Biochemical and molecular evidence of azadirachtin binding to insect actins

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Azadirachtin, a neem seed terpenoid is effective against serious lepidopteran crop pests like *Plutella xylostella* and *Spodoptera litura*. The mode of action of azadirachtin and its cellular target in insects has been largely unexplored, although physiological and morphological defects have been documented. Understanding the mechanism of action of this complex natural molecule at the cellular level is crucial to define safety and entomo-specific affinity. Actin has been recently indicated as a putative target of azadirachtin *in vivo* and *in silico* using *Drosophila melanogaster*. In this communication, the target of azadirachtin in *Plutella* and *Drosophila* has been validated by *in vitro* polymerization assays of actin and its mRNA transcript profiles of Act5C, a ubiquitous cytoplasmic and Act57B, a muscle-specific isoform. Further, phalloidin labelling of *Spodoptera* haemocytes identifies the exact passage of azadirachtin on the highly proliferating developmental tissues.

Keywords: Azadirachtin, cellular target, *Drosophila*, *Plutella*, *Spodoptera*.

AZADIRACHTIN, a tetranortriterpenoid neem seed kernel component and the bitter principle of neem, is active over nearly 550 species of insects. Pin-pointing the actual target is crucial for such a complex biological molecule with unique broad-spectrum activity. This uniqueness renders azadirachtin to be a feeding deterrent[1,2], systemic poison[3] and a moult inhibitor[4,5]. It is well known to cause delay or disruption in the post-embryonic development[6,7], ovipositional defects[8,9], anti-fertility effects[4,10–15], and chitin and enzyme inhibition[5,7,16–18]. Amongst the different insect species, it is suggested that lepidopterans are more susceptible to azadirachtin than homopterans[19,20]. The diamondback moth, *Plutella xylostella* selectively attacks cruciferous crops and control of this pest is seemingly difficult.

With no available genetic tools to understand the complex mechanism of action of azadirachtin in pest species, the discovery of *Drosophila melanogaster* (Dipteran) as a good genetic-model organism with conserved signal transduction pathways was significant. Although the effects of azadirachtin on the pest species have been reported[21,22], the exact cellular target has not been ascertained. It is also known to be affected by the cytotoxic action of azadirachtin causing developmental delay[23]. However, there is compelling evidence to show that azadirachtin affects rapidly dividing cells and causes cell-cycle arrest[24] by preventing the orientation of the microtubule during spindle assembly in the axonemes[25]. The earliest efforts to identify the mode of action of azadirachtin using Sf9 cells was that of Rembold and Annadurai[26], indicating that the concentration required for the cytotoxic effect of Azadirachtin in insect cell lines is negligible compared to mammalian cells and that it binds to the nuclear fraction components[27–29]. This was followed by the identification of tubulin as a primary target of azadirachtin *in vitro*[30]. Reports suggest that azadirachtin alters or prevents the formation of new assemblages of organelles or cytoskeleton[31].

So far there is no direct evidence to show the missing link for the protein biosynthesis being affected in *Tetrahymena thermophilia*[31] and the disappearance of protein spots in locusts treated with azadirachtin[32], but a putative azadirachtin-binding complex hsp60 was identified in *Drosophila* Kc167 cells[33]. The possibility of azadirachtin effect at transcriptional and translational level during particular stages of cell cycle was however not ruled out[34]. More recently, azadirachtin was reported to target actin in the differentiating cells of *D. melanogaster*, especially the eye and wing imaginal discs of third instar larva leading to non-caspase mediated apoptosis of the cell. It has also been confirmed using *in silico* docking analysis that azadirachtin binds with high affinity to insect actins compared to mammalian actins. However, the lead target identified in a genetic model system like *Drosophila* needs validation in an azadirachtin-sensitive pest system. Hence, in this communication, we report the *in vitro* binding efficiency of azadirachtin to actin proteins in *Drosophila* and *Plutella*, which would further strengthen the azadirachtin-actin binding theory and indicate the possible route of mechanism of action using *Spodoptera litura* haemocytes.

