on survival by difference in survival curve; $p > 0.05$). The GNA carrier protein showed significant effects on *M. persicae* survival (difference in survival curve; $p < 0.05$), in agreement with previous reports that this protein is weakly insecticidal towards aphids[29]; it also caused growth retardation at the beginning in the bioassays,. although aphids were able to recover from the effects and produced nymphs. There were no significant differences in the effects of GNA between aphid strains. At the concentrations used, the Hv1a toxin showed significant effects on *M. persicae* (30% mortality after 7 days; effect on survival by difference in survival curve $p<0.05$), whereas Pl1a did not have a significant effect, although both toxins have been shown previously to have some effect on aphids when fed in diet. Once again, no significant differences between aphid strains were observed in these assays. These data confirm previous observations that the separate components of insecticidal fusion proteins have only limited insecticidal effects when fed to *M. persicae*.

3.2 Toxicity of Pl1a/GNA recombinant fusion protein

Purified recombinant Pl1a/GNA fusion protein was fed to each *M. persicae* strain at a range of concentrations, and survival curves were plotted for all treatments. Results for feeding at 1 mg ml^{-1} are shown in Fig 2A. At this level, the fusion protein caused complete mortality to strain 4106A (*wild-type*) after 7 days, but not in any of the insecticide resistant strains, even after 11 days. The survival curves show significant differences between strains 4106A (*wild-type*), 794J (*kdr*) and UKO (*super-kdr*) and the controls not fed fusion protein (≥90% survival) ($p < 0.05$), confirming the insecticidal activity of the treatment. However, the survival curve for strain 4824J (*kdr* + *super-kdr*: 90% survival over the assay) fed Pl1a/GNA at 1 mg ml^{-1} is not significantly different to that for aphids fed control diet containing no fusion protein ($p < 0.05$). Survival curves

for strains 794J (*kdr*) and UKO (*super-kdr*), which both show 40% survival over the assay, differ significantly from controls, from wild-type survival, and from strain 4824J survival ($p < 0.05$). Growth retardation was observed in all aphids exposed to fusion proteins, but was least in strain 4824J (Fig. 2B), where aphids were able to produce nymphs during the assay period, as did the controls. No other aphid strain exposed to treatment was able to produce nymphs. The data demonstrate a differential effect of the fusion protein on the different aphid strains, with *wild-type* strains fully susceptible to the toxin at this concentration, whereas the *kdr* and *super-kdr* strains are partially tolerant, and the *kdr* + *super-kdr* strain is almost completely tolerant.

By analysing survival curves for aphids exposed to different concentrations of Pl1a/GNA, LC_{50} values for the different strains could be deduced. The values obtained range from 0.35 to 1.76 mg ml^{-1}, and are shown in Table 1. There is a strong correlation between insecticide resistance of aphid strains and the estimated LC_{50} values; wild-type susceptible aphids have the lowest LC_{50}, and the order of insecticide tolerance (*wild-type* < *kdr* < *super-kdr* < *kdr* + *super-kdr*) is reflected in the LC_{50} values (*wild-type* < *kdr* < *super-kdr* < *kdr* + *super-kdr*). The *kdr* + *super-kdr* strain 4824J has an estimated LC_{50} of 1.76 mg ml^{-1} for Pl1a/GNA; recombinant protein at 2.0 mg ml^{-1} caused significant effects on survival, and treatment with 2.5 or 3.0 mg ml^{-1} of Pl1a/GNA resulted in complete mortality (Fig. 2C).

3.3 Toxicity of Hv1a/GNA recombinant fusion protein

An insecticidal fusion protein containing the calcium-channel specific toxin Hv1a was used as a control to identify non-specific effects on sensitivity towards insecticidal compounds in the pyrethroid-resistant *M. persicae* strains. Purified recombinant Hv1a/GNA fusion protein was fed to each strain at a range of concentrations, and survival curves were plotted for all treatments. Results for feeding at 1 mg ml^{-1} are shown in Fig.3A. Hv1a/GNA fusion protein at this concentration caused complete mortality to strains 4106A (*wild-type*) and UKO (*super-kdr*) after 6 days, and to strains

794J (*kdr*) and 4824J (*kdr* + *super-kdr*) after 9 days. The survival curves show significant differences between all strains fed fusion protein and the controls not fed fusion protein (100% survival over 11 days) ($p < 0.05$), in agreement with previous assays showing that this fusion protein is insecticidal. Growth retardation was observed in all aphids exposed to fusion proteins (Fig 3B), and no aphids exposed to treatment were able to produce nymphs. Comparison of individual survival curves when Hv1a/GNA was fed at 1 mg ml^{-1} suggested that strain 4824J (*kdr* + *super-kdr*) was more tolerant to Hv1a/GNA than *wild-type* aphids (strain 4106A), (difference between survival curves at $p < 0.05$) but that other differences were not significant. Assays at other concentrations of Hv1a/GNA did not give consistently significant differences between treatments, although the *wild-type* strain always showed greater susceptibility to the fusion protein than the pyrethroid-resistant strains.

