CHAPTER III

RESULTS
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3.1. Mode of action and cellular targets of Azadirachtin A

3.1.1.1. Introduction

The response elicited by Azadirachtin treatment varies between different species of insects. Lepidopterans were found to be highly susceptible to the anti-feedant properties of the compound than the hemipterans\(^{211, 212}\) and inversely related to the larval stage\(^{213, 214}\). Hence, extensive research was done on the action of Azadirachtin on *Schistocerca gregaria* and *Locusta migratoria*, *Spodoptera littura* and *Helicoverpa armigera*. Earlier reports suggest the anti-feedant properties of Azadirachtin\(^{215, 216}\), interfere with the development\(^{213}\) and delays ecdysis in *Drosophila melanogaster*, *Aedes aegypti* and *Manduca sexta*\(^{217}\). The IGR related properties has been attributed to the disturbance in the molting hormone. A fine interplay of Juvenile Hormone from *Corpora allata* and 20-OH ecdysone from prothoracic glands is essential for a proper molt which is controlled by the prothoracicotropic hormone (PTTH). PTTH released from the Pars intercerebralis neurosecretory complex present in the *Corpus Cardiacum* stimulates the synthesis and release of ecdysone from the Prothoracic glands. The allatotropins from the *Corpus Cardiacum* stimulate the release of the Juvenile Hormone which is inturn inhibited by the allatostatins secreted by the lateral neurosecretory cells of the brain. Not only was Azadirachtin found to inhibit the release of steroidal hormone ecdysone from brain/ring gland complex but also a reduction in the hydroxylation of ecdysone to 20-hydroxyecdysone in the larval fat body of blowfly *Calliphora vicina*\(^{218}\). Similar results were obtained from *L. migratoria* in which altered titers of ecdysteroids was observed\(^{219}\). Juvenile hormone level which is required for vitellogenin biosynthesis in the fat body is abolished by Azadirachtin which indirectly inhibits oogenesis\(^{220}\). Therefore, Azadirachtin interference with PTTH and allotropic hormone synthesis was believed to be the primary effect leading to the inhibitory effects on the two morphogenous hormones. Disintegration and defective cuticle secretion was observed in histopathological sections of epidermis of third instar larva of Mexican bean beetle *Epilachna varivestis* larva following Azadirachtin treatment\(^{33}\). It is believed that the delayed or reduced titers of Juvenile Hormone cause changes in the larval cuticular structures. The molt inhibition caused due to the disruption of neurosecretory release was primarily due to the alterations in the profiles of proteins/ peptides\(^{121}\).
However, it is not known if Azadirachtin interferes with the biosynthesis of the neurosecretory hormones/proteins although its interference with protein biosynthesis has been reported in *Tetrahymena thermophila*. Histological studies with the $^3$H-dihydroazadirachtin or immunohistochemistry with peptide hormones suggest that it disrupts transport and release of Peptide hormones like PTTH, allotoinhibins and allotostatins. The report on insect cells being more susceptible to Azadirachtin than mammalian cells suggests that Azadirachtin might be targeting a protein that is common but has a different conformation or differentially expressed between the two systems. It has been shown that Azadirachtin binds to the axonemes and blocks the sinusoidal movement of flagellar axonemes. As binding of Azadirachtin to axonemes causes disruption of microtubular cytoskeleton, ß-tubulin is thought to be one of the targets of Azadirachtin. The cellular cytotoxic effect of Azadirachtin is due to the block in the active cell division followed by massive tissue degeneration.

3.1.1.2. Results

Equal number of males and virgin females were released into the Aza-treated medium of various concentrations. The females laid their eggs in the treated media and the resultant progeny were continuously exposed to Azadirachtin till the third instar stage. The progeny were screened for pupal lethality, adult emergence and adult eye phenotypes. Although there was a developmental delay at different stages from egg laying to pupal development, larvae of the same developmental stage based on the (ii) wandering stage and (ii) the number of mouth hooks were used for immunohistochemistry.

A) Azadirachtin-induced phenotypes in *Drosophila*

Aza induced moderate-to-severe phenotypes in different tissues in a dose-dependent manner (see Fig. 5 for graphical representations). Typically, Aza-treated wild type larvae showed delay in development (3–4 days) and larval or early (at 5 ppm concentration) to late (at lower concentrations) pupal lethality. Flies that survived up to the adult stage often showed eye phenotypes. At 3 ppm concentration, approximately 60% of the eclosed flies (n=62) showed mild-to-severe eye phenotypes (Figs. 1B–I, 2B, C).
Figure 1. Aza-induced eye phenotypes in Canton-S flies. (A) Untreated control. (B)–(I) Range of eye phenotypes in flies grown in the presence of 3 ppm Aza. Commonly observed phenotypes are mild roughness (B), (C), reduced number of ommatidia (D)–(F), outgrowth of non-eye tissue (G), split eyes (H) and total loss of eyes (I).

Figure 2. Scanning electron microscope images of Aza-induced eye phenotypes in more detail. (A) Untreated control. (B)-(C) Eyes of flies grown in the presence of 3 ppm Aza. (D)–(F) Higher magnification images of the eyes of untreated (D) and 3 ppm Aza treated flies. Normally one bristle arises in each inter-ommatidial space, while in Aza-treated flies multiple inter-ommatidial bristles are seen (arrows in (E) and (F)). Often, inter-ommatidial bristles are bent (arrow-head in (F)).

Scanning Electron microscopy of compound eyes revealed irregular organization of ommatidia, duplicated, bent and forked interommatidial bristles (Fig. 2E, F). At 5 ppm, most of the
animals died at early pupal stages with few escaping adult flies having eye phenotypes. The progeny could never reach even pupal stages at concentrations higher than 5 ppm. A small subset of population had thoracic legs missing, rudimentary legs, and duplication of A3 lobe of antenna, short arista, maxillary palp and haltere missing (data not shown). All the larval and adult phenotypes described here are from larvae fed with Aza at 3 ppm concentration, unless stated otherwise.

B) Azadirachtin-induced apoptotic cell death

![Figure 3](image)

Figure 3. Aza-induced eye phenotypes are manifested in the larval stages. (A)–(C) Eye imaginal discs stained for mAb22C10 (a marker of differentiating photoreceptor cells) of untreated control (A), 3 ppm Aza-treated larvae (B) and (C). Note reduced number of photoreceptor cells in eye discs of Aza-treated larvae. (D)–(E) Higher magnification images of photoreceptor cells of the eye discs shown in (A) and (B), respectively.

As loss of ommatidia was the most prominent phenotype observed, we used Aza-induced eye defects as an assay to investigate the mechanism by which Aza exerts its effect. Staining eye imaginal discs with the photoreceptor specific marker mAb22C10 suggested severe loss of photoreceptors at the disc level itself (Fig. 3B, C). The size of the eye disc was relatively small when compared to the controls (Fig.3C). However, the photoreceptor neuron formation and their arrangement per se were unaffected (Fig.3E). Furthermore, neuronal projections including the Bolwig’s nerve were intact. Most of the imaginal discs in Aza-treated larvae are much smaller in size (Fig. 4B, C; data shown only for wing discs). Possible apoptotic death by staining discs with acridine orange was examined.
Large number of cells were noticed undergoing apoptosis in the larval central nervous system (CNS; Fig. 4E) and in all imaginal discs (Fig. 4C, G, I; data shown for wing and eye discs). The apoptosis was evident even at a lower concentration of 2 ppm. The degree of apoptosis increased with the increased dosage of Aza (data not shown). These observations suggest that loss of ommatidia in Aza-treated flies could be due to apoptosis. Interestingly, mostly eye phenotypes in the adult flies were evident. Although a large population of cells undergo apoptosis in the wing imaginal discs too (Fig. 4G), a majority of the emerged flies had normal wings. This could be attributed to the compensatory proliferation mechanism operating in wing discs, wherein cells undergoing apoptosis release growth signals to the neighbouring cells to divide and fill the gap. However, Phosphohistone H3 labeling suggested reduced number of mitotically active cells in Aza-treated wing imaginal discs (Fig. 5B). It is possible that compensatory proliferation may occur at pupal stages during metamorphosis.
**Figure 5.** Aza-induced apoptosis is not suppressed by the caspase-inhibitor Baculovirus p35. (A) Wing imaginal disc of untreated control larva stained with antibodies against Phosphohistone H3, which marks cells undergoing mitotic divisions. (B) Wing imaginal disc of 2 ppm Aza-treated larva stained with antibodies against Phosphohistone H3. Note that cells undergoing mitosis are fewer in Aza-treated discs. (C) Wing imaginal disc of 2 ppm Aza-treated CS larva stained with Acridine Orange. (D) Wing imaginal disc of 2 ppm Aza-treated ptc-GAL4/UAS-p35 larva stained with Acridine Orange.

