Jonsson, N. N., Klafke, G., Corley, S. W., Tidwell, J., Berry, C. M. and Koh-Tan, H.H. C. (2018) Molecular biology of amitraz resistance in cattle ticks of the genus Rhipicephalus. Frontiers in Bioscience, 23(2), pp. 796-810.

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Deposited on: 23 November 2017

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Molecular biology of amitraz resistance in cattle ticks of the genus *Rhipicephalus*

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TABLE OF CONTENTS

1. ABSTRACT

Amitraz is an important product for the control of cattle ticks around the world. In comparison with other products for the control of ticks, it is quite affordable and it has a rapid knock-down effect. It binds with and activates adrenergic neuro-receptors of animals and it inhibits the action of monoamine oxidases (MAO). Resistance to amitraz has been documented in *Rhipicephalus microplus*, *R. decoloratus* and *R. appendiculatus*. Four mechanisms of resistance have been proposed, each of which is supported by evidence but none of which has been definitively confirmed as the cause of resistance in the field. The proposed mechanisms include genetic target site insensitivity in two G protein-coupled receptors, the beta-adrenergic octopamine receptor (*BAOR*) and the octopamine/tyramine receptor (*OCT/Tyr*), increased expression or activity of monoamine oxidases and increased expression or activity of the ATP binding cassette transporter.

2. INTRODUCTION

Amitraz is a widely used, affordable and relatively safe short-acting acaricide for use by spray or plunge dip. It has been available for over 40 years and resistance has been reported with increasing frequency in recent years. Research into the mechanisms of acaricide resistance has generated several lines of enquiry but so far none of the proposed mechanisms has been confirmed using functional studies. This review is intended to provide an overview of current knowledge and understanding of the possible mechanisms of amitraz resistance in rhipicephaline ticks.

3. AMITRAZ

3.1. Structure and chemical characteristics

Amitraz is listed and described on PubChem Open Chemistry Database as CID36324 or BTS-27419. Its molecular formula is $C_{19}H_{23}N_3$ and its structure is as shown in Figure 1. It has many synonyms, including U-36059. Amitraz has been widely used as an acaricide since its discovery and reporting in 1972 by the Boots Company (1). Although pour-on products with amitraz have been developed for *Sarcoptes* mites affecting pigs, in cattle it is formulated for use as an aqueous dip or spray, at a

concentration of 0.2.5 g/l (2). Although soluble in most organic solvents, it is not soluble in water, which leads to some challenges with formulation. Amitraz is rapidly absorbed and is primarily excreted in the urine in the form of 5 degradation products, of which N′-(2,4-dimethylphenyl)-Nmethylformamidine (BTS-2721) and 2,4-dimethylformanilide (BTS-27919) predominate, both of which are regarded as potentially having genotoxic effects (3).

3.2. Amitraz as an acaricide

For application to cattle using aqueous sprays or dips, it is formulated as either an "emulsifiable concentrate" (EC), often used in spray races, or as a "wettable powder" (WP), often used in dipping vats. The WP formulation, in particular, is prone to sedimentation in the dipping vat and requires vigorous and regular agitation to ensure that the active compound is in suspension. To add to the challenges of its use, amitraz is not stable under acidic pH conditions (4) and it requires buffering with lime to maintain an alkaline pH, ideally of pH = 12 (5). Whereas the compound is relatively stable at neutral pH in sterile solutions, in the pH range 7 to 11, amitraz is co-metabolised by bacteria in fouled dipping solutions. These processes are blocked at alkaline pH >11.5.(5).

The efficacy of amitraz as an acaricide for several species of tick including *R. microplus* and *R. annulatus* has been established (eg (6)). Treatment with 0.1.25 g/l and 0.2.5 g/l amitraz both provided > 97% control of ticks, accompanied by a reduction in the weights of and reduced egg production by any surviving female ticks. The application of amitraz to animals for the control of ticks is characterised by rapid detachment of all stages of the tick from the host, with subsequent mortality. Ticks begin to detach from the host within one hour of application and are completely or mostly cleared from the host within 7 hours (6, 7). Amitraz is considered to be a short-acting acaricide but it does have residual efficacy for 7 to 10 days (6, 7).