For our experimental work we used Canton-S or wild-type stock of *D. melanogaster*. Larvae of *S. litura* and *P. xylostella* were obtained from the Project Directorate of Biological Control, Bangalore. Canton-S flies were fed on different concentrations of azadirachtin mixed with standard 3% fly-food composition. *Spodoptera* and *Plutella* second instar larvae were reared on castor leaves and tender cabbage leaves respectively, coated with different concentrations of azadirachtin.

Actin was isolated from the total protein by mini-prep method[35]. Approximately 60 µg of protein sample of *Drosophila*, *Plutella* and *Spodoptera* was taken in a fresh eppendorf tube and incubated with 30 pM of phalloidin for ~45 min at RT, along with polymerization buffer. Different concentrations of azadirachtin (1, 2, 3, 5×10^{-7} µM) were added and left overnight for polymerization at 4°C. The polymerized samples were added with 12 µl of 4 M KCl and left for 30 min at 37°C. The samples were later spun at 100 K for 10 min at 4°C. The super-

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natant with unpolymerized G-actin was checked on 10% SDS–PAGE gel and stained with Commassie-Blue.

RNA (~1 µg) of azadirachtin treated and untreated samples of *Drosophila*, *Spodoptera* and *Plutella* was incubated with 10^{-3} M concentration of actin 5C (Forward – 5′CAGATCATGTTCGAGACCTTCAA3′; Reverse – 5′ATCTTCATCAGGTAGTCGGTCAA3′) or Act57B (Forward – 5′ACATCTGCTGGAAGGTGGAC3′; Reverse – 5′ATCCGCAAGGATCTGTATGC3′). A 10X, 25 mM MmLV RT reaction mixture with dNTPs and enzyme was added to the samples and incubated at 42°C for 1 h. PCR was carried out using *Taq* polymerase enzyme and the standard reaction conditions were maintained. The samples were subjected to the following PCR conditions: (i) Initial denaturation at 95°C for 8 min; (ii) 94°C, 40 cycles for 45 s; (ii) 60°C, 40 cycles for 30 s; (iii) 72°C, 40 cycles for 30 s and (iv) final extension of 72°C for 10 min for *Drosophila* and *Plutella* samples. The samples were checked on the 1.5% agarose gel. A 1 kb *Hin*f-I digested product of pBS was loaded as a marker. The gel was imaged on UVIPro Platinum gel documentation system with UVIpro Platinum version 2.0 software[36] and analysed on UVISoft UVI Band Map Windows application.

The haemocytes were collected from azadirachtin-treated and control samples from the abdominal pro-leg of *Spodoptera*, *Plutella* and posterior side of *Drosophila* third instar larva with a capillary tube[37]. The haemolymph was collected in 100 µl of 1X PBS. The haemocytes were incubated at 25°C to spread onto a clean cover-slip for 1 h. Cells were washed twice with 1X PBS and fixed with 3.7% formaldehyde for 10 min. The cells were permeabilized with 100 µl of 1X PBS + 0.2% Triton X-100 for 10 min and washed several times with 1X PBS + 0.2% Triton X-100 before labelling with phalloidin-TRITCC (1 : 1000) for 40 min. The cells were rinsed twice with 1X PBS + 0.2% Triton X-100 and mounted in 1 : 1 glycerol/PBS after final wash with 1X PBS. The phalloidin labelled haemocytes were imaged on Olympus 1X70 using ProgRes CaputrePro 2.1 software[38].