C) Azadirachtin induced apoptosis is caspase independent

Over-expression of *Drosophila* Reaper (Rpr) and Headinvolution defective (Hid) induce caspase-dependent apoptotic cell death in wing and eye imaginal discs whereas p35, an anti-apoptotic protein from the Baculovirus *Autographica californica* acts downstream of Rpr and Hid to inhibit apoptosis. Apoptosis induced by Aza, however, was not rescued by the over-expression of p35 with ey-GAL4 or ptc-GAL4 driver (Fig. 5D; data shown only for ptc-GAL4). Ubiquitous over-expression of p35 from very early stages of development using arm-GAL4 too failed to rescue apoptotic cell death in eye and wing imaginal discs (Fig 5D). Thus, it seems plausible that Aza-induced cell death is not mediated through caspases. This is consistent with the observation that there was no compensatory proliferation in Aza-treated wing imaginal discs at a larval stage (Fig. 5B), which is normally induced by caspase-mediated apoptosis.
Figure 6. Over-expression of CycE and String suppresses the effect of Aza. The effect of different concentrations of Aza was tested in wild-type (CS flies) and in the background of gain-of-function of Cell-cycle components, Cyclin E and String. Over-expression of CycE and String considerably suppressed the phenotypes induced by Aza at 3 and 5 ppm concentrations. As larval lethality was not estimated, the total number of progeny included only number of pharate adults (or dead pupae) and number of eclosed flies. For better comparison, distribution of flies with and without phenotype and degree of pupal lethality for each experiment is graphically represented as percent of total number of progeny of that experiment. The actual numbers (average of four experiments) are shown in Supplement Table 1 (* indicates values that are $P \leq 0.01$). Average values of CycE++ and String++ experiments are compared to those of CS experiments by using Student $t$-test.

D) Over-expression of Cyclin E or string rescues the Azadirachtin-induced cell death

Often apoptotic cell death is caused by defective cell-cycle such as irreversible cell-cycle arrest. Thus, possible cell-cycle arrest in G1/S and G2/M transition phases was examined. To relieve any possible arrest of cells in the G1 phase induced by Aza, CycE required for G1-S transition was over-expressed. Similarly, cdc25/ string (a regulator of G2/M transition) was over expressed to relieve any possible arrest of cells in G2/M transition phase. This led to the suppression of Aza-induced eye phenotypes coupled with improved adult emergence, when either of them was over-
expressed (Fig. 6C). However, Aza did not have any effect on CycE or string mutants (data not shown), suggesting that CycE may not be a direct target. Mutants or over-expression of other positive and negative components of the cell cycle such as dap (cdk inhibitor; inhibits G1-S transition), dE2F (required for cells to enter S phase) and RBF (inhibitor of E2F; inhibits entry into S phase) too had no effect (data not shown).

As there was no specific correlation to the hierarchy of genes regulating cell-cycle and the rescue of eye-phenotypes, it is possible that rescue observed in these experiments were due to compensation by over-proliferation and they rule out the possibility that Aza directly affects any phase of the cell-cycle.

E) Azadirachtin targets actin

Earlier reports indicate that Aza inhibits polymerization of β-tubulin in vitro. Similarly, down regulation of β-tubulin levels in Aza-treated wing discs (Fig. 7B) were observed. However, closer examination of wing epithelial cells along the Z-axis revealed increased accumulation of β-tubulin at the basal region of the epithelial cells (Fig. 7B), suggesting defective localization rather than decrease in β-tubulin levels per se. These observations were true for both normal-looking wing discs as well as severely deformed wing discs. Similar phenotypes with a tubulin::GFP fusion construct (Fig. 7D) were identified. The overall effect on cytoskeleton was evident in the shape of cell nuclei and in the way they were positioned along the Z-axis of the epithelial cells. Nuclei were more elongated and often positioned more basally than in untreated cells (Fig. 7B’). This could be due to collapse of cytoskeleton along its breadth. Aza-induced phenotypes at the level of interommatidial bristles are similar to those observed for the actin phenotypes by known inhibitors of actin polymerization such as Cytochalasin D and Latrunculin. Therefore, the effect of Aza on actin cytoskeleton was examined. F-actin is mainly localized to the apical end of the epithelial cells. The effect on the pattern of Phalloidin staining, that binds to F-actin was observed to provide insights into the actin polymerization status in vivo. This revealed a complete loss of Phalloidin staining in Aza-treated wing discs (Fig. 8B). However, localization of other apico-basal markers such as Discs large (Dlg), Fasciclin III (Fas III) or Armadillo (Arm) were unaffected (Fig. 8B’; data shown only for Dlg). Thus, despite severe defects in cytoskeletal organization, Aza-treated disc cells still retained their apico-basal polarity. The effect of Aza on actin per se was investigated. Anti-actin antibodies were used to monitor the effect of Aza. A severe depletion in
actin levels in Aza-treated wing disc cells was noticed (Fig. 8D, F) suggesting Aza has much pronounced effect on actin than β-tubulin. The effect of Aza on actin::GFP fusion construct was studied. Although, a considerable depletion of actin::GFP in Aza-treated disc cells was observed, the effect was less severe compared to the effect on endogenous actin.

Figure 7. Aza-induced decrease in β-tubulin levels. (A) Optical cross-section along the Z-axis of wing disc of untreated control larva stained for β-tubulin (red; A) and Lamin B (green; A’). A” is the merged image of A and A’. Normally, β-tubulin is present all along the apicobasal axis of the cell, although its levels are considerably higher in the apical end. Cell nuclei (marked by Lamin B staining) are mostly located more apically and they are spherical in shape. (B) Optical cross-section along the Z-axis of wing disc of 3-ppm Aza-treated larva stained for β-tubulin (red; B) and Lamin B (green; B’). B” is the merged image of B and B’. Note decreased levels of β-tubulin and its mis-localization to more basal regions. Nuclei are mis-localized in different regions along the apico-basal axis. Nuclei are also more elongated. (C-D) Optical cross-section along the Z-axis of arm-GAL4/UAS-β-tubulin::GFP wing discs of untreated control larva (C) and 3-ppm Aza-treated larva (D) stained for β-tubulin::GFP. Note decreased levels of β-tubulin::GFP. (E) Optical cross-section along the Z-axis of ptc-GAL4/UAS-Cyclin E wing disc of 3-ppm Aza-treated larva stained for β-tubulin. Note that Cyclin E over-expression has no effect on β-tubulin levels in Aza-treated larvae (compare with B).
Figure 8. Aza has pronounced effect on actin cytoskeleton. (A) Optical cross-section along the Z-axis of wing disc of untreated control larva stained for Rhodamine-conjugated Phalloidin (red; A) and Dlg (green; A’). A’’ is the merged image of A and A’. Normally, actin is present all along the apico-basal axis of the cell. Phalloidin, however, mainly binds to F-actin, which is predominantly localized to the apical end (marked by Dlg staining), of the epithelial cells. (B) Optical cross-section along the Z-axis of wing disc of 3 ppm Aza-treated larva stained for Rhodamine-conjugated Phalloidin (red; B) and Dlg (green; B’). B’’ is the merged image of B and B’. Note complete loss of Phalloidin staining. Interestingly, Dlg staining remains normal suggesting that apico-basal axis of the cells is intact. (C)–(D) Optical cross-section along the Z-axis of wing discs of untreated control larva (C) and 3 ppm Aza-treated larva (D) stained with anti-actin antibodies. Note complete loss of actin protein suggesting that Aza-targets actin itself and not its localization. (E)–(F) Optical cross-section along the Z-axis of arm-GAL4/UAS-actin::GFP wing discs of untreated control larva (E) and 3 ppm Aza-treated larva (F) stained for actin::GFP. Note considerable decrease in the levels of actin::GFP. (G)–(H) Optical cross-section along the Z-axis of ptc-GAL4/UAS-CycE wing discs of untreated control larva (g) and 3 ppm Aza-treated larva (H) stained with anti-actin antibodies. Note that CycE over-expression reverses the Aza-mediated depletion of actin levels (compare D and H).
Figure 9. Over-expression of CycE restores Aza-induced damage to the actin cytoskeleton. Figure shows Optical cross-section along the Z-axis of eye discs of CS and ey-GAL4/UAS-CycE eye discs of control and treated (with Aza at 3 ppm) and stained for either Rhodamine-conjugated Phalloidin or with anti β-tubulin antibodies as indicated on the panels. The effect of Aza on tubulin is less pronounced (compare A and C) and Cyclin E has only marginal effect (compare C and D). In contrast, the effect of Aza is more pronounced on actin (compare E and G) and the restoration of actin cytoskeleton by CycE is also significant (compare G and H).