Although it is not currently a recommended or legal strategy, Harrison and Palmer (2) describe the use of amitraz in pasture and on vegetation to control *R. microplus* and *Haemaphysalis longicornis*. Application of 0.5. g/l amitraz to pastures (equivalent to 667 g/ha) resulted in >90% mortality of free-living *R. microplus* larvae. Application of 200 to 800 g/ha of amitraz by aerial spraying resulted in a 50 to 80% reduction in nymphs of *H. longicornis*.

In comparison with commercially available pour-on and injectable products, amitraz is cheap and where there are no resistance problems the correctly formulated and applied product provides a rapid knockdown effect. Amitraz is commonly used by small-holder cattle producers in Africa, where hand-sprays are often used, and macrocyclic lactones are unaffordable for smallholders. A recent study in Uganda (8) showed that 37% of all acaricides used for tick control were amitraz. In Zambia, 27% of treatments against ticks were with amitraz (9).

3.3. Biological effects of amitraz

The application of amitraz to ticks on cattle is followed by behavioural changes including detachment, then by mortality or reduced weight of ticks and reduced egg production (6, 7). Hence, amitraz causes a wide range of effects in mammals and arthropods and it appears that its effects are mediated by enzyme inhibition and by binding with neuroreceptors.

The first proposed mechanism for acaricidal effects of the related compound chlordimeform was the inhibition of monoamine oxidase (MAO) (10). Amitraz (described in the paper as U-36059) was subsequently shown to be a more potent inhibitor of monoamine oxidases in rat liver than chlordimeform (11). The I_{50} concentration (the concentration providing 50% inhibition of MAO activity) of amitraz (U-36059) was $6.6. \times 10^{-7}$, which suggests that it has 20-fold higher activity than chlordimeform, which had an I_{50} value of $1.4. \times 10^{-5}$. Yim *et al.* (12) subsequently showed that the amidine acaricides chlordimeform and amitraz were potent anti-inflammatory compounds in rats and suppress prostaglandin synthesis by inhibition of prostaglandin synthetase activity. Compared with indomethacin and aspirin in a rat model of prostaglandin synthetase inhibition, the order of potency was indomethacin > chlordimeform > amitraz > aspirin.

Amitraz is identified on *PubChem* (13) as an adrenergic alpha-2 receptor agonist, and there is a considerable body of evidence that G protein-coupled receptors are the main targets of the compound in mammals. Shin & Hsu (14) clearly showed that amitraz acts via alpha-adrenoceptors and that the effect can be blocked by yohimbine, an alpha-adrenoceptor-like antagonist. Amitraz also behaves as a histamine H1 receptor antagonist (15). In insects and acari, it has been proposed to bind with the octopamine receptor (16, 17) or the octopamine/tyramine receptor (18, 19).

4. RESISTANCE TO AMITRAZ IN TICKS

4.1. Bioassays for the diagnosis of amitraz resistance

Diagnosis of amitraz resistance in *R. microplus* is based on *in vitro* bioassays, of which there are four main variants in common use at present. The adult immersion test (AIT) is the simplest of these assays and involves immersion of fully engorged female ticks in commercial acaricide for 30 minutes and subsequent incubation for 7 days (20). The Shaw larval immersion test (SLIT) uses 300-500 larvae of 14 – 21 days old, which are placed in a filter paper sandwich and washed with 10 ml of the test compound for 10 minutes, after which larvae are incubated in filter paper tubes for 17-18 h, when mortality is assessed (21). The larval packet test of Stone and Haydock (LPT) has been the most widely used test, in which filter paper envelopes are impregnated with technical grade acaricide in oil, filled with 100 larvae of 14 - 21 days old and incubated for either 24 or 48 h before assessment of mortality (22). The LPT has been modified to improve its performance with amitraz (23) and its

performance has been shown to be superior to the AIT, which should be regarded as an unsatisfactory bioassay for amitraz resistance (20). A recent innovation has been the development of the larval tarsal test (LTT) in which 50 eggs are placed in each of the required number of wells of a 96-well plate after pre-treating the wells with the acaricide in oil. The plate is sealed and incubated for two weeks prior to visual assessment of mortality (24). This bioassay enables the testing of a large number of replicates with no handling of larvae at all. The predictive value of a positive diagnosis using the LPT and the SLIT has been questioned in the past (25-27).