The dose-dependent effect of azadirachtin on *S. litura*, *P. xylostella* and Canton-S of *D. melanogaster* was distinctly different. *Spodoptera* fourth instar larvae normally could not develop into their next metamorphic stage and were often seen with constricted cuticle. A series of publications related to azadirachtin describe the phenotypic effects of neem on lepidopterans. Cuticle constriction is regarded as molt inhibition, often seen among Lepidoptera as a result of azadirachtin treatment[39,40]. The larvae were thus unable to survive through the successive stages of metamorphosis. Though *Plutella* is susceptible to azadirachtin, the phenotypic effects observed preceding the mortality of the larva seem to be distinctly different from other azadirachtin-sensitive lepidopterans. The larvae of *Plutella* which were unable to enter into their next metamorphic stage underwent melanization and ultimately died, as previously reported[41]. Similarly, *Drosophila* did not show any indication of moult inhibition on the third day of Azadirachtin treatment, but at higher concentrations beyond 5 ppm, the progeny were larval lethals (data not shown). The average weight gain of larvae of *Plutella* treated with azadirachtin retarded gradually (Figure 1 *a*) and a marked reduction in their body weight was observed on the third day, with increase in concentration. The Canton-S or the wild-type strain of *Drosophila* was observed with a delay in post-embryonic development (3–4 days). The late third instar larvae of *Drosophila*, which constitute the non-feeding stage (wandering stage) and crawl onto the surface of the glass vial, were observed with abnormal eye imaginal discs of reduced size[23] and emerged as adult flies. These flies were noticed with defective ommatidial arrangement at lower concentration which was severe at 3 ppm concentration of azadirachtin (Figure 2 *b*). Rarely, some of the azadirachtin-treated flies had defective thoracic closure due to loss of wing (Figure 2 *c*), similar to mutants having abnormal dorsal closure as a result of actin cytoskeletal collapse generally required for epithelial cell movement[42]. The pupal lethals obtained at 5 ppm were usually early larval lethals with moderate-to-severe histolysis (Figure 2 *e* and *f*). The effect was well pronounced when the young larvae were challenged with azadirachtin (first instar) in *Spodoptera* and *Plutella*. Later instars showed a less pronounced effect. This could be probably due to the presence of undifferentiated cells in primordial structures like wing, leg and eye discs during early instars, which are highly sensitive. With the growth of the larvae the cells become differentiated and the molecule may not bind to the target/s. A similar scenario may exist in the immune system, where the earlier instars were more vulnerable than the older instars. This answers the question as to why older instars are resistant under field conditions to azadirachtin? Hence, for our experiments late third instar larvae, collected at the end of the third day after continuous exposure to azadirachtin, were used for immunohistochemistry of haemocytes and for detecting the transcript profiles of cytoplasmic and muscle-specific actin of *Drosophila* and *Plutella*.

We have already reported that actin is a putative target of azadirachtin in *Drosophila* and the cellular consequences following cytoskeletal collapse[23]. Since no distinct phenotypic anomalies were noticed in *Spodoptera* at lower concentrations, except for inter-molt defects at the larval stage, unlike *Drosophila* which die as larval lethals only at higher concentrations of azadirachtin, the effect was seen tending to be primarily routed through the haemolymph. The haemolymph of insects comprises of different cell types known as haemocytes (Figure 3 *a*). These haemocytes labelled with phalloidin-TRITCC (stains F-actin) clearly reveal the actin localization in different cell types (Figure 3 *a*). Each cell type has a distinct cell morphology and actin localized in the lamellar extensions of the cell. Plasmatocytes occur in many morphological