This could be due to partial compensation by the over-expression of actin::GFP. Finally, the question arises whether over-expression of CycE, which rescues Aza-induced phenotypes, restores actin cytoskeleton. When CycE was over expressed using ptc-GAL4 and ey-GAL4 drivers and monitored the levels and distribution of actin and β-tubulin were monitored, a significant restoration of actin levels as well as actin cytoskeleton in both wing and eye imaginal discs, but not in the levels of β-tubulin (Figs. 8H, 9H) was observed. As CycE rescues Aza-induced eye phenotypes as well as lethality, it appears that the primary effect of Aza is on actin cytoskeleton, which in turn causes several developmental and cellular defects including apoptosis.

3.2. Biochemical and molecular evidence of Azadirachtin binding to insect actins
3.2.1. Introduction
The first scientific report on neem’s ability to repel insects was reported in the year 1928 when two Indian scientists, R.N.Chopra and M.A.Hussain used aqueous suspensions of ground neem kernels to repel desert locusts. In Southern India, traditionally, dried neem leaves were mixed with grains or placed in the clothes and woolen blankets to repel insects. It was later that the anti-feedant properties of the neem tree in desert locusts were described\textsuperscript{240}.

Studies conducted on different economically important pests indicate at least 550 pest species are affected due to exposure to neem products. It acts as a feeding deterrent particularly on Orthopteran insect species (grasshoppers, crickets, locusts etc). Homopteran pests (aphids, leafhoppers, psyllids, white-flies, scale-insects etc) are sensitive to neem especially to Azadirachtin to varying degrees. Azadirachtin in these pest species contribute to anti-feedant and growth regulating effects. While leafhoppers and plant hoppers are more sensitive, aphids and scale insects are a less sensitive to the effects of neem. Amongst the sap-sucking aphids, susceptibility varies as there maybe exceptions in the systemic transport of Azadirachtin through different plants. Lepidopteran pests (armyworms, fruit-borers, corn borers etc) are highly sensitive to neem. Generally, Lepidopterans are considered highly susceptible to Azadirachtin than Homopterans. Phytophagous Coccinellids of Coleopteran class (Mexican bean beetle and cucumber beetle) and Chrysomelids (Colorado potato beetle and others) are equally sensitive to neem. Both Hymenoptera and Heteroptera (rice bug, the green vegetable bug and East African coffee bug) are affected due to feeding disruption, growth and developmental retardation. Amongst the Dipters (fruit fly, face-fly, bot-fly, horn-fly and house-fly are the targets of neem. The Thysanoptera group of insects which includes the thrips also shows appreciable sensitivity to Azadirachtin\textsuperscript{241}. Thus different insect groups are sensitive to Azadirachtin to varying degrees.

Neem compounds are thought to act on the insects through one of the following ways:

a) Delay or disruption in any of the developmental stages (egg, larva or pupal stage)\textsuperscript{125, 242, 243}  
b) Feeding deterrent\textsuperscript{211, 112, 244, 245, 246, 211}  
c) Systemic poisoning\textsuperscript{224}  
d) Hormonal inhibition/imbalance and Moult defects\textsuperscript{33, 226, 247, 248, 249}  
e) Ovipositional defects\textsuperscript{242, 250}  
f) Anti-fertility effects\textsuperscript{219, 247, 251, 252, 253, 254, 255}  
g) Enzyme inhibition\textsuperscript{213, 218, 226, 242, 256, 257}.

Tobacco cut-worms and adult Japanese beetles are habituated to repeated or continuous exposures to Azadirachtin\textsuperscript{3}. The North American grasshoppers feed on the neem-treated plants
despite most insects later suffer from prolonged physiological effects\textsuperscript{258}. Insect growth regulating activities of Azadirachtin and neem extracts is reported amongst different species of aphids, \textit{Nasonovia ribis-nigri} (Currant Lettuce aphid) and \textit{Myzus persicae} (Green Peach aphid)\textsuperscript{125}. Although a few insects are relatively tolerant to neem, a large number of insects are still vulnerable to the toxic actions of Azadiarchtin and other neem limonoids. Metamorphic effects of Azadirachtin on \textit{Plutella xylostella} larva\textsuperscript{259} were classified into (i) fourth instar larva died before or during pupation, (ii) malformed pupa, (iii) larva successfully pupates but dies after emergence (iv) malformed adult/s do not eclose completely from the pupal case. Reduced activity of alanine amino transferase and aspartate amino transferase were detected in the ovary of an Orthopteran Cricket, \textit{Teleogryllus mitratus}\textsuperscript{257} and exhibit reduced ecdysteroidal synthesis in the integument and cultured ovaries of Cricket \textit{Gryllus bimaculatus de geer in vitro}\textsuperscript{249}. The anti-fertility effect of Azadirachtin is documented in a number of insect species. It inhibits phospholipid transfer from lipophorin to the oocytes thereby reducing the growth of oocytes in \textit{Rhodnius prolixus}\textsuperscript{253}. Continuous exposure to NeemAzal causes molt inhibition and reduced fecundity effects in \textit{Aedes aegypti}\textsuperscript{247}. The development of oocytes in \textit{Spodoptera litura} is indicated by the histological defects like irregular shape, lesser yolk deposition and greater vacuolization in ooplasm\textsuperscript{251}. Immunocytochemical studies of serotonin contribute to the hypothesis of Azadirachtin interference of hormonal release with growth and metamorphosis in locusts, \textit{L. migratoria} and \textit{S. gregaria}\textsuperscript{248}. Azadirachtin was reported to lower the 20-monooxygenase activity required for the conversion of ecdysone into 20-hydroxyecdysone in the fat body (\textit{Aedes, Drosophila, Manduca}) and midgut of \textit{Manduca}. The lead target identified in a genetic model system like \textit{Drosophila} needs validation in an Azadirachtin-sensitive pest system. \textit{Plutella xylostella} is a selectively attacks cruciferous crops and the control of this pest is seemingly difficult with synthetic pesticides.

Hence, in this chapter, the \textit{in vitro} binding efficiency of Aza to Actin proteins in \textit{Drosophila} and \textit{Plutella} is reported which would further strengthen the Aza-actin binding theory and indicate the possible route of mechanism of action using \textit{Spodoptera litura} haemocytes. These results would link the targets found in the pest species and that ones found in \textit{Drosophila} to give a comprehensive idea of the whole mechanism of action of Azadirachtin in insects.

\textbf{3.2.2. Results}
A) Effect of Azadirachtin on post-embryonic development of *Plutella, Drosophila* and *Spodoptera*

The dose-dependent effect of Azadirachtin in *Spodoptera litura, Plutella xylostella* and *Canton-S* of *Drosophila melanogaster* were distinctly different. *Spodoptera* fourth instar larvae normally could not develop into their next metamorphic stage and are often seen with constricted cuticle. Cuticle constriction is regarded as molt inhibition, often seen among Lepidoptera as a result of Azadirachtin treatment. The larvae thus could not survive through 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 Aza-sensitive Lepidopterans. Similarly, *Drosophila* did not show any indication of molt inhibition on the third day of Azadirachtin treatment but at higher concentrations beyond 5ppm they were larval lethal.

