Insect cuticle: a critical determinant of insecticide resistance

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Abstract

Intense use of insecticides has resulted in the selection of extreme levels of resistance in insect populations. Therefore understanding the molecular basis of insecticide resistance mechanisms becomes critical. Penetration resistance refers to modifications in the cuticle that will eventually slow down the penetration of insecticide molecules within insects' body. So far, two mechanisms of penetration resistance have been described, the cuticle thickening and the altering of cuticle composition. Cuticular modifications are attributed to the over-expression of diversified genes or proteins, which belong to structural components (cuticular proteins mainly), enzymes that catalyze enzymatic reactions (CYP4G16 and laccase 2) or ABC transporters that promote cuticular translocation. In the present review we summarize recent studies and discuss future perspectives.

Introduction

Protection of food sources and control of vector borne diseases relies heavily on insecticides. However, the prolonged and intense use of a limited number of different compounds has generated a massive selection pressure for resistance to evolve. Extreme cases, in which resistance has developed against multiple insecticide classes [1] are particularly worrying.
The best studied mechanisms of resistance involve modifications of the insecticide target protein which affect the molecules binding efficiency and over-expression of detoxification enzymes that inactivate the insecticide by metabolism or sequestration. However, other resistance mechanisms exist outside of this paradigm. One such process involves reducing penetration of insecticide into the body by modifying the composition of the insect cuticle. For example, in *Cimex lectularius* the cuticle thickness was positively correlated with time-to-knockdown effect [2]. Importantly, slowing down the rate of penetration is thought to allow detoxification enzymes more time to act multiplying their effect and resulting in stronger resistance phenotypes. Penetration resistance can also broaden insecticide resistance across different chemical classes (multiple resistance), with substantial practical implications for commonly used insecticide resistance management (IRM) practices, such as rotations [3]. In this review we summarize the biochemistry and genetics underpinning penetration based resistance with a focus on both disease vectors and agricultural pests.

Function, structure and composition of the insect cuticle

The cuticle, also known as the exoskeleton, is the outermost part of the insect body. It serves a variety of functions such as protection from desiccation, sensory perception of the environment, mechanical support and means of locomotion [4]). Moreover, the cuticle is the first and major barrier, protecting the insect from penetration of external compounds. Its structure is generally well preserved among insect species and consists of different layers (Figure 1) with distinct composition and properties. The outermost layer is called epicuticle and is commonly covered by a film of wax and cement. Underneath the epicuticle is the procuticle which accounts for most of the cuticular mass and can be divided into the exo-cuticle (upper and harder part) and the endo-cuticle (lower and softer part). Lastly, a single layer of epidermal cells that secrete many of the cuticular components lies at the base of the cuticle.

The epicuticle is mainly composed of hydrocarbons, proteins and lipids, the majority of which are free fatty acids and wax esters. Insect hydrocarbons are highly variable elements, varying from n-alkanes and methyl- branched alkanes to unsaturated hydrocarbons. Hydrocarbons are produced by a specific cell type called oenocytes. Different hydrocarbon blends are characteristic for different insect orders, while their composition is also affected by factors like age, sex and environmental conditions [5]. The procuticle is mainly composed of chitin fibers and proteins [6]. Genome analyses in arthropods have revealed a large number of genes coding for structural cuticular proteins (CPs) or putative CPs [7-9]. For example in *Anopheles gambiae* over 2% of the protein coding genes belong to the family of CPs [10]. Although the function of the majority of predicted structural proteins remains unknown, chitin binding has been demonstrated for some of them [11, 12], suggesting their role in interacting with the polysaccharide to form a highly organized structure.