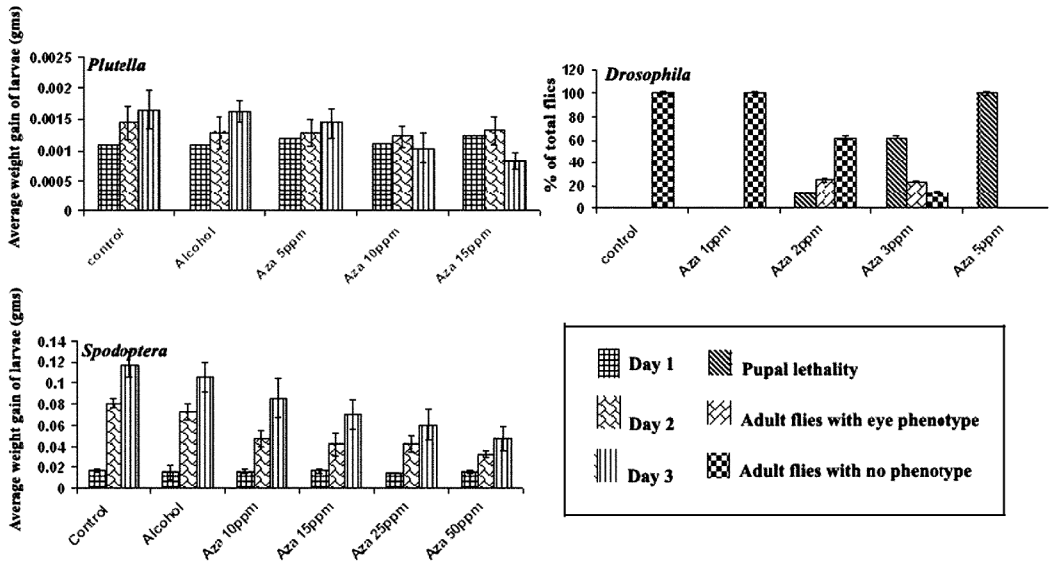

Figure 1. Average weight gain of five replicates of *Plutella*, *Spodoptera* and % total of flies emerged in *Drosophila* (n = 56) post-azadirachtin treatment with $P < 0.001$. Standard error bars are indicated against each histogram.

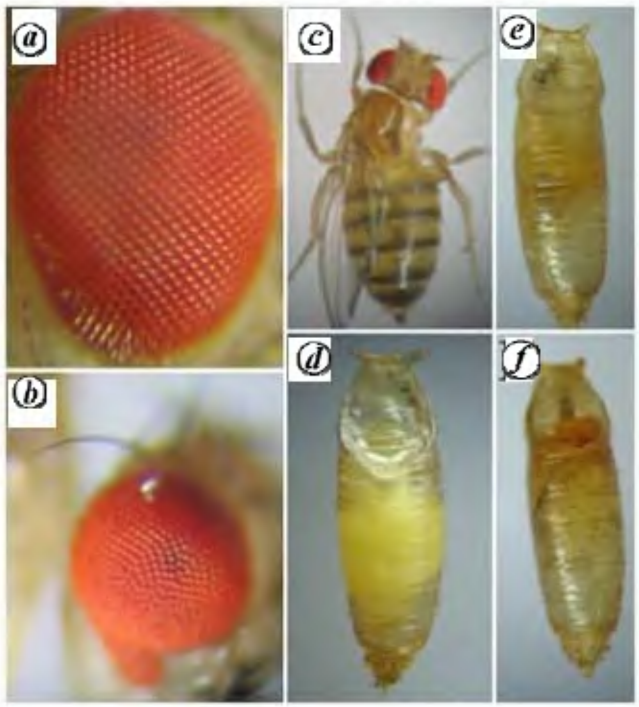

Figure 2. Effect of azadirachtin on different stages of *Drosophila melanogaster* (wild-type) development. ***a***, Eye of untreated control fly; ***b***, Small eye in 3 ppm azadirachtin-treated fly; ***c***, Defective dorsal closure and wing missing in 3 ppm azadirachtin-treated fly; ***d***, Normal untreated pupa, and ***e***, ***f***, Early pupal lethals with severe histolysis.

shapes and sizes (Figure 3 *a*). The actin levels are down-regulated in the haemocytes of the larvae treated with azadirachtin (Figure 3 *b*), a clear indication that the cells might have lost their adherent properties. Also, the cells tend to lose their morphological features, which are distinct for each cell type (compare Figure 3 *a* and *b*). Although we have shown the maximum resolution achieved with *Spodoptera* haemocytes, loss of phalloidin staining was observed even in the haemocytes of *Drosophila* and *Plutella*. Our results suggest that all the three species undergo a similar mechanism of action via the haemolymph to target the dividing cells, leading to morphological deformities.