![Graph](image1)

**Figure 1.** Average weight gain of 6 larval replicates of *Plutella, Spodoptera* and % total of flies emerged (n=59) in *Drosophila* post-Azadirachtin treatment with a P-value < 0.01

The average weight gain of larvae of *Plutella* treated with Azadirachtin is retarded gradually (Fig 1A) and a marked reduction in their body weight is observed on the third day with increase in concentration. The *Canton-S* or the wild –type strain of *Drosophila* are observed with delay in
post-embryonic development (3-4 days). The late third instar larvae of *Drosophila* which are the non-feeding stages that crawl onto the surface of the glass vial were observed with abnormal eye imaginal discs of reduced size\(^{260}\) and still emerged as adult flies. These flies are noticed with defective ommatidial arrangement at lower concentration and are severe at 3 ppm.

![Image of flies and developmental stages](image)

**Figure 2.** Effect of Azadirachtin on wild-type (Canton-S) *Drosophila melanogaster* at different stages of development. (A) Eye of untreated control fly; (B) small eye in 3 ppm Aza-treated fly; (C) defective dorsal closure and wing missing in 3 ppm Aza-treated fly; (D) normal untreated pupa; (E&F) early pupal lethals with severe histolysis.

concentration of Aza (Fig 2B). Rarely, some of the Aza-treated flies had defective thoracic closure due to loss of wing (Fig 2C), similar to the mutants that show abnormal dorsal closure as a result of actin cytoskeletal collapse generally required for epithelial cell movement\(^{261}\). The pupal lethals obtained at 3 ppm were either with eye deformities or headless (Fig 2D) and usually died as early larval lethals with degenerating mass of tissue at 5 ppm (Fig 2F). The effect of Azadirachtin is very well pronounced when the larvae were challenged with Azadirachtin as young larvae (1st instar) in *Spodoptera* and *Plutella*. Later instars showed 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 larva (ie., late third instar) the cells of the imaginal disc are already differentiated and the molecule may not bind to the target/s. A similar scenario may happen in the immune system where the earlier instars, are much more vulnerable than the older instars. This answers why older instars are resistant in field conditions to Azadirachtin. Hence, for our experimental purpose 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*.

**B) Azadirachtin causes loss of Phalloidin in the haemocytes of *Spodoptera***

In a recent publication, it was reported for the first time, actin as a putative target of Azadirachtin in *Drosophila* and the cellular consequences following cytoskeletal collapse\(^\text{259}\). 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 seen tending to be primarily routed through the haemolymph. Haemolymph of insect comprises of different cell types known as haemocytes. Based on their morphological features, the haemocytes are characterized as prohaemocytes, plasmatocytes, granular haemocytes and spherule cells (Fig 3A). These haemocytes labelled with Phalloidin-TRITCC (stains f-actin) clearly reveal the actin localization in different cell types (Fig 3A). Each of the cell types have a distinct cell morphology and actin localized in the lamellar extensions of the cell. The plasmatocytes occur in many morphological shapes and sizes. The actin levels are down regulated in the haemocytes of the larvae treated with Azadirachtin and the cells are devoid of the lamellar extensions (Fig 3B), a clear indication that the cells tend to lose their adherent properties which are normally facilitated by the lamellar protrusions, to allow cell-to-cell contact. This is possible because the cytomechanics of actin is required for lamellelipodial formation during cell-to-cell communication and perturbation in the actin cytoskeleton leads to withdrawal of the forces that drive the extensions of the lamellipodia of the cell\(^\text{217}\). Also, the cells have lost their morphological features which are distinct for each cell type (Compare Fig 3A and B). Although, loss of Phalloidin staining was observed in the haemocytes of *Drosophila* and *Plutella*, due to technical difficulties involved in resolving the cell structures during imaging due to their small size, the supporting data was not provided. Through our findings, we suggest that all the three species of insects undergo a similar mechanism of action *via* the haemolymph to target the dividing cells which lead to morphological deformities\(^\text{262}\).
Figure 3. Haemocytes of third instar larva of Spodoptera litura treated with 10ppm of Azadirachtin and labeled with Phalloidin-TRITCC. (A) Haemocytes of untreated control larva (a-Prohaemocytes; b-granular haemocytes; c-Spherule cell; d-Plasmatocyte-indicated by arrow heads) (B) Haemocytes of Aza-treated larva with structural deformities due to loss of Phalloidin.

C) Down-regulation of transcripts of Actin isoforms in the larvae of Plutella and Drosophila challenged with Azadirachtin

Reports available contribute to the existing knowledge of tubulin depolymerisation and the 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 was unaltered in Plutella (Fig 4A) and Drosophila (Fig 4B). However, though the expression of Act57B transcript was moderately downregulated in D. melanogaster at 2 and 3ppm of Azadirachtin (Fig 4B), the effect seemed many-fold in P. xylostella at 15ppm (Fig 4A). 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 Aza-induced cell death. Since muscle-specific transcript is present only in a small population of cells, any effect observed maybe amplified unlike the Act5C mRNA expression. So, the effect of Azadirachtin may be entirely at the protein level and does not reflect at the transcript level. It is quite intriguing to know that larva of insect 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 molt-defects which may ultimately lead to larval lethality.
Figure 4. mRNA expression profile of Act5C (ubiquitous and cytoplasmic) and Act57B (muscle-specific) isoforms of Actin in third instar larva of (A) Drosophila (B) Plutella post-Azadirachtin treatment.

D) Azadirachtin de-polymerises *Plutella* and *Drosophila* actin *in vitro*

![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.](image)

*In vitro* polymerization of Actin isolated from *Plutella* and *Drosophila* incubated with Azadirachtin reveal that G-actin accumulates in the supernatant in a dose-dependent manner (Fig 5). The polymerized G-actins (or F-actins) in *Plutella* and *Drosophila* controls settles at the bottom as no G-actin band is detected on the SDS gel. Polymerisation has to be initiated at room temperature prior to Azadirachtin incorporation to observe the dose-dependent effect.
3.3. Mode of action of Vinca alkaloids

3.3.1. Introduction

A) Vinblastine

Microtubules are cytoskeleton structures required for basic fundamental processes of a cell. Microtubules are involved in the axon guidance, axonogenesis, microtubule-based process, larval behavior, response to light stimulus, heart development, chromosome segregation, intracellular protein transport, mitosis, microtubule-based movement, muscle attachment, cytoskeleton organization and biogenesis. Disruption of microtubule function can cause inhibition of all important cellular processes. Amongst the many microtubule disrupters available, vinka alkaloids are well studied in clinical biology.

The mode of action of vinka alkaloids was extensively worked at in the early 90’s. The anti-proliferative properties of vinka alkaloids were well characterized through the biochemical assays. It was reported that Vinblastine reversibly binds to the tubulin polymers and decreases the dissociation rate constant at the assembly or the “A” site as compared to the dissociation or the “D” site causing decrease in dynamic instability (growth and shortening phase) and modulates polymerization dynamics rather than depolymerisation of the microtubule assembly. Using Bovine brain microtubules which were either depleted of microtubule-associated proteins (MAPs) or rich in MAPs, it was demonstrated that low concentrations of Vinblastine (0.2 microM-1 microM) suppressed the growing and shortening rates and increased the percentage of time microtubules spent in a state of attenuated activity, neither growing nor shortening detectably. Their results suggest that Vinblastine kinetically stabilizes microtubule ends by modulating the gain and loss of the stabilizing GTP or GDP-Pi "cap", which is believed to be responsible for the transitions between the growing and shortening phases. Further, it was hypothesized that (1) low concentrations of Vinblastine inhibit mitosis by kinetically stabilizing the polymerization dynamics of spindle microtubules and that (2) the dynamics of spindle microtubules are critical for the proper progression of mitosis. Following this report, the differential effect of Vinblastine on microtubule polymerization and dynamics at “plus” and “minus” ends was characterized. Their work significantly contributes to the understanding of overall dynamicity of the growing microtubule in presence of Vinblastine. It was inferred through their findings that Vinblastine moderately increases the overall dynamicity at “minus” ends while strongly suppressing...
dynamicity at “plus” ends. Vinblastine contributes to the state of attenuated dynamics where both the average duration of a pause (a state where neither growth nor shortening could be detected) and the percentage of total time spent in pause were shown to increase significantly in vitro\textsuperscript{266}. Vinblastine was also found to induce rapid assembly of focal adhesions and microfilament bundles in Swiss 3T3 cells, tyrosine phosphorylation of FAK and paxillin (part of integrin-dependent cascade) and subsequent enhancement of DNA synthesis\textsuperscript{267}.

