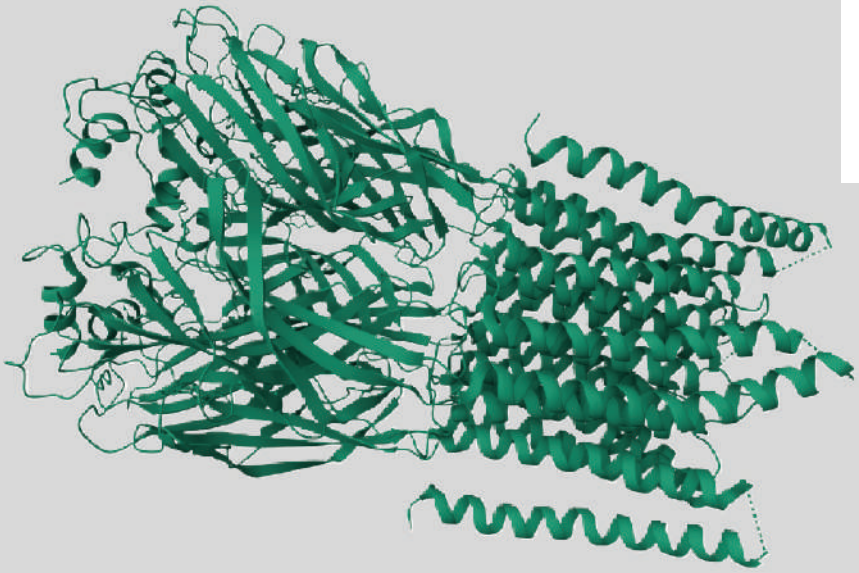

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A Reliable Discovery Platform

PENTAGRIT
ZEBRAFISH CRO

A SCREENING SYSTEM DESIGNED SPECIALLY TO IDENTIFY RESISTANCE PROOF INSECTICIDES.

WHITE PAPER: OCTOBER 2024

Evolutionary mutations of drug targets allows insects to develop resistance to even new insecticides quickly. Now, new scientific methods can qualify insecticide candidates if they continue to possess bioactivity against a target that mutates and change's structure. This ensures insecticide resistance is contained or delayed by several years.

Authors
P. Kalaichitra, S. Sandhiya

INTRODUCTION:

GABA receptors in insects are primary targets for Insecticides. Fundamentally these receptors are GABA gated chloride channel and hence classified as inotropic receptors. In insects three genes' codes for GABA receptors namely Resistance to dieldrin, GABA and glycine-like receptor of Drosophila and Ligand-gated chloride channel homologue three. Based on the binding sites of insecticides on these receptors, the insecticides impart pharmacological properties.

However, increasing resistance to insecticides have been reported due to variants within the GABA receptors. These variants could be due to an extra copy of GABA receptor gene, a point mutation in the receptor or a sub type of the receptor.

Primarily, variants within the target gene sequence are responsible for insecticide resistance.

To address this gap of translating field studies to market success, new discovery research models are required. Therefore, our goal was to develop a target variant screening system that selects a robust test candidate that can survive a wide range of target variation. We generated genetic variants of GABA receptor through targeted mutagenesis of the zebrafish Gamma-aminobutyric acid type B receptor. We set up a study group of variants and screened them with existing insecticides for hyperactivity response under stimuli. Hyperactivity behaviour levels inversely corelates with GABA receptor agonism. Therefore, test candidate treated animals that react hyperactively indicate strong antagonism of GABA receptor, whereas those variants that do not respond hyperactively indicate lack of GABA antagonism.

METHODS:

SELECTION OF GABA RECEPTOR VARIANTS:

Variants were identified from retrospective studies of established mutations but also from evolutionary tree analysis that pointed out prospective variants that would render resistance.

Figure 1: : Amino acid sequence showing sites of key amino acid substitution in zebrafish gamma-aminobutyric acid type B receptor that were identified to impart resistance in insects.

Anopheles sinensis Mutation Site	Corresponding Zebrafish Mutation Site	*Anopheles sinensis* Mutation Site	Corresponding Zebrafish Mutation Site
R119G	R107G	M349L	-
L176V	L171V	R356G	R352G
L162V	-	K357L	K351L
V327L	V326L	F360L	F362L
L278V	L270V	Q408H	Q410H
A296S	-	G473S	G483S
T345S	T341S	K357L	K351M

Table 1: Sites of amino acid substitution for zebrafish that were corelated from RDL sequence in *Anopheles sinensis*.