Association of insecticide resistance with reduced insecticide penetration

Correlation of insecticide resistance with reduced insecticide penetration through the cuticle has been reported in a variety of insect species (Table 1). A common method used to monitor the rate of insecticide internalization involves exposure of insects to insecticides for a certain time followed by extraction and comparison of the insecticide that remained on the cuticle surface (non-penetrated) to the insecticide recovered from the whole insect homogenate (internalized). In this way a 30% reduction in the ratio of internalized:cuticular radiolabeled DDT, was observed for the 91-R *Drosophila melanogaster* strain which shows high levels of resistance (>1500 fold) [13]. Likewise, in a *Helicoverpa armigera* deltamethrin-resistant strain, the time required for 50% penetration of the topically-applied radiolabeled deltemethrin was 6h compared to 1h for the susceptible strain [14].

Several studies have used this methodology to examine cuticular resistance (Table 1), however, in some insects the actual route of the insecticide starts from the appendages, mainly the legs. Thus, to resemble the route of the insecticide it is more relevant to apply the insecticide on surfaces rather than dropping it directly on the insect body. This approach was followed in a recent study showing a 50% reduction in the penetration of radiolabeled deltamethrin in a multi-resistant *Anopheles gambiae* population compared to the susceptible strain after exposure to ^{14}C-deltamethrin impregnated WHO filter papers [15]. Less commonly the role of the cuticle in conferring resistance has been examined by injecting the insecticide to by-pass the cuticular barrier and comparing resistance with that of external application. This approach was followed in the bed bug (*Cimex lectularius*) and revealed that injecting the insecticide reduced the levels of resistance [16].

Penetration resistance mechanisms

Cuticular changes underlying insecticide resistance involve two main parameters: the thickness or composition of the cuticle. Cuticular thickening has been more commonly associated with resistance, but there have been recent reports linking cuticular composition to decreased xenobiotic penetration [17]. A small number of genes associated with both mechanisms have been identified (Table 2).

Thickening of the cuticle

Detailed Transmission Electron Microscopy of mosquito legs revealed that a multi-resistant *Anopheles gambiae* (*An gambiae*) mosquito population, which exhibited remarkable tolerance to multiple insecticide classes, possessed thicker leg cuticles, due to enriched deposition of hydrocarbons to their epicuticle. Underpinning this phenotype was the over-expression of two cytochrome P450s (CYP4G16 and CYP4G17) in the abdominal oenocytes, which synthesized the extra hydrocarbons [15]. Constitutive over-expression of CYP4G16, together with other genes implicated to hydrocarbon biosynthesis pathway, was identified in *Anopheles arabiensis* resistant populations from Zanzibar islands in Africa [18].

A different population of *An gambiae* originated from West Africa, also resistant to pyrethroids and DDT, adopted an alternative cuticle-thickening mechanism, by thickening all the chitin layers (exo-, meso- and endocuticle). These mosquitoes were found to over-express a suite of CP proteins (CPLCG3, CPR124, CPR127, CPR129) with characteristic chitin-binding motifs, which could explain the reinforcement of all procuticle chitin layers [19]. Previously, CPLCG3 (and G4) was found expressed at higher levels in pyrethroid-resistant compared to susceptiple *An gambiae* mosquitoes [20, 21]. Localization of these proteins in limbs and particularly in the endocuticle, is consistent with the cuticle-thickening resistance mechanism [22]. A third multi-resistant *An gambiae* mosquito strain, from Tanzania had elevated expression of CPLCG4, G5 and CPR131, suggestive of a putative cuticle-thickening mechanism [23]. Cuticular resistance has also been implicated in other malaria vectors. For example, comparison of legs of laboratory strains of *An funestus* revealed thicker cuticles in the resistant strain than seen in a lab susceptible strain [24].

A deltamethrin resistant *Cx. Pipiens pallens* mosquito population showed elevated expression of 14 CP proteins compared to a susceptible lab strain [24]. Moreover silencing of a single gene, CpCPLCG5, increased insecticide susceptibility of the resistant strain.