**B) Vincristine**

Vincristine, a microtubule inhibitor causes mitotic catastrophe and activation of apoptosis in the interphase cells in a p53 independent manner\textsuperscript{268}. Electrophoretic mobility shift assays and associated antibody super-shift experiments suggests that Vincristine promotes release of p53 protein from the binding site of mTUBB2 (a class II β-tubulin isotype) and stimulates increase in mTUBB2 mRNA expression around the nuclei of mouse melanoma cells\textsuperscript{269}. Separate experiments with human breast adenocarcinoma (MCF-7) carrying mutant p53 showed resistance to the cytotoxicity of microtubule inhibitors (Galamarani et al., 2003). Subsequently, it was found that both Vincristine and Vinblastine decrease Estrogen receptor alpha protein (ERalpha) and mRNA levels in the human breast cancer cell line MCF-7 through the inhibition of estradiol (E2) mediated transactivation at ERE-driven promoters\textsuperscript{271}. Vincristine disrupts microtubule cytoskeleton causing HIF-1 protein and not mRNA down-regulation, thus inhibiting tumor angiogenesis via the hypoxia-inducible factor-1 (HIF-1) pathway\textsuperscript{272}. Tumor suppressor protein p53 associates with MTs and uses the MT-dependent motor complex dynein/dynactin for nuclear targeting, e.g., after DNA damage\textsuperscript{273}. Disruption of the MT network polymerization, or depolymerization with vincristine (VCR) impedes p53 translocation to the nucleus and in turn inhibits activation of downstream targets by p53\textsuperscript{274}.

**3.3.2. Results**

Equal number of males and virgin females were released into the Aza-treated medium of various concentrations. The females laid their eggs in the treated media and the resultant progeny were continuously exposed to Azadirachtin till the third instar stage. The progeny were screened for pupal lethality, adult emergence and adult eye phenotypes. Although there was a developmental
delay at different stages from egg laying to pupal development, larvae of the same developmental stage based on the (ii) wandering stage and (ii) the number of mouth hooks were used for immunohistochemistry.

A) Eye and wing phenotypes induced by Vinblastine and Vincristine in the wild-type

Figure 1. Adult eye phenotypes in Canton-S flies treated with Vincristine and Vinblastine. (A) Adult eye of untreated Canton-S (wild-type) fly. (B-C) Adult eyes of Vincristine-treated flies (B) Roughoid eye phenotype (C) Distorted eye. (D-F) Adult eyes of Vinblastine treated flies (D) Small roughoid eye (E) Tiny eye (F) Eyeless phenotype.

The phenotypes of Vinblastine treated Canton-S flies were much stronger as compared to Vincristine treated flies (Compare 1B, 1C with 1D, 1E, 1F). Scanning electron microscopic imaging of the Vincristine treated eyes revealed bent bristle phenotype at interommatidial facets (Compare Fig 2C and 2D). The bent bristle morphology was also noticed in Azadirachtin treated flies together with multiple bristle phenotype which are absent in the Vincristine-treated condition. This is best explained as Vincristine is a tubulin inhibitor and may not target Actin as observed in case of Azadirachitin treated condition which targets both tubulin as well as actin cytoskeleton.

B) CycE and tubulin rescues Vincristine and Vinblastine phenotypes

To understand the mechanism of action of the two vinka alkaloids and their involvement in arresting cell cycle, CycE (a cdk sub-unit) was over expressed in the eye using ey-Ga4. CycE rescues Vincristine (Fig 4C) and moderately rescues Vinblastine induced lethality (Fig 3C). Tubulin::GFP fusion protein when over expressed using arm-Ga4 could rescue Vincristine and not Vinblastine induced lethality (Fig 3C; 4C). Vinblastine-induced lethality was rescued by CycE
Figure 2. Scanning Electron Microscopy images of Vincristine-treated eye. (A) Compound adult eye of Canton-S fly (wild-type) (B) Vincristine-treated adult eye (C) Bristles around the ommatidial facets of untreated wild-type at higher magnification (D) Bent bristle phenotype in Vincristine-treated adult eye.

only upto 1ppm beyond which over expressing CycE does not override the Vinblastine effect completely (Compare Fig 3C and 3D). At higher concentration i.e., 2ppm only CycE rescues marginally and tubulin does not contribute to the rescue effect (Fig 3D).

Figure 3. CycE moderately rescues Vinblastine induced lethality. The effect of different concentrations of Vinblastine was tested in wild-type (CS flies) and in the background of gain-of-function of Cell-cycle components, Cyclin E and Tub::GFP. Over-expression of CycE considerably suppressed the phenotypes induced by Vinblastine at 2ppm concentration.
Figure 4: CycE and Tubulin rescues Vincristine induced lethality. The effect of different concentrations of Vincristine was tested in wild-type (CS flies) and in the background of gain-of-function of Cell-cycle components, Cyclin E and Tub::GFP. Over-expression of CycE and Tub::GFP considerably suppressed the phenotypes induced by Vincristine at 15 and 20ppm concentration.

C) Vinblastine and Vincristine induces enhancement of phenotype in a tubulin deficiency mutant

Since, β-tubulin was already identified to be a target of Vinca alkaloids, the effect of these alkaloids in a tubulin deficiency background was tested. β-tubulin exists in two isoforms in the somatic tissue; the ubiquitous β1 tubulin or βTub60D which is cytoplasmic and a structural constituent of cytoskeleton and β3 tubulin or βTub56C which is expressed in the late third instar larva, both of which has a GTP and a tubulin binding site. Our experiments indicate that Vincristine and Vinblastine-induced phenotypes were enhanced in both the tubulin isoform deficiency background (Fig 5C; 6C). But at higher concentrations both Vinblastine and Vincristine treated progeny were pupal lethal (Fig 5D; 6D). In Vinblastine experiments, the lethality was enhanced in a Df (2R) BSC26 tubulin mutant background in a dose-dependent manner unlike in the Df (2R) PX2 tubulin mutant (Fig 5C; 5D). But such distinguished effect is not observed in Df (2R) PX2 mutant isoforms in Vincristine (Fig 6C) as compared to the Vinblastine experiments (Fig 5C).
Figure 5. Vinblastine enhances β-tubulin deficiency phenotype. The effect of different concentrations of Vinblastine was tested in wild-type (CS flies) and in the tubulin deficiency background, Df (2R) BSC26 and Df (2R) PX2. The phenotype of Df (2R) BSC26 was enhanced at Vinblastine 1ppm concentration.

Figure 6. Vincristine enhances β-tubulin phenotype. The effect of different concentrations of Vincristine was tested in wild-type (CS flies) and in the tubulin deficiency background, Df (2R) BSC26 and Df (2R) PX2. The phenotype of Df (2R) BSC26 and Df (2R) PX2 were enhanced at Vincristine 15 and 20ppm concentrations.
Figure 7. CycA and CycD Over-expression causes mild rescue of Vinblastine induced lethality. The effect of different concentrations of Vinblastine was tested in wild-type (CS flies) and in the CycA and CycD Over-expression background.

Figure 8. CycD and CycA do not rescue Vincristine-induced phenotype. The effect of different concentrations of Vincristine was tested in wild-type (CS flies) and in the CycA and CycD Over-expression background. Wing phenotypes were noticed along with the eye phenotypes.
D) Cyclin A and Cyclin D moderately rescues Vinblastine and not Vincristine induced lethality

Two important cyclins that are involved in cell-cycle are Cyclin D and Cyclin A, which regulate the G1 phase and G2-M phase respectively. Over-expression of CycD with ptc-Ga4 and CycA with arm-Ga4 does not rescue the Vinblastine-induced effect completely. But at higher concentrations i.e., 1ppm, a moderate rescue of Vinblastine-induced phenotype was noticed in CycD and CycA over-expression background (Fig 7D). The effect of CycA and CycD over-expression in Vincristine experiments was however inconclusive.