4.2. Prevalence of resistance to amitraz

Resistance to amitraz has been documented in several tick species. A recent study from Uganda (8) demonstrated resistance to amitraz in *R. decoloratus* and *R. appendiculatus* using the larval packet test and using a discriminating concentration of double the LC_{99} for susceptible populations. The prevalence of amitraz resistance in *R. microplus* has been documented in many studies conducted around the world (9, 28-32).

4.3. Proposed mechanisms of resistance to amitraz

Polymorphism and consequent target site insensitivity of two G protein-coupled receptors (GPCR) has been associated with amitraz resistance in *R. microplus*. Chen *et al.* (18) identified two amino acid substitutions that are present in the octopamine/tyramine receptor of amitraz-resistant isolates but not in susceptible isolates. These results were confirmed by Baron *et al.* (19). Corley *et al.* (33) identified an amino acid substitution in the resistant populations of *R. microplus* and showed that the proportion of this variant increased in populations subjected to selection with amitraz at a rate that was proportional to the increase in resistance as measured in the LPT bioassay for amitraz. A variant of R. microplus beta-adrenergic octopamine receptor (*RmBAOR)* was amplified from an amitraz-resistant tick strain (Colombia). The variant contained a 36 bp insertion in the first trans-membrane domain, resulting in a predicted extension to the extracellular N-terminal chain, was identified in cell culture from an amitraz-resistant isolate (34). The same study also found what appeared to be a duplicate Rm*BAOR* gene only in one of the amitraz-resistant isolates, but its sequence identity is unknown. Expression of the ATP binding cassette B10 transcript (*ABCB10)* was also elevated in the same cell-line, which originated from ticks that were cross-resistant to organophosphate and organochlorine acaricides. This is consistent with the work of Lara *et al.* (35), who showed that amitraz-resistant *R. microplus* ticks detoxified amitraz more efficiently than amitraz-susceptible ticks and this was associated with increased levels of *ABCB10* expression. There are no published reports of the relationship between polymorphism and/or transcript expression levels of monoamine oxidase and resistance to amitraz. However, there are preliminary data from two studies on the subject. The first comes from an MSc project conducted by Xiarong Jiang in 2006 (36). The second work is a small, exploratory study by Jonsson & Corley, conducted in 2009. The results of these studies are inconclusive.

5. TARGET-SITE INSENSITIVITY - G PROTEIN-COUPLED RECEPTORS AND THEIR POTENTIAL ROLE IN AMITRAZ RESISTANCE

5.1. G protein-coupled receptors – structure and function

Some knowledge of GPCR structure and function is essential for the understanding of the potential roles in amitraz resistance of the *BAOR*, of *OCT/Tyr* receptor and of MAO. The GPCRs are diverse proteins that mediate second-messenger signalling at the cell membrane and constitute the largest protein superfamily of mammalian genomes (37). Ligands or triggers for GPCRs are diverse and range from photons through ions, amines, nucleotides, peptides, lipids and proteins (38). All members of the GPCR superfamily have seven hydrophobic, membrane-spanning alpha-helices, each of about 25-35 residues, which form a receptor or recognition and connection unit, which is coupled with and can interact with a G protein (38). Beyond these essential characteristics, there is considerable diversity in structure and function, particularly in relation to the alternative signalling pathways that are triggered by activation of the associated G proteins. On the basis of DNA sequence analysis of the genes coding the receptors, which are not well conserved among homologs (or paralogs) in different taxa, there are five main families of GPCR: secreting receptor family, adhesion receptor family, glutamate receptor family, frizzled/taste2 receptor family, and the rhodopsin family (38). In this classification scheme, octopamine, serotonin, tyramine, dopamine and octopamine/tyramine receptors all belong to the amine receptor cluster within the alpha-group of rhodopsin receptors within the rhodopsin family (38). Although the 7 transmembrane domain structure is consistent, there is much diversity in the N-terminal chain and in the extracellular loops, resulting in substantial variation in the size, shape and electrostatic properties of the ligand binding pockets, particularly among GPCR subfamilies (37).