LC_{50} values for Hv1a/GNA in the different aphid strains were deduced by analysis of survival curves for aphids exposed to different concentrations fusion protein. The values obtained range from 0.19 to 0.28 mg ml^{-1}, and are shown in Table 1. The estimated LC_{50} values show no significant differences between any of the aphid strains although the *wild-type* strain, 4106A, has a lowest LC_{50} value. The uncertainties in estimated LC_{50} values are relatively large compared to the differences, but the fitted dose-response curve for the *wild-type* strain differs significantly from the other curves ($p < 0.05$), supporting the conclusion that this strain is more susceptible to Hv1a/GNA.

4 DISCUSSION

The insect sodium channel is a major target for conventional pesticides, such as pyrethroids. The Pl1a toxin, which acts on the same target, could represent a novel type of insecticidal component as a substitute to pyrethroids. The mode of binding of this toxin would be expected to differ significantly from binding a small molecule channel blocker like a pyrethroid, with contacts between the toxin and the channel potentially extending over a wider area. However, Pl1a/GNA fusion protein exhibits

reduced toxicity towards pyrethroid-resistant peach-potato aphid (*Myzus persicae*) strains, showing that the mutations, which remove sensitivity to pyrethroids, also affect the binding of Pl1a. The mutations which give pyrethroid sensitivity are in domain II of the sodium channel, with the mutation at L1014 in helix S6 and the mutation at M918 in the linker between helices S4-S5. Changes to the spatial structure of domain II as a result of these mutations presumably also disturb the binding of Pl1a to receptor site 4, in domain II. However, although the bioassays show that mutations in domain II of the insect sodium channel affect the insecticidal activity of the Pl1a/GNA fusion protein, some toxicity is still observed, with a higher concentration of fusion protein required to cause mortality in the pyrethroid-resistant *kdr* and *super-kdr* strains. This result implies that either some interactions still exist between Pl1a and domain IIS6 or domain IIS4-S5 linker of the mutated sodium channel, or that Pl1a also binds to other sites on the sodium channel to cause inactivation. The extracellular loops of IIS1-S2, IIS3-S4 are thought to be the main binding sites of Pl1a, which are distinct from the pyrethroid binding site but contribute to receptor site 4 for toxins. The change in the spatial structure of domain II as a result of the *kdr* and *super-kdr* mutations may have a relatively small effect on toxin binding in the interaction between Pl1a and the sodium channel but may prevent the toxin inactivating the channel. The greater effect on channel structure caused by combining the mutations at L1014 and M918 would be expected to affect Pl1a binding more than single mutations, in agreement with the lack of sensitivity to Pl1a/GNA shown by aphid strain 4824J.

As expected, when fusion protein containing the calcium channel-specific toxin Hv1a is fed to aphids, there is no evidence for significant differential sensitivity between insecticide-resistant aphid strains, since the strains differ in mutations to the sodium channel. However, the observation that wild-type aphids are more susceptible to this toxin is unexpected. Mutations in sodium channels present in strains 794J, UKO and 4824J would be expected to result in a fitness cost to *M. persicae*, similar to that observed both for other insect-resistant aphids of this species,[30] and for other insect