Reports available contribute to the existing knowledge of tubulin depolymerization and mRNA expression. The levels of tubulin monomers in the cytoplasmic pool decide the expression of the transcript within the cell, thus compensating for the loss of tubulin to attain steady-state equilibrium. Since we report actin to be the primary target of azadirachtin, the mRNA expression of actin is expected to maintain steady-state equilibrium within the cell. The transcripts levels of Act5C, a cytoplasmic ubiquitous isoform of actin were unaltered in *Plutella* (Figure 4 *a*) and *Drosophila* (Figure 4 *b*). However, though the expression of Act57B transcript was moderately down-regulated in *D. melanogaster* at 2 and 3 ppm of azadirachtin (Figure 4 *b*), the effect seemed many-fold in *P. xylostella* at 15 ppm (Figure 4 *a*). This trend observed with the mRNA expression of muscle-specific actin is striking; a reason to believe that decline in the muscle-specific actin profile may be due to azadirachtin-induced cell death. Since muscle-specific transcript is present only in a small population of cells, any effect observed may be amplified. So the effect of azadirachtin may be entirely at the protein level and does not reflect itself at the transcript level. It is quite intriguing to know that the insect larva undergoes metamorphosis by shredding its old cuticle by muscle contractions under the influence of the ecdysis hormones.

A decline in the muscle-specific actin transcript due to cell death may be corroborated with moult defects, which may ultimately lead to larval lethality.

In vitro polymerization of actin isolated from *Plutella* and *Drosophila* incubated with azadirachtin revealed that G-actin accumulates in the supernatant in a dose-dependent manner (Figure 5). The polymerized G-actins (or F-actins) in *Plutella* and *Drosophila* controls settle at the bottom as no G-actin band was detected on the SDS gel. Polymerization has to be initiated at room temperature prior to azadirachtin incorporation to observe the dose-dependent effect.

Increasing reports on the importance of actin reinstate its status in diverse cellular functions in animals[42–45] and plants. It is primarily involved in transcription[46,47], especially F-actin has been indicated to be part of the nuclear matrix[48–50], the substrate on which replication and transcription of DNA occurs[51–53]. Amongst insects, the lepidopterans have been regarded as an ideal system to understand the actin dynamics in response to a baculovirus and polydnavirus infection. The AcMNPV baculoviruses infect the lepidopteran pests by gaining entry into the cell through stabilization and re-arrangement of actin cables to facilitate replication within the cell[54]. Similarly, re-arrangement of actin in the haemocytes is observed in *Heliothis virescens*, when *Campoletis sonorensis* infects the host with ichneumonid virus, suppressing the host immune-system through secreted viral proteins[42]. The effect of methanolic neem solution on a 43 kDa protein expression in the haemolymph of females of *Epilachna varivestis* has already been demonstrated[55]. Besides insects, even plants like *Arabidopsis*, reorganize actin cytoskeleton in response to the nematode-induced isotropic growth of hypertrophied feeding cells. These actin cables serve as tracks for vesicle trafficking needed for extensive plasma membrane and cell-wall biogenesis[45]. The evidence thus gathered in the present scenario in the case of *S. litura*, *P. xylostella* and *D. melanogaster* converges towards the speculation that azadirachtin or any naturally originating pesticidal molecule may exert its activity by targeting actins. However, this needs to be investigated with more pesticidal molecules of botanical origin. A number of isoforms of actin have been identified in *Drosophila*[56,57] that could be good candidate targets for insecticide binding causing global impact on the overall development of the pest in a stage-specific (larva, pupa, adult) manner, rendering the pest vulnerable at any stage of development. Considering the relative similarity of binding in *Drosophila* and lepidopterans, the strategy could be exploited towards development of new biorational molecules with highly precise targets.

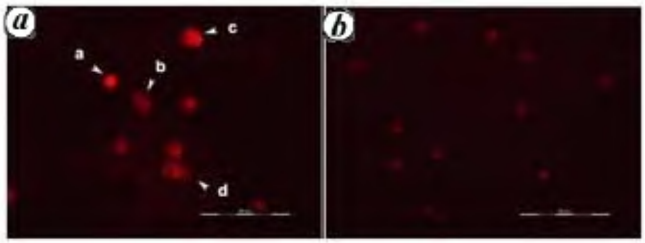

Figure 3. Haemocytes of azadirachtin-treated (10 ppm) third instar larva of *Spodoptera litura* labelled with phalloidin-TRITCC. ***a***, Haemocytes of untreated larva (a – prohaemocytes; b – granular haemocytes; c – Spherule cell and d – plasmatocyte); ***b***, Haemocytes of azadirachtin-treated larva with structural deformities due to loss of phalloidin. Scale bar is 50 μm.