The structure and function of GPCRs have been described in detail (37). In brief, inactive and active GPCR in the cell membrane are bound with heterotrimeric G protein (G_{alpha}, G_{beta} and G_{gamma} subunits). Binding of a ligand results in varying degrees of conformational change to one or more of the 7 transmembrane alpha-helices, which enables interaction of the the G_{alpha} subunit with a domain of the GPCR that functions as a guanine nucleotide exchange factor (GEF). This interaction results in the exchange of GDP that is bound to the G_{alpha} subunit for GTP. This process is the activation of the G protein, which results in dissociation of G_{alpha} from $G_{betagamma}$. The activated G_{alpha} subunit then goes on to activate one of several possible signalling pathways. The dominant two pathways are via adenylyl cyclase (AC), generating cAMP, and via phospholipase C-beta, generating inositol (1,4,5) triphosphate (IP3) and diacylglycerol (DAG). The IP3 binds with receptors on the membrane of the endoplasmic reticulum to cause intracellular release of Ca^{2+} and the DAG binds with a member of the protein kinase C (PKC)

family. These pathways and the relative dispositions of octopamine and tyramine signalling are nicely illustrated by Farooqui (39).

5.2. Classification of G protein-coupled receptors in ticks

Invertebrate GPCR share very low levels of identity with mammalian GPCR, although their ligands are more similar. Insect octopamine receptor classification has been recently revised, as shown in Table 1 (39,40). This classification seems broadly compatible with the limited information available for ticks, although the convention for naming receptors has not been applied consistently. Corley *et al.* (41) generated full length cDNA sequences for 8 putative GPCR from *R. microplus*, including one OCTbeta2-R and one OCTalpha-R, which were referred to as Rm_beta2AOR (*Rhipicephalus microplus* beta-2 adrenergic-like octopamine receptor) and Rm_alpha2AOR (*Rhipicephalus microplus* alpha-2 adrenergic-like octopamine receptor). In subsequent work examining SNPs in the Rm_beta2AOR associated with amitraz resistance, it was referred to as *RmBAOR* (33). The gene that Baxter & Barker (17) and subsequent workers examined (18, 19, 42, 43) was initially described as an octopamine-like GPCR. Subsequently, Chen *et al.* (18) referred to it simply as a putative octopamine receptor. The phylogenetic analysis of Corley *et al.* (41) classed it as a tyramine/octopamine receptor. Baron *et al.* (19) referred to it as OCT/Tyr receptor and Gross *et al.* (42) followed the approach of Verlinden *et al.* (44) and Farooqui (39) by referring to it as a tyramine receptor (*TAR1*). By definition, classification implies comparison among genes, and the rapid and somewhat confusing evolution of nomenclature is a result of ambiguities/errors in annotations that persist in public databases. Given their importance in acaricide development and drug resistance, an attempt should be made by the acarine community to standardise the nomenclature of acarine GPCRs. One important consideration in relation to the proposed mechanisms of resistance discussed below is ligand-receptor specificity, which is not often a binary characteristic. Tyramine is the final biosynthetic intermediate in octopamine production and as discussed by Lange (45), this has led to the view that its importance as a neurotransmitter in its own right is somewhat less than that of octopamine, being largely considered to be a partial agonist for octopamine at octopamine receptors. However, there is a spectrum of activity shown by octopamine and tyramine, with sometimes differential effects on the same receptor, as shown in Table 1. There are few recent pharmacological studies of acarine GPCRs, however Gross *et al.* (42) clearly demonstrated that although the effect of tyramine at the TAR1 receptor is 39 times more potent than the effect of octopamine, both are agonists.

5.3. Octopamine and tyramine and their receptors in ticks – evidence for their role in amitraz resistance

Octopamine has a very wide range of physiological and behavioural functions, which are discussed extensively elsewhere (39). In arthropods, as a neurotransmitter, it modulates neuromuscular transmission, muscle metabolism and sensory signals and influences the functional responses of innervated organs. In the haemolymph, it functions as a neurohormone, modulating haemocyte recruitment and phagocytic ability in the face of microbial challenge, and lipid mobilisation during extended motor activity. Amitraz affects all of these known physiological functions of octopamine; thus it is hypothesized that resistance to amitraz is mediated by a) alterations to amitraz degradation leading to its inactivation before interacting with octopamine or tyramine neuroreceptors (metabolic resistance), b) alterations to these neuroreceptors resulting in reduced amitraz binding (target site insensitivity), and c) modified reuptake of amitraz from the synaptic space. Currently there is evidence in support of a) and b) above. Among GPCRs of the biogenic amine group, transmission is largely modulated by the activity at the neuronal cell membrane of transporter proteins, which resorb ("re-uptake") free neurotransmitters from the synaptic cleft. Examinations of this process in acarine systems have not been published to date.