The tubulin inhibitors, Vinblastine and Vincristine were good negative controls for the Actin cytoskeleton inhibitors like Azadirachtin and EGFR tyrosine inhibitors, Gefitinib and Erlotinib.
3.4 Mode of action of anilinoquinazolines

3.4.1. Introduction

Epidermal growth factor receptor protein (EGFR) is a 170kDa transmembrane glycoprotein which is expressed in almost all the epithelial cells. It consists of two domains, (i) the extracellular ligand binding domain and (ii) intracellular tyrosine kinase domain. Auto-phosphorylation at the tyrosine kinase domain of the EGFR is a key step for the activation of the pathway which is required for many cellular functions. EGFR/ Ras pathway is conserved during evolution\(^{146}\); a striking similarity exists in the tyrosine kinase domain of EGFR between humans and *Drosophila melanogaster*, which makes *Drosophila* an ideal system for *in vivo* drug screening\(^{104}\) of small molecule tyrosine kinase inhibitors. As large number of transgenics and mutants are available for these pathways, the system is useful for target identification and validation studies. Previously, target identification has been reported using *Drosophila* eye as an assay to study the effect of non-steroidal anti-inflammatory drug, Indomethacin on *Adenomatous polyposis coli*\(^{194}\) and Azadiractin, a neem limonoid on actin cytoskeleton\(^{260}\).

EGFR signaling in *Drosophila* is a pre-requisite for the morphogenesis of the eye\(^{275, 276}\) and wing development\(^{177, 277}\). In the eye, EGFR plays multiple roles\(^{278}\) which include control of cell proliferation, neuronal differentiation in the posterior margin of the imaginal disc\(^{279}\), cell recruitment\(^{280}\), cluster/ founder cell spacing\(^{124, 281}\), spacing of the R8 cells, cell survival and later recruitment of non-R8 cells. Besides, EGFR has a specific role in the cell size control of most epidermal cells\(^{205}\).

EGFR is also required for early development of the wing and for notum specification\(^{177}\). Dp-ERK1/2 which represents EGFR signaling is expressed as a nuclear staining in the peripodial cells of the late third instar wing imaginal discs both in the wing pouch and notum, exemplifying the role of EGFR in the wing and notum specification.

In humans, over-expression or mutation of EGF-receptor tyrosine kinase or dysregulation of the ligand binding to the receptor can cause cancer in humans due to the expression of pro-proliferative gene products in the nucleus. Constitutive activation of ligand-independent mutant receptor was reported in many cell types such as gliomas, NSCLC, prostate, breast, ovary and stomach cancers\(^{85, 86, 87}\). Ligand binding to the extracellular domain of the receptor causes conformational change that promotes dimerisation with other EGFR monomers. 50% of gliomas
and 60-85% of NSCLC are known to be caused due to aberrant EGFR signaling. Homodimerisation results in transphosphorylation of tyrosine residues within the cytoplasmic domain of the receptor which provides scaffold for many adaptor proteins and kinases. On the other hand, EGFR can heterodimerize with other members of the HER-2 and HER-3 family members. Erb2 receptor lacks ligand (rather is not reported) but consists of a kinase domain. On the other hand Erb-3 receptor lacks the kinase domain but has a ligand binding domain which compensates for the Erb-2 receptor activity during heterodimerisation. Downstream to the receptor is a membrane-associated Ras, a G protein with intrinsic GTPase activity which exists in either an active GTP-bound form or an inactive GDP-bound form. When SOS, a GTP–GDP nucleotide exchange factor, is recruited to the receptor complex through its association with Grb2 and it catalyzes the exchange of GDP for GTP, resulting in an increase in the active GTP-bound form of Ras. Clinically, Ras is implicated in 90% of pancreatic cancers, 50% of colorectal cancers, 30% of lung cancers, and 15–30% of melanomas. Out of the three forms of Ras known in humans (K-ras, H-ras and N-Ras), the K-ras secondary mutations are known to be of concern as it causes impaired GTP hydrolysis causing a shift from a GDP-form to a GTP-form which leads to constitutive activation of the intracellular signal. Thus, EGFR activation of Ras relays signals through the Raf-mitogen-activated protein kinase (MAPK)/ ERK kinase-ERK pathway, culminating in the regulation of cell-cycle. Raf translocates to the plasma membrane and gets phosphorylated by other kinases (PAK, Src and JAK). In the wake of growing awareness of human cancers in relation to Raf particularly its implication in 65% of melanomas, 40-70% of Papillary thyroid cancers, 60% of low-grade ovarian tumors, and 4-16% of colorectal cancers. Alternately, EGFR activation of protein kinase C can promote ERK activation by directly phosphorylating Raf. The most prevalent point mutation in B-Raf, V600E, results in a constitutively active B-Raf. It is presumed that the negatively charged glutamate mimics the phosphorylation of a neighboring serine or threonine residue required for activation. Raf phosphorylates MEK1, 2 which in turn activates serine/ threonine residues of ERK1 and 2 which later translocates to the nucleus and causes specific changes in the gene transcription. Elevated ERK1/2 phosphorylation coincides with the cancer and reflects the convergence of various oncogenic signals. However mutations of MEK1 and 2 in human cancer have not been reported so far. Over-expression or mutation of receptor tyrosine kinase or dysregulation of the ligand binding to the receptor can cause cancer in humans due to the expression of pro-proliferative gene products in the nucleus.
Clinically, to treat patients with cancer caused due to the activation of EGFR, Gefitinib and Erlotinib are used in the chemotherapeutic regimens to treat metastatic NSCLC. These are small molecule tyrosine kinase inhibitors of the anilinoquinazoline class, having cytostatic effect. Clinical data suggests that Gefitinib inhibits the tyrosine kinase activity and thus block the signaling by inhibiting the pAkt leading to apoptosis of the cells. Several mutations have been identified which alter the activity of Gefitinib\textsuperscript{91, 250, 287}. Recently, it was reported that EGFR mutations include small in-frame deletions and mis-sense mutations of the ATP binding pocket that decide the binding efficiency of Gefitinib to EGFR\textsuperscript{91}. K-Ras secondary mutation are also an observable phenomenon in Gefitinib acquired resistant cases. Thus, it is considered that phosphorylation status of K-Ras mutation can predict the responsiveness of Gefitinib in blocking the EGFR singaling in NSCLC\textsuperscript{287}.

Due to the prevalence of EGFR-induced tumors worldwide accounting for nearly 80% of all lung cancers, it is imperative to design highly potent small molecule inhibitors that specifically target the tyrosine kinase domain of EGFR. Clinically, Gefitinib and Erlotinib are widely used in the chemotherapeutic regimens for treating patients with EGFR induced tumors. These small molecule tyrosine kinase inhibitors of the anilinoquinazoline class possess cytostatic effect.

In this chapter, the scope of using an \textit{in vivo} system like \textit{Drosophila} is discussed to study the mode of action of tyrosine kinase inhibitors primarily through enhancer-suppressor assays. As complex genetic pathways are much more discernable \textit{in vivo} as compared to \textit{in vitro}, the system is ideal for screening small molecule tyrosine kinase inhibitors, validating their targets and evaluation of secondary effects.

By using enhancer-suppressor assays and \textit{in-silico} analysis, the probable mechanism through which TKI Gefitinib and Erlotinib block EGFR signaling is reported. Binding affinities of tyrosine kinase inhibitors to the modeled \textit{Drosophila} EGFR protein are also discussed.

\textbf{3.4.2. Results}

Having experimented some of the phytochemicals with \textit{Drosophila}, inorder to find the suitability of the system for evaluating synthetic drug molecules, the following was attempted.
A) Homology of EGFR-TK domain in *Drosophila*

In order to identify the suitability of the *Drosophila* model system for screening tyrosine kinase inhibitors, sequence similarity of tyrosine kinase domain of EGFR between humans and *Drosophila melanogaster* was compared. The protein sequence alignment studies of the TK domain indicated 59% identity between *Drosophila melanogaster* and *Homo sapiens*. The TK model of *Drosophila melanogaster* revealed 99% sequence and structural identity to *Homo sapiens* in the active site of the protein.