253 species (e.g. when comparing insecticide-resistant and insecticide-susceptible German
254 cockroaches, *Blattella germanica*[31]) A fitness cost for insecticide resistance can be
255 inferred in *M. persicae* from population data; if there were no fitness cost, the
256 population of resistant *M. persicae* should be much larger than wild type before
257 selection occurs.[32] The fitness cost would be expected to make insecticide-resistant
258 strains of *M. persicae* more susceptible to Hv1a/GNA, but this is not the case. Possibly,
259 other changes to the phenotype of insecticide-resistant aphids are affecting
260 susceptibility to this fusion protein; a transcriptomic study[33] has suggested that
261 insecticide resistance in *M. persicae* is complex, and involves a broad array of
262 resistance mechanisms. The present results support that conclusion.
263 The *kdr* strain of *M. persicae* is resistant to all pyrethroids, showing 23-to 73-fold
264 increased resistance[1] and the *kdr* +*super-kdr* strain is virtually immune to all the
265 pyrethroids.[34] A fusion protein containing the sodium-channel specific Pl1a toxin can
266 cause 100% mortality towards pyrethroid-resistant aphids containing a single mutation
267 in the sodium channel if administered at concentrations increased only 3-fold, but is not
268 effective towards aphids containing a double mutation in the sodium channel. However,
269 insecticide-resistant aphids are still sensitive towards a calcium channel-specific toxin,
270 albeit at higher doses than wild-type aphids. These experiments demonstrate the
271 potential for fusion protein-based biopesticides to complement existing pesticides, and
272 to be used in the management of insecticide-resistant insect strains; the Hv1a/GNA
273 fusion protein is currently undergoing trials leading to commercial use as a biopesticide.
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393 **Table 1.** Estimated LC_{50} values for fusion proteins towards wild-type and pyrethroid
394 tolerant strains of *M. persicae*. Values were calculated from dose-response curves
395 fitted to survival data after 9 days' exposure to diets containing fusion proteins at
396 varying concentrations.

397

Genotype (Strain)	LC_{50} (mg ml^{-1}) Pl1a/GNA	LC_{50} (mg ml^{-1}) Hv1a/GNA
4106A (wild type)	0.35	0.19
794J (*kdr*)	0.60	0.28
UKO (*super-kdr*)	0.83	0.25
4824J (*kdr* + *super-kdr*)	1.76	0.20

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399

400 **Figure Legends**

401 **Figure 1.** Toxicity of fusion protein components towards *M. persicae*. Graph shows
402 survival after 7 days of pyrethroid-tolerant *M. persicae* strains (794J*, kdr*; UKO, *super-*
403 *kdr*; and 4824J, *kdr*+*super-kdr*) and wild type 4106A strain after feeding artificial diet
404 containing 0.4 mg ml^{-1} Pl1a, 0.46 mg ml^{-1} Hv1a or 0.6 mg ml^{-1} GNA. Survival on control
405 diet was 100% for all aphid strains over this interval. n = 20 aphids per replicate.
406
407 **Figure 2.**
408 **(A):** Toxicity of Pl1a/GNA fusion protein towards *M. persicae*. Graph shows survival
409 curves of pyrethroid-tolerant *M. persicae* strains (794J*, kdr*; UKO, *super-kdr*; and 4824J,
410 *kdr*+*super-kdr*), and wild type 4106A strain fed Pl1a/GNA at 1 mg ml^{-1}. All aphid strains
411 on control diet showed survival similar to that presented for 4106A strain. n = 20 aphids
412 per replicate.
413 **(B):** Growth suppression by Pl1a/GNA fusion protein. Graph shows lengths of aphid
414 strains 794J*, kdr*; UKO, *super-kdr*; and 4824J, *kdr*+*super-kdr* and 4106A (wild type)
415 from neonate to adult (9 days) after feeding on artificial diet containing 1mg/ml
416 Pl1a/GNA (n=3 per treatment). 100 % mortality for strain 4106A prevented analysis for
417 day 9. Data for strain 4842J fed on control diet is shown, but all aphid strains fed on
418 control diet were of comparable size at each time point.
419 **(C):** Dose-response effects of Pl1a/GNA. Graph shows survival curves of 4824J
420 (*kdr*+*super-kdr*) *M. persicae* strain fed diets containing different concentrations of
421 Pl1a/GNA in the range 0 - 3.0 mg ml^{-1}. n=20 aphids per replicate.
422
423 **Figure 3.**
424 (**A):** Toxicity of Hv1a/GNA fusion protein towards *M. persicae*. Graph shows survival of
425 pyrethroid-tolerant *M. persicae* strains (794J*, kdr*; UKO, *super-kdr*; and 4824J,
426 *kdr*+*super-kdr*) and wild type 4106A strain fed on diet containing 1 mg ml^{-1} Hv1a/GNA.
427 All aphid strains on control diet showed survival similar to that presented for 4106A

strain. n = 20 aphids per replicate.

(B): Growth suppression by PHv1a/GNA fusion protein. Graph shows lengths of aphid strains 794J*, kdr*; UKO, *super-kdr*; and 4824J, *kdr*+*super-kdr* and 4106A (wild type) from neonate to adult after feeding on artificial diet containing 1mg/ml Hv1a/GNA (n=3 per treatment). 100 % mortality for strains UKO, 4824J and 4106A prevented analysis for day 9. Data for strain 4842J fed on control diet is shown, but all aphid strains fed on control diet were of comparable size at each time point.