5.3.1. Octopamine/tyramine receptor

The potential role of this receptor was first examined by Baxter and Barker, who found that there was no association between cDNA sequence of the gene and resistance to amitraz in two strains of ticks in Australia (17). The susceptible N-strain had exactly the same sequence as the amitraz-resistant Ultimo strain and it was concluded that resistance to amitraz was not mediated by polymorphisms in this gene. Subsequent work conducted using North American populations of susceptible and resistant *R. microplus* identified two SNPs that were only present in amitraz-resistant isolates (18). The strains were Gonzalez (susceptible), Santa Luiza (selected with amitraz and resistant, originally from Brazil), San Alfonso (highly resistant to amitraz, from Mexico), Pesqueria (low level of resistance to amitraz – RR = 4 relative to Gonzalez, also from Mexico), Coatzacoalcos (amitraz susceptible but synthetic pyrethroid resistant), Tuxpan (organophosphate resistant but amitraz susceptible) and Corrales (amitraz susceptible but synthetic pyrethroid resistant). The complete open reading frame cDNA sequences from each were compared with that of the Australian sequences (17) and there were 37 nucleotide substitutions (~ 97% identity) and 7 amino acid substitutions (~98% identity). The San Alfonso and Santa Luiza strains shared two non-synonymous SNPs (A22C – T8P; T65C – L22S) that were absent from the other tick strains, including the Australian strains. Both SNPs were in the N-terminal, first extracellular domain, suggesting a possible role for conformational change in the octopamine/tyramine receptor in resistance to amitraz.

Baron *et al.* (19) examined South African field samples of *R. microplus* and demonstrated that the SNPs causing amino acid substitutions T8P and L22S, as previously identified in North America (18), were present in the resistant samples. They also showed a very tight relationship between survival of larvae in the LPT and the genotype. Every one of seven larvae that survived was homozygous for the putative resistance-conferring allele at both loci and none of the six larvae that did not survive the LPT were homozygous for the putative resistance-conferring alleles at the two loci. This clearly strengthens the evidence that polymorphism in the octopamine/tyramine gene contributes to the amitraz-resistance phenotype. These workers showed that the two SNPs were in linkage disequilibrium and suggested that balancing selection arising from a fitness cost of the resistant alleles

might contribute to the observed allelic frequencies. They also developed an RFLP-based assay for amitraz resistance and used it successfully in a field survey (43).

5.3.2. Beta-2-adrenergic-like octopamine receptor

Several mutations have been detected in this gene that have been associated with resistance to amitraz. Corley *et al.* (33) identified a non-synonymous mutation in the beta-2-adrenergic-like octopamine receptor in the same amitraz-resistant Ultimo strain of ticks as used by Baxter and Barker (17, 18), which resulted in a I61F amino acid substitution. In a three-year field trial in which varying degrees of selection with amitraz was applied to cattle with accompanying genetically isolated tick populations on replicate small farms (farmlets), the frequency of the homozygote putative resistance-conferring homozygous (FF) genotype closely matched the observed proportion of resistance to amitraz by the LPT. In the tick populations on the two farmlets to which amitraz selection was applied most intensively, the frequency of the putative resistance allele increased from 10 and 21% to 97 and 93% respectively over two years. On the two farmlets in which amitraz was not used there were reductions in the frequency of the putative resistance-conferring alleles. The correlation between percentage resistance in the LPT and frequency of the FF genotype was 0.9.0 and between the percentage resistance and the homozygous II genotype was -96%. An additional SNP in the *RmBAOR* was reported in a field sample at the next amino acid residue (I62T), however the resistance status of this population was not known.

Work with cell lines from *R. microplus*, five other Rhipicephalinae and three other genera (*Amblyomma variegatum, Ixodes ricinus, Hyalomma anatolicum*) recently resulted in the discovery of further polymorphism associated with amitraz resistance in the *RmBAOR* (34). Two of the cell lines (BME/CTVM6 and BME/CTVM5) were initiated in 1983 from a single isolate of ticks (Paso Ancho from Colombia), considered to be resistant to amitraz. Both of these cell lines yielded gDNA and cDNA amplicons that were larger (~245 and ~220 bp) than the 183 bp generated from each of the amitraz-susceptible populations. The 220 bp products from the BME/CTVM5 and BME/CTVM6 cell lines were sequenced and both revealed a 36 bp duplication insertion at position 190, which was predicted to result in an extended N-terminal extracellular domain (Figure 4). The approximate 245 bp product was not sequenced due to multiple primer binding sites, leading to the interpretation that there is an additional, related gene.