STUDY SET UP AND EXPOSURE:

Targeted mutagenesis was confirmed by Sanger sequencing and lines were ensured for stability through repeated genetic analysis. Animals within the group were also graded for size and physiological response markers to ensure the only difference between each of the animals were the target sequence. Established mutants were transferred to well plates with one fish per well for the screening panel. Overall, 30 variants of the receptor have been identified, of which three variants were employed for the current study holding triplicates for each. Animals were dosed with test candidates at 5,6 and 7 days post fertilization (dpf) and screened on 8 dpf. Dosing was through water dissolution.

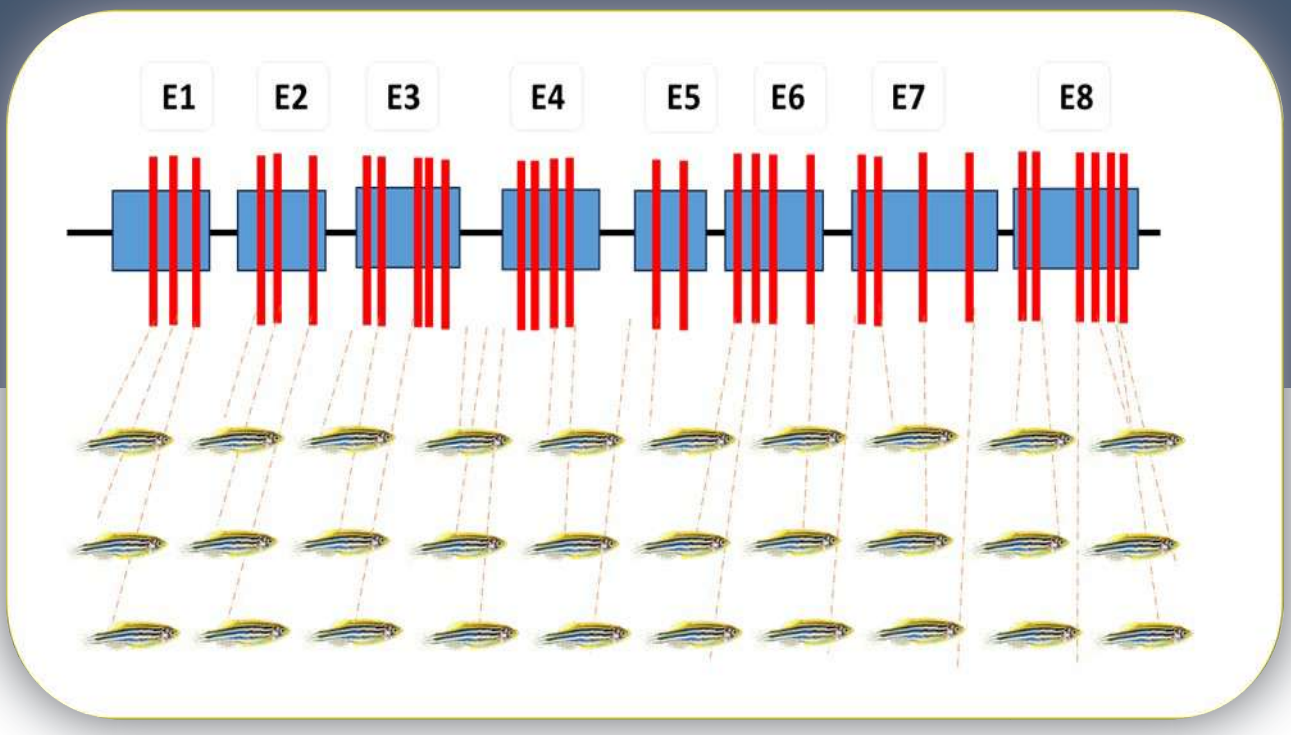

Figure 2: Schema showing a group set up of study animals with each animal encoding one genetic variant of the GABA receptor gabbr1b.

READ OUTS:

Dosed larvae were exposed to a hyperactivity inducing stimuli to measure hyperactivity induced behavior. The hyperactivity inducing stimuli included a controlled audio stimuli from M-Audio systems USA, at 300 decibels and controlled white light stimuli from Zhongshan SOBO Electric Co., Ltd, China at 2500 lumens. The exposure for pre-stimuli was audio for a period of 1 minute with a 4 minute observation period, followed by a refractory phase of 5 minutes; this was further followed by both audio and white light stimuli for a period of 1 minute with a 14 minute observation period. Response to white light and audio stimuli were recorded with motion tracker software and analyzed by ImageJ, National Institute of Health, USA to quantify hyperactivity response.