A Triatoma infestans pyrethroid-resistant population had an impressive increased amount of cuticular hydrocarbons (CHCs) when compared to pyrethroid-susceptible populations (50% more CHCs). Scanning Electron Microscopy revealed that resistant insects had significantly thicker cuticles versus the susceptible ones [25]. Since Triatoma bearing HC-free cuticles were more susceptible to drug penetration [26], it was proposed that enrichment in CHCs delays the uptake of pyrethroids through the insects cuticle.

The *91-R* strain *of Drosophila melanogaster*, which was extremely tolerant to DDT compared to the susceptible *Canton-S* strain, showed a 30% less contact penetration of DDT through the pronotum. The same strain expressed significantly more cuticular hydrocarbons and a 4% thicker more laminated cuticle compared to the susceptible flies. These differences were suggested to contribute to the impressive DDT resistance levels, by reducing penetration into the body [13]. Similarly a resistant strain of *Bactrocera dorsalis* possessed not only thicker cuticle, but also more chitin layers and laminated structures in the endocuticle. The epidermal cells of the corresponding strain had thicker interspaces· coiled filaments and thicker interspaces of epidermal cells could correlate with enhanced secretion of chitin and cuticular proteins [27].

Altered cuticle composition

Adaptation of cuticular composition often leads to increased insecticide resistance due to inhibition of insecticide penetration capacity. Among several hypotheses, two specific types of compositional changes have been reported, one mediated by *laccase 2* leading to cuticular hardening and a second mediated by ABC transporters:

I. Over-expression of laccase 2.

Cuticle tanning (or sclerotization and pigmentation) is a multi-step extracellular process,that affects hardening and pigmentation of insect cuticle. Two oxidases of the phenoloxidase group, a laccase and a tyrosinase, are involved in the cross-linking of adjacent polypeptide chains from cuticular proteins through the oxidative conjugation of quinones and quinones methides [28]. Very recently, the important role of laccase 2 in the tanning process of *T.castaneum* cuticles was revealed [29]. Similarly to *Tribolium*, beetles of *M. alternatus* suffered from abnormal, soft and enlarged cuticles, upon *lac2* silencing, while building a much thinner procuticle, when compared to the control insects [30]. Concomitantly, Cp*Lac2* gene was found significantly up-regulated in a *Cx. Pipiens pallens* fenvalerate-resistant mosquito population, compared to the susceptible. Cp*Lac2* induction was observed both in 4th instar larvae and pupae stage, which could support extra sclerotized cuticles of resistant mosquitoes [31].

II. Over-expression of ABC transporters and cuticular translocation.

ABC transporters act as efflux pumps in the eukaryotic cells and their expression in the epidermis could facilitate the export of cuticular components towards the cuticle. For example in plants and flour beetle ABC transporters from subfamily G are correlated with the transport of cuticular lipids in the epidermis [32]. Enriched expression of several ABCGs in mosquito legs, which in parallel found over-represented in different insecticide resistant strain populations, could account for elevated CHC deposits, and therefore penetration resistance [33]. It is conceivable that enriched cuticular translocation can affect not only composition but also cuticle thickness. In tobacco budworm pesticide resistant populations a p-glycoprotein located mainly to the cuticle, was found up-regulated compared to susceptible strains [34]. As ABC transporters have well annotated roles in the transport of both insecticides and hydrocarbons [35], the precise role played by these transporters in resistance is not straightforward.

Overall, besides thickness, cuticular restructuring affects other biomechanical properties, including reduced modulus and hardness [36]. Very recently, it was proposed that the epicuticle regulates the mechanical properties of the cuticle by fine-tuning the water balance [37]. Moreover, CHCs in Drosophila species found to play dual role as waterproofing agents and pheromonal signals [38]. Cuticle is a well-organized structure which can orchestrate diverse physiological functions, such as desiccation resistance and chemical communication. Therefore, cuticle modifications identified in resistant insects besides reduced drug penetration, could extend to alterations affecting the whole organisms' physiology and fitness, with possible implications in the spread of resistance.