In subsequent work that is being prepared for publication, we have analysed gDNA sequences of *RmBAOR* from samples of ticks from diverse locations around the world, including Brazil, Mexico, Australia, Thailand, and South Africa. We used the primers described previously (forward: GAAATCTGACGGACGAGGAA; reverse: GCGACACGATGAAGTAGTTG; (34)) and have identified several SNPs listed in Table 2 and shown graphically in Figures 3 and Figure 4, which are specific to populations in which amitraz resistance was confirmed. This work has confirmed the presence of the I61F mutation in amitraz-resistant South American isolates of *R. microplus* and strengthens the belief that variation in *RmBAOR* might contribute to resistance to amitraz.

6. DETOXIFICATION MECHANISMS

It is likely that detoxification plays a role in resistance to amitraz in a similar manner to the roles that detoxification mechanisms contribute to the well-documented resistance to synthetic pyrethroids (SP). In the SP model, three mutations have been identified in the *para*-sodium channel gene (46-48), yet there is a mixed picture in the field, with some cases of resistance to SPs (generally with lower resistance ratios) being attributable to elevated esterase activity (49).

6.1. Monoamine oxidases

Variation in the structure or level of expression of monoamine oxidases has been considered as a possible mechanism of resistance in *R. microplus*, although little or no information is available in the public domain. There are several pathways of enzymatic degradation of octopamine in insects, including beta-alanine, glutamate and sulfate conjugation (39). Whereas MAO is believed to be of little importance in insects (50), its activity has been demonstrated in *R. microplus* by Holden & Hadfield (51), who showed that although MAO was inhibited by chlordimeform, this was not the main mechanism of toxicity. Further, identification of the correct gene encoding MAO has proven to be a challenge. The early work by Jiang (36), targeting a putative monoamine oxidase, appears to have amplified a putative lysine-specific histone demethylase, based on sequence comparison with the publicly available *Ixodes scapularis* genome. More recently, we undertook a small, exploratory sequencing and gene expression study, comparing 6 ticks of the amitraz-resistant Ultimo strain with 6 ticks of the amitraz-susceptible Mount Alford strain from Australia. We targeted a putative monoamine oxidase using primers derived from *R. microplus* ESTs (Table 3, by Dr Paula Moolhuijzen of the Centre for Comparative Genomics, Murdoch University) and found that there was no variation in sequence among the samples. Gene expression using high resolution melt qRT PCR showed no significant difference in the level of expression of the two strains, however as seen in Figure 5, from our data, the observed trend to higher expression in resistant ticks suggests that there might be some value in investigating this further.

6.2. ATP binding cassette transporter activity

ATP-binding cassette (ABC) transporters are an important family of integral membrane glycoproteins that are implicated in the elimination of xenobiotic lipophilic substances from cells after prior chemical modification and conjugation to an anionic moiety (52). ABC transporters mostly function to pump xenobiotic compounds out of the cytoplasm and into the extracellular space (or more generally across the membranes of other organelles), and they have been associated with

anthelmintic resistance in parasitic nematodes (53). A recent study by Lara *et al.* (35) reported on the expression and activity of *RmABCB10*, a PgP-1-type ABC transporter, and the metabolism of amitraz in amitraz-resistant and amitraz-susceptible strains of *R. microplus*. They found that the ABCB10 glycoprotein is responsible for the transport of heme from the digestive vesicle to the hemosome and that amitraz was similarly dependent on ABCB10 for its incorporation into the hemosome. The expression of *RmABCB10* and the cyclosporine A-sensitive uptake of a labelled protoporphyrin marker were both elevated in ticks that were resistant to amitraz (Ibirapua strain) compared with those that were susceptible (Porto Alegre or POA strain). These results are consistent with studies using cell cultures from *R. microplus (34). RmABCB10* expression was significantly higher in a cell line (BME/CTVM6) that was derived from *R. microplus* ticks that were resistant to amitraz, organophosphates and organocholorine compounds, relative to five other cell lines. However, in another cell line (BME/CTVM5) derived from the same original Colombian isolate (Paso Ancho), the expression of *RmABCB10* did not differ from that of the other cell lines. Taken together, these two studies (34, 35) suggest that the effects of amitraz on ticks are likely to be at least partially mediated by ABCB10 activity.