Table 1. Templates used for protein sequence alignment of *Drosophila* TK domain.

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![Protein sequence alignment of Drosophila EGFR TK-domain with mammalian crystal structures. Drosophila TK sequence is followed by four human sequences with crystallized TK-Domain structures obtained from PDB which are in complex with different drug except for 1M14 (the list is elaborated in table 1). The top bordered line in the alignment window shows the sequence identity among the species and the alignment region encapsulated in the boxes is the active site of TK-Domain.](image-url)
B) Gefitinib and Erlotinib induce wing and eye phenotypes

The CS strain of *Drosophila melanogaster* was used to observe the dose-dependent effect of the TKIs. The LD_{50} for Gefitinib and Erlotinib (concentration mixed in the food) were 100 and 500 ppm, respectively. Flies treated with Gefitinib showed a range of wing phenotypes (Fig. 2B–E). At higher concentrations of Gefitinib, i.e. 150 and 200 ppm, duplicated wings with complete lack of venation (Fig. 2C–E) was noticeable.

![Figure 2. Gefitinib induces duplicated wing phenotypes. (A) Wing of Canton-S flies or untreated wild type. (B-E) Gefitinib treated wild type flies with moderate-to-severe wing phenotypes. (B) Wing with a necrotic patch (C-E) Adult wings with duplicated pattern and veins missing](image)

Necrotic patches were seen in few wings (Fig. 2B). A mild eye phenotype in the wild-type adult flies (n=7) was rarely observed at a concentration of Gefitinib of 25ppm (Fig. 3B). The effect was more severe with an increased number of flies having strong eye phenotypes at higher concentrations (data not shown). At a concentration of 250 ppm, a few of the progeny were late pupal lethals with a headless phenotype. On the other hand, Erlotinib–treated flies had less severe eye phenotypes (Fig. 3C) as compared with Gefitinib–treated ones at concentrations as high as 200ppm (data not shown). Although, the sublethal dose of Erlotinib was very high when compared with Gefitinib, the phenotypes appeared to be similar. Hence, for the experimental studies, sublethal concentrations of Gefitinib (25 ppm) and Erlotinib (200 ppm) which induced very mild phenotypes in the wild-type but a good response in a sensitized background was used.
C) Gefitinib and Erlotinib suppressed the activated EGFR induced eye phenotypes

The significance of the EGFR pathway in morphogenesis of the eye has been previously reported\textsuperscript{146}. Over-expression of EGFR\textsuperscript{\lambda\text{top}} with ey–Gal4 (an early expressing Gal4) causes a roughoid eye phenotype in untreated adult flies (figure 3G). Gefitinib and Erlotinib were able to suppress the roughoid phenotype induced by EGFR\textsuperscript{\lambda\text{top}} (Figs. 3H, and I; 4) in 100\% of flies (n = 33 [Gefitinib]; n = 23 [Erlotinib]), indicating that both the molecules were able to downregulate the EGFR pathway signaling in an EGFR gain-of-function mutant background. Further, it was examined if the TKIs could cause any phenotype in Ras’e’B, a recessive-lethal allele of Ras, which acts downstream from EGFR. A mild enhancement of wild-type eye phenotype in a Ras mutant was noticed in Gefitinib-(n=10) and Erlotinib-(n=21) treated adult flies (Figs. 3E and F; 4).

Figure 3. An enhancer-suppressor screen for Gefitinib and Erlotinib induced eye phenotypes: (A) Compound eye of an untreated wild type Canton-s fly. The compound eye of Drosophila is made up of 700-800 facets of ommatidia in a precise array with a pseudo pupil in the centre. (B&C) A mild eye phenotype induced after anilinoquinazoline treatment (B) Gefitinib-treated fly at 25 ppm. (C) Erlotinib-treated fly with phenotype at 200 ppm. (D-F) Compound eye of a recessive-lethal allele of Ras, Ras’e’B. (D) untreated (E) Gefitinib-induced eye phenotype at 25 ppm (F) Erlotinib-induced eye phenotype at 200 ppm (G-I) EGFR\textsuperscript{\lambda\text{top}} over expressed with ey-GAL4. (G-I) EGFR over expressed gain-of-function mutants (G) untreated fly with roughoid phenotype. (H) Gefitinib rescues EGFR over expressed phenotype to a wild type state at 25 ppm (I) Erlotinib rescues EGFR over expressed phenotype at 200 ppm.
Lethality was observed to increase in a dose-dependent manner in Ras mutants in the presence of the inhibitors at higher concentrations (data not shown). Although enhancement of eye phenotypes was not perceptible in flies with Over-expression of Argos (ligand antagonist of EGFR) driven with ey-Gal4, the fewer flies that emerged after treatment had missing antennae ($n = 11$ [Gefitinib]; $n = 12$ [Erlotinib]). Most of the Argos-overexpressed progeny in both the treatments were late larval lethals. Also, the effect of the drugs on the over-expression of Raf, a downstream effector of the Ras pathway was investigated. Over-expression of Raf using ey-Gal4 induced small eye phenotypes, which were not rescued with either of the anilinoquinazolines, although a noticeable down-regulation of dp-ERK1/2 levels in the eye imaginal discs was obvious (Fig. 6N, O). The EGFR pathway provides inputs to the cell cycle during eye morphogenesis. The specificity of both the molecules was validated using cell-cycle regulators. To identify if anilinoquinazoline-induced phenotypes could be rescued, CycE, a cyclin-dependent kinase (cdk) subunit that regulates G1–S

![Graphs showing phenotypic effects of Gefitinib and Erlotinib](image)

*Figure 4. Enhancement of lethality in a Ras mutant and argos over expressed with ey-Gal4. Gefitinib and Erlotinib suppress activated EGFR phenotype driven by ey-Gal4 at 25 and 200ppm respectively.*
transition of cells in a cell cycle, was overexpressed using ey–Gal4. Over-expression of CycE did not reverse the effect induced by Gefitinib and Erlotinib indicating that the impact might occur prior to the cell-cycle event.

D) Gefitinib–suppressed wing phenotypes induced by activated EGFR

In the gain-of-function experiments, Gefitinib suppressed UAS–EGFR^{Δtop} /vg–Gal4-induced serrate wing phenotype (n = 36) in the adult flies (Fig. 5A) to a normal wild-type wing (Fig. 5B). Over-expression of EGFR^{Δtop} with Omb–Gal4 (late expressing Gal4) which expresses in a broader domain along the anterior-posterior (A/P) axis in the wing pouch induced small and dupli-

![Image](image_url)

**Figure 5.** Gefitinib suppresses EGFR^{Δtop}-induced wing and leg phenotype. (A) Adult wing in an activated EGFR over-expression background. Often the mechanosensory bristles are found missing both on the dorsal and ventral margins of the wing when UAS-EGFR^{Δtop} is overexpressed with vg-GAL4. (B) Suppression of EGFR wing phenotype with Gefitinib. Note that the wing is completely restored to a wild type state. (C) Pupal wing phenotype when UAS-EGFR^{Δtop} is over expressed with omb-GAL4. The pupae do not eclose completely and die as pharate adults. The wings are small and often duplicated with no venation. Though a few mechanosensory bristles are missing on the dorsal margin, ectopic bristles are seen on the wing blade. (D) Gefitinib causes partial rescue of the wing phenotype although veins are not formed. Also the ectopic bristles of the adult flies are reduced. (E) Leg phenotype of EGFR^{Δtop} over
expressed untreated uneclosed flies driven by Omb-Gal4. Thoracic legs are severely deformed with fused tarsal segments and relatively short femur. (F) Gefitinib rescued activated EGFR phenotype to a near wild type state. (G) The wing of untreated Argos flies. (H) Gefitinib enhances Argos phenotype. Duplicated wings with no proper venation are observed in Argos-treated condition.

cated wings. These wings often had ectopic bristles on the wing blade along with a few mechanosensory bristles missing on both the dorsal and ventral sides (Fig. 5C) and the progeny died as pharate adults. Gefitinib suppressed the duplicated wing phenotype (Fig. 5D) and increased the survivability (decreased pupal lethality) of the flies. A partial rescue of wings in Gefitinib-treated activated EGFR adult flies was observed. These wings were relatively larger with regular arrangement of the mechanosensory bristles along the margins of the wing (Fig. 5D). There was a significant reduction in the number of ectopic bristles but rescue of wing venation was not observed. Gefitinib also suppressed the leg phenotype in the same group of flies (Fig. 5, compare 5G and H).