Concluding remarks and future perspectives

The emergence of pest and vector populations with remarkable levels of insecticide resistance against all classes of insecticides tested, implies the existence of broad spectrum resistance mechanisms. One such mechanism is cuticular resistance which can confer multi-insecticide resistance by changing the thickness or composition of the cuticle (Table 1). In all cases, cuticle changes are attributed to the over-expression of genes such as P450s (CYP4G16), CPs, a laccase and ABC transporters, but their precise role in cuticular resistance remains elusive. The methodologies described so far in the literature to study altered cuticle-based insecticide resistance need additional consideration, while new approaches (as high-throughput proteomics) are necessary (BOX 1). Given the high complexity of the cuticle structure, is not surprising that still many questions need to be addressed.

The limited armory of chemicals for insect control is very rapidly being eroded by the emergence of insecticide resistance in many of the agricultural and public health pests. The cuticle is an ideal target for novel insecticides also because cuticular components are usually absent in higher eukaryotes.

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Figure and Figure legends

Figure 1: Penetration resistance mechanisms. All cuticular modifications that have been proposed to underlay reduced insecticide uptake are depicted. *Resistant: thickening of the epicuticle, **Resistant: thickening of the procuticle, ***Resistant: altered cuticle composition.

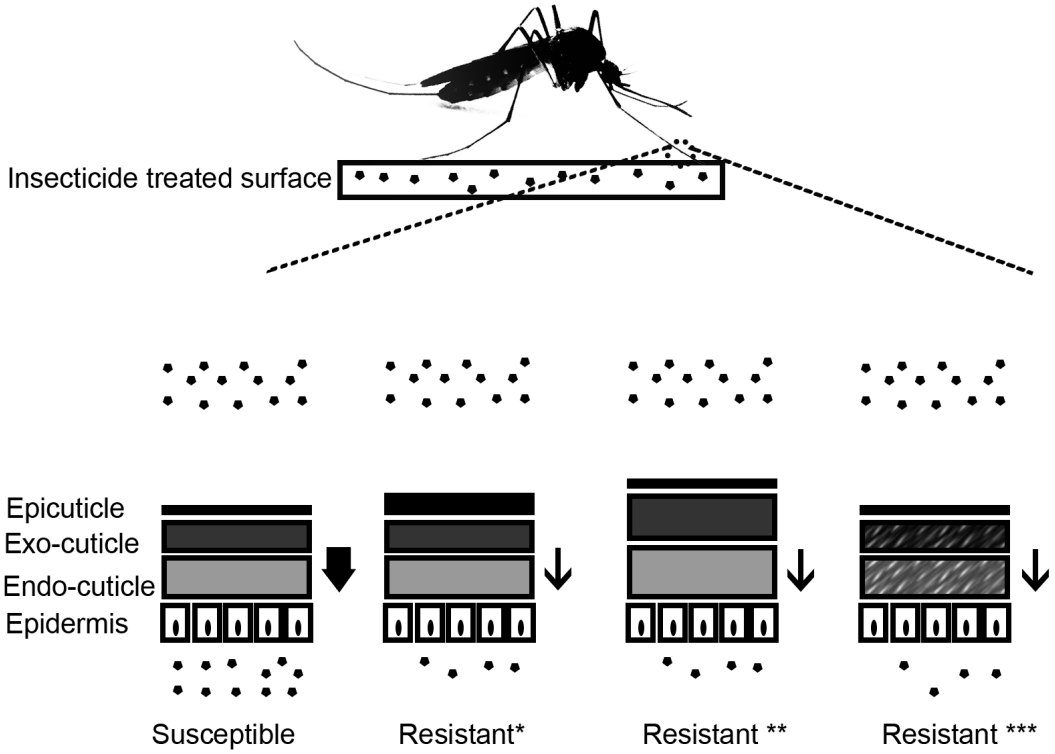

Table 1: Studies reporting evidence for reduced insecticide penetration and/or cuticular modifications, as mechanisms for insecticide resistance.