7. SUMMARY AND PERSPECTIVES

Although at present there is no unambiguous proof of the role of any one specific mechanism in conferring resistance to amitraz in *R. microplus*, there are consistent and strong associations between mutations of GPCRs and resistance to amitraz in *R. microplus*. Amitraz-resistant isolates from Australia and Latin America share mutations in the N-terminal extracellular domain and in the first few amino acid residues of the first transmembrane domain of *RmBAOR*. Similarly, amitraz-resistant isolates from the USA, Latin America and South Africa share mutations in the octopamine/tyramine receptor. It is possible that mutation of either of these GPCR could independently interfere with the action of amitraz on ticks or that the two genes are in tight linkage disequilibrium, such that one is a predictor for the other. Unfortunately, no studies have been reported yet in which both of these GPCR have been genotyped in the same individual, leaving these possibilities unresolved. Clear definition of the roles of the genes would be dependent on *in vitro* studies using a standardised reporter system, in which the pharmacological properties of putative susceptible and resistant receptors can be compared. Given that several mutations have been detected to date and more are possible, the development of a single, comprehensive diagnostic test based on DNA sequence variation alone is likely to be difficult. It is possible that resistance to amitraz is mediated by ABC transporter proteins and by monoamine oxidases, among other enzymes; however these mechanisms, if present, would result in lower resistance ratios than those expected from target site insensitivity.

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Key Words: amitraz; amitraz resistance; *Rhipicephalus microplus;* octopamine receptor; monoaminooxidases.

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Table 1. Classification scheme of octopamine receptors according to intracellular signalling, ligand affinity and DNA sequence

OCTα-R (α-adrenergic-like)	**OCTβ-R (β-adrenergic-like)**	**TYR1-R**	**TYR2-R**
↑ Ca^{2+} {↑} cAMP OCT > TYR	↑ cAMP OCT > TYR	↓ cAMP (TYR > OCT) ↑ Ca^{2+} (OCT > TYR)	↑ Ca^{2+} TYR
	These split into OCTβ1-R OCTβ2-R OCTβ3-R		

Table 2. SNPs identified in *RmβAOR* sequences

Position	**SNP**	**Amino Acid**
123	T → C	Synonymous
126	C → T	Synonymous
181	A → T	I → F
185	T → C	I → T
225	A → G	Synonymous
263	A → C	Y → S
264	C → A	Y → S

Table 3. Sequencing and RT-PCR primers for putative monoamine oxidase

Name	**Primer sequence**	**Location and Product**
MAOB-F1	GACGGACAGTACCCGGCCA	Pos 19 (619 bp product with MAOB-R1)
MAOB-F2	TATCGTCGATAACGTCCCTAGA	Pos 565 (707 bp product with MAOB-R2)
MAOB-R1	CTACCAAGGCTACCATGCATG	Pos 638 (619 bp product with MAOB-F1)
MAOB-R2	TAATAATTCGGTGAGGCACACAA	Pos 1272 (707 bp product with MAOB-F2)
MOAB RT-F	CATTGGGCGGCTTCACTTC	67 bp product with MAOB RT-R
MOAB RT-R	GCTTGGATGGCACCGTTGA	

Figure 1. Two-dimensional (L) and three-dimensional (R) structure of the amitraz molecule (Source: PubChem URL: https://pubchem.ncbi.nlm.nih.gov). Data deposited in or computed by PubChem.

Figure 2. Location of putative amitraz resistance-conferring amino acid substitutions T8P and L22S in the octopamine/tyramine receptor, as described previously (18, 19).

Figure 3. Location of putative amitraz resistance-conferring amino acid substitutions in the RmβAOR.

Figure 4. Location of the 12 amino acid insertion and SNP in the BME/CTVM6 cell line of the putative beta-adrenergic-like octopamine receptor of *R. microplus*

Figure 5. Relative gene expression of monoamine oxidase in 6 adult female *R. microplus* ticks from the Mt Alford strain (MTA), known to be susceptible to amitraz and in 6 adult female ticks from the Ultimo strain, a population that is selected with amitraz and known to include individuals that are resistant to amitraz, but also to show some heterogeneity of response in bioassays.

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