Gefitinib enhanced the wing phenotype when Argos, a negative regulator of EGFR was overexpressed with vg–Gal4 (n=16). The flies showed a duplicated wing pattern (Fig. 5F). Similarly, Gefitinib enhanced the adult wing phenotype in Ras mutants (n = 10) demonstrating that duplication of wing is due to the downregulation of the Ras pathway.

Due to the patterning defect induced by TKI through the down regulation of EGFR signalling, the intrinsic property of the cells is to undergo apoptosis preceded by developmental arrest. In this context, the role of an anti-apoptotic protein P35 was examined when over expressed with arm–Gal4. Gefitinib-treated P35–overexpressed adult flies did not rescue the phenotype, indicating that the effect is caspase-independent. It is probable that the cells undergo a different mechanism of cell death in this case, as evident from the appearance of necrotic patches in the adult wing (Fig. 2B).

E) Gefitinib and Erlotinib suppress enhanced dp-ERK1/2 levels in the background of activated Egfr/Ras signaling.

To understand the signal transduction profile of the EGFR pathway after exposure to the two experimental drugs, the imaginal discs of late third instar larvae were stained with an antibody that
recognizes the diphosphorylated forms of ERK 1/2. ERK, a mitogen activated kinase (MAPK), gets phosphorylated in the presence of the MAP at the conjunction of the EGFR and Ras signalling pathways. The eye and wing imaginal discs of untreated wild-type larva were arranged with well-spaced dp-ERK1/2 cells (Fig. 6A; 7A). Gefitinib and Erlotinib were able to downregulate the dp-ERK1/2 levels in the eye and wing imaginal discs of wild-type larvae (Figs. 6B, C; 7B, C). The drugs were also potent enough to downregulate the dp-ERK1/2 expression in the eye and wing

Figure 6. Gefitinib and Erlotinib down-regulates dp-ERK1/2 in the eye imaginal disc of the late third instar larvae of wild type and EGFR/ Ras mutant background. (A-C) Eye imaginal disc of late third instar larvae of Canton-S or wild type with an antibody rose against dp-ERK1/2. (A) Eye imaginal disc of untreated wild type control. Dp-ERK1/2 staining is seen in a few rows of cells with regular spacing between each ommatidial pre-cluster. (B) Eye imaginal disc of Gefitinib-treated wild type larva with down-regulation of dp-ERK1/2 (see arrowheads). (C) Eye imaginal disc of Erlotinib-treated larva with down-regulation of dp-ERK1/2 (see arrowhead). (D-F) Eye imaginal discs of late third instar larvae of Ras mutant labeled for d-ERK1/2. (D) Eye imaginal disc of untreated Ras mutant larva. Dp-ERK1/2 staining is seen in few rows of cells in the ommatidial pre-clusters which are disorganised. (E) Eye imaginal disc of Gefitinib-treated Ras mutant larva with down-regulation of dp-ERK1/2 (see arrow heads). (F) Eye imaginal disc of Erlotinib-treated Ras mutant larva with dp-ERK1/2 positive cells arranged in a disorganized pattern. Note the arrangement of cells completely gets disrupted in a Ras mutant with dp-ERK1/2 levels down-regulated and many pre-clusters missing in both Gefitinib and Erlotinib-
treated conditions. (G-I) Dp-ERK1/2 staining in the eye disc of activated EGFR larvae. (G) Eye imaginal disc of untreated activated EGFR. Dp-ERK1/2 positive cells are arranged in a disorganized manner in a cluster (see arrowhead). (H) Eye imaginal disc of Gefitinib-treated activated EGFR larva stained for dp-ERK1/2. (I) Cells of eye imaginal disc of Erlotinib-treated activated EGFR larva stained with dp-ERK1/2. (J-L) Eye imaginal discs of third instar larvae of UAS-Argos over expressed with ey-Gal4 and stained for dp-ERK1/2. (J) Eye imaginal disc of untreated Argos over expressed larva labeled for dp-ERK1/2. (K) Eye imaginal disc of Gefitinib-treated Argos over expressed larva. (L) Eye imaginal disc of Erlotinib-treated Argos over expressed larva. (G-I) Eye imaginal discs of Argos over expressed late third instar larva stained for dp-ERK1/2. (M-O) Eye imaginal disc of untreated Raf over expressed with ey-Gal4 and labeled for dp-ERK1/2. (M) Untreated Raf over expressed eye imaginal disc. (N) Down regulation of dp-ERK1/2 (see arrowhead) in the Gefitinib-treated Raf over expressed eye imaginal disc. (O) Down-regulation of dp-ERK1/2 in Erlotinib-treated Raf over expressed eye imaginal disc (see arrowheads).

Figure 7. Gefitinib down-regulates dp-ERK1/2 in the peripodial cells of activated EGFR, Argos gain-of-function and Ras loss-of-function mutants. (A-C) Wing imaginal disc of Canton-S (wild type) late third instar larvae stained for dp-ERK1/2. (A) Untreated wild type larval wing disc (B) Gefitinib-treated wild type larval wing disc (C) Erlotinib-treated wild type larval wing disc. Note the dp-ERK1/2 staining is down regulated in the wing imaginal disc in Gefitinib and Erlotinib-
treated imaginal discs except for a few areas (see arrow heads) which was unaltered. (D-F) Wing imaginal disc of wild type late third instar larvae of Ras loss-of-function mutant with dp-ERK1/2 staining lost in the pouch and notum of wing imaginal disc (see arrowheads). (D) Wing imaginal disc of untreated Ras mutant larva (E) Wing imaginal disc of Gefitinib-treated Ras mutant larva (F) Wing imaginal disc of the Erlotinib-treated Ras mutant larva. (G-I) Wing imaginal discs of third instar larvae of UAS-EGFR^{top} crossed to vg-Gal4 indicating with enhanced dp-ERK1/2 staining. (G) Wing imaginal disc of dp-ERK1/2 over expressed in the vestigial domain of untreated EGFR^{top} mutant larva (see arrow heads). (H) Loss of dp-ERK1/2 staining in the wing imaginal disc of Gefitinib-treated EGFR^{top} mutant larva. (I) Loss of dp-ERK1/2 signal in the wing imaginal disc of Erlotinib-treated EGFR^{top} mutant larva. (J-L) Down-regulation of dp-ERK1/2 in the wing imaginal disc of Argos over expressed third instar larvae. (J) Dp-ERK1/2 expression in the wing imaginal disc of untreated Argos over expressed mutant larva (K) Loss of dp-ERK1/2 expression in the wing imaginal disc of Gefitinib-treated Argos over expressed mutant larva (see arrowheads). (L) Wing imaginal disc of Erlotinib-treated Argos over expressed larva with loss of dp-ERK1/2 expression (see arrowhead).

imaginal discs of late third instar larvae from different genetic backgrounds. Enhanced dp-ERK1/2 levels observed in the eye and wing imaginal discs of untreated Ras’e’B (Figs. 6D; 7D) and UAS–EGFR^{top} overexpressed with either ey–Gal4 (Fig. 6G) or vg–Gal4, (Fig. 7G)) were suppressed in the presence of the TKIs (Figs. 6E, F, H, I; 7H, I, E, F). Conversely, these TKIs were able to downregulate the dp-ERK1/2 levels significantly in the eye and wing imaginal discs of late third instar larvae from certain other genetic backgrounds, such as, UAS–Argos over expressed either with either ey–Gal4 (Fig. 6K, L) or vg–Gal4 (Fig. 7K, L) and UAS–Raf with ey–Gal4(Fig. 6N; 6O). Taken together, these results suggest that Gefitinib and Erlotinib are potent inhibitors of EGFR signalling in Drosophila too. In silico analysis suggests that EGFR itself appears to be the target of these two anticancer molecules.

Taken together, these results suggest that Gefitinib and Erlotinib are potent inhibitors of EGFR signaling in Drosophila too. The in silico analysis suggest that EGFR itself appears to be the target of these two anti-cancer molecules.