Figure 4. mRNA expression profile of Act5C (ubiquitous and cytoplasmic) and Act57B (muscle-specific) isoforms of actin of third instar larva of (***a***) *Drosophila* and (***b***) *Plutella*, post-azadirachtin treatment.

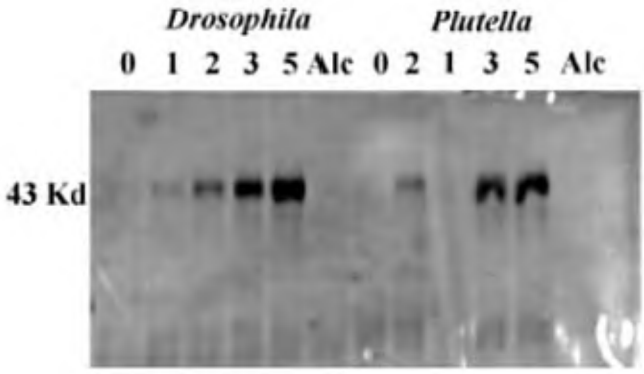

Figure 5. SDS–PAGE of G-actin obtained from *in vitro* polymerization assay of *Drosophila* and *Plutella* actin incubated with different concentrations of azadirachtin. G-actin accumulates in the supernatant with increase in the concentration of azadirachtin.

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Orthodontic arch wires for seismic risk reduction

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Earthquakes are naturally occurring events demonstrating the power of nature and the catastrophic impact of such power on normal life. Development of new techniques and opting for new materials which are not traditionally used in civil-engineering structures, offer significant promise in reducing seismic risk. Super-elastic Nitinol in the form of wires (orthodontic wires) is a common and well-known engineering material available with dentists all over the world. It belongs to the class of shape memory alloys (SMAs) bearing unique properties such as super elasticity and shape memory effect. The greater flexibility of the material drives many of its applications in the medical industry, but the use of this material in other fields is less known. This communication seeks the suitability of this material for structural applications, especially earthquake risk reduction. A study has been conducted to find the suitability of orthodontic wires in passive vibration control of structures. The super-elastic properties found in these wires are made use of in the development of vibration control devices of re-centering type. Protection of structures from damage during earthquakes can be addressed using passive protection devices designed to provide energy dissipation with re-centering capabilities. Possible application lies in reducing the seismic risk of multi-span bridges, rehabilitation of heritage structures and protection of special structures of national importance.

Keywords: Earthquake risk reduction, orthodontic arch wires, passive vibration control, re-centering devices.

ORTHODONTIC wires, known as Nitinol, are made of NiTi alloy. Nitinol alloys are most commonly known for their super elasticity and thermal shape memory[1]. While the term shape memory is used to describe the phenomenon of restoring a pre-determined shape through heating, after having plastically deformed that shape, the term super elasticity refers to the enormous elasticity of the alloys. An important feature of the super-elastic Nitinol alloys is that their loading and unloading curves are substantially flat over large strains. This allows the design of devices that apply a constant stress over a wide range of shapes[2]. The orthodontic arch wire was the first wire to use this property, more specifically the constant unloading stresses. Nitinol wires unlike stainless steel wires are able to move with the teeth, applying a constant force over a broad treatment time and tooth position.

Due to distinctive macroscopic behaviour like super elasticity, shape memory alloys (SMAs) are the basis for innovative applications ranging from devices for the correction of tooth mal positions (orthodontic arch wires) to those for protecting structures from structural vibrations. The super elasticity-based applications take advantage of the following features: (1) the possibility of recovering large deformations and (2) the existence of a transformation stress plateau, which guarantees constant stress over non-negligible strain intervals. Super-elastic properties of orthodontic wires have been established from experiments conducted and the salient features are highlighted here. The parameters influencing the seismic response have been tested and the material is found suitable for response-control applications in devices. Among the various types of passive vibration-control devices, re-centering

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