			Reduced insecticide penetration	**Cuticle Modifications**		
Species	Developmental Stage	Assay used	Insecticide tested	Cuticle Thickening (Method used)	Cuticle Hardening (or other modifications)	Reference
Anopheles gambiae	Adult	1,3	Deltamethrin	v (TEM)		Balabanidou et al., 2016
Anopheles gambiae	Adult	2	Deltamethrin	v (TEM)		Yahouedo et al., 2017
Anopheles funestus	Adult		Permethrin	v (SEM)		Wood et al., 2010
Culex pipiens pallens	Larvae and pupae				v	Pan et al., 2008
Culex pipiens pallens	Adult	Contact assay	Deltamethrin	v		Fang et al., 2015
Cimex lectularius	Adult	Contact assay	lambda-cyhalothrin	v (SEM)		Lilly et al., 2016
Cimex lectularius	Adult	4	Deltamethrin β -cyfluthrin			Koganemaru et al., 2013
Helicoverpa armigera	Larvae	2	Deltamethrin	v		Ahmad et al., 2006

Bactrocera dorsalis	Larvae	2	β-cypermethrin	v (TEM)	(more laminated structures in the endocuticle)	Lin et al., 2012
Drosophila melanogaster	Adult	2	DDT	v (TEM)	(more laminated structures in the endocuticle and more coiled filaments in the epidermis)	Strycharz et al., 2013
Plutella xylostella	Larvae	2	S-Fenvalerate			Noppun et al., 1989
Triatoma infestans	Nymphs	2	Deltamethrin	v (SEM)		Pedrini et al., 2009 and Juarez, 1994

1. Contact assay with ^{14}C radiolabeled insecticide and penetration rate comparison between resistant and susceptible strains
2. Topical application of radiolabeled insecticide and penetration rate comparison between resistant and susceptible strains
3. Comparison of insecticide toxicity (efficiency) between Topical application and Contact assay in Resistant versus Susceptible strains
4. Comparison of insecticide toxicity (efficiency) between Injection and Topical assay in Resistant versus Susceptible strains

SEM: Scanning Electron Microscopy; TEM: Transmission Electron Microscopy

Table 2: Genes involved in cuticle composition or synthesis that have been found up-regulated in insecticide resistant strains.

Strain	Up-regulated genes related to the cuticle	Reference
Anopheles gambiae	CYP4G16 CPAP3-E, CPLCX1, CPLCG3, CPR124, CPR129 CPR127,CYP4G16 CPLC8, CPLC# CPR30 (and more 19 CPs) CPLCG4, G5 and CPR131 ABC transporters	Balabanidou et al., 2016 Yahouedo et al., 2017, Awolola et al., 2009 Bonizonini et al., 2012 Nkya et al., 2014 Pignatelli et al., 2017
Anopheles stephensi	CPLC8	Vontas et al., 2007
Anopheles funestus		Gregory et al., 2011
Anopheles arabiensis	CYP4G16	Jones et al., 2013
Culex pipiens pallens	CpLac2	Pan et al., 2008
Culex pipiens pallens	CpCPLCG5	Fang et al., 2015
Cimex lectularius	CPR-type	Koganemaru et al., 2013
Heliothis virescens	P-glycoprotein	Lanning et al., 1996

Box 1: Methods to identify and characterize altered cuticle-based insecticide resistance mechanisms

Classical assays

- Compare bioassay response of topically applied insecticide in organic solvent versus contact application and/or microinjection of insecticide inside hemolymph
- Direct measure of insecticide uptake using C^{14} radiolabeled molecules or sensitive analytical techniques
- Measure cuticle thickness in insecticide uptake relevant insect parts by Electron Microscopy

Molecular and analytical assays

- Transcriptomic approaches (new generation sequencing, microarrays, targeted RT-PCR), to determine levels of "cuticle" related genes
- Proteomic approaches to determine levels of "cuticle" related proteins
- Measure chitin content
- Determine epicuticular lipids