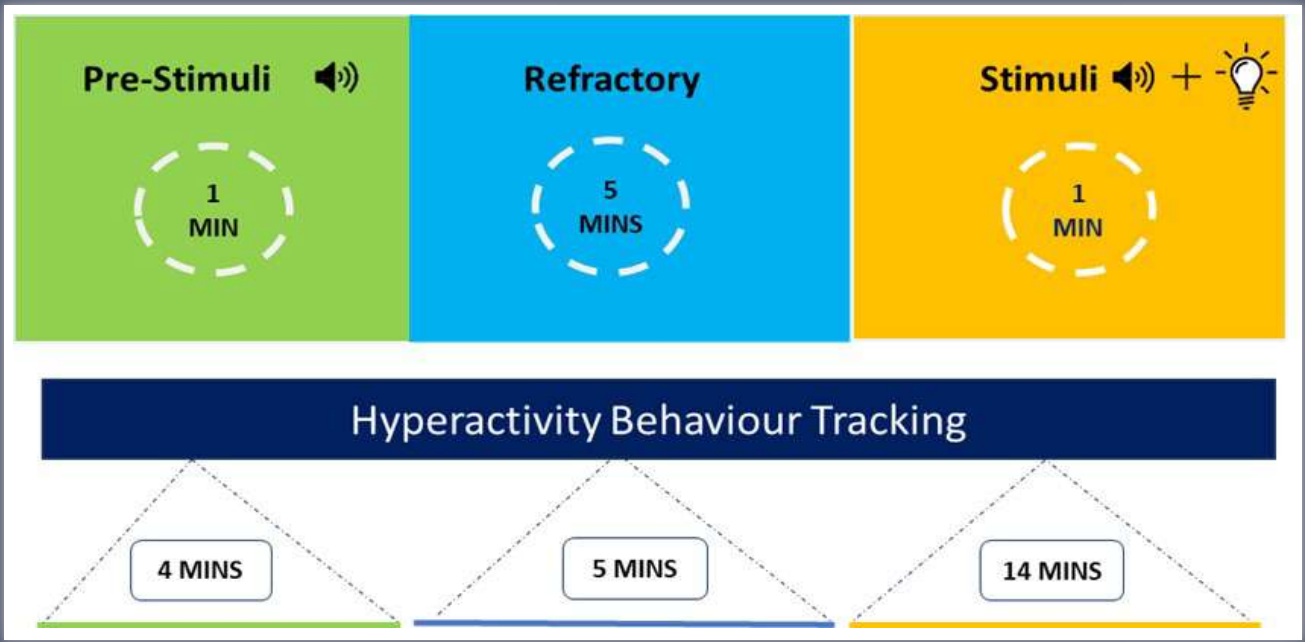

Figure 3:
Illustration showing hyperactivity induction and behavioral tracking phases.

Assay Principle:

GABA is stored in the post synaptic terminal and released into the synaptic cleft upon response from an action potential. GABA travels across the synapse to the post synaptic membrane and bind with GABA receptors. Upon binding, it exerts its inhibitory action on the nerves. When GABA receptors are occupied by an insecticide, GABA cannot exert its action leading to excessive CNS stimuli and death of the insect. In the current assay GABA is released moments after the stimuli, animals that have not acclimatized to the pre-stimuli or find the stimuli too strong continue to be in a hyperactive state indicating lack of GABA biding to the GABA receptor.

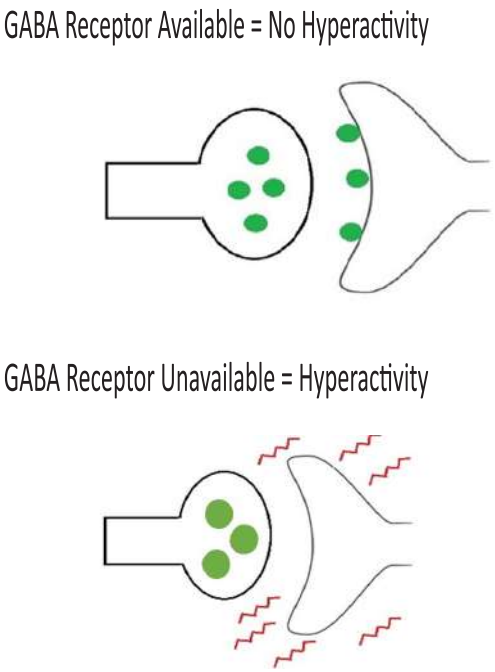

Figure 4: Schema of GABA activity in the post synaptic cleft with and without GABA receptor availability.

Scoring:

Read outs were converted to z-scores based on the behavioural response and bioactivity scores were plotted on a heat map. Test candidates with maximum bioactivity response to variants were identified with a score of +3 and those that had no activity against variants were identified with a score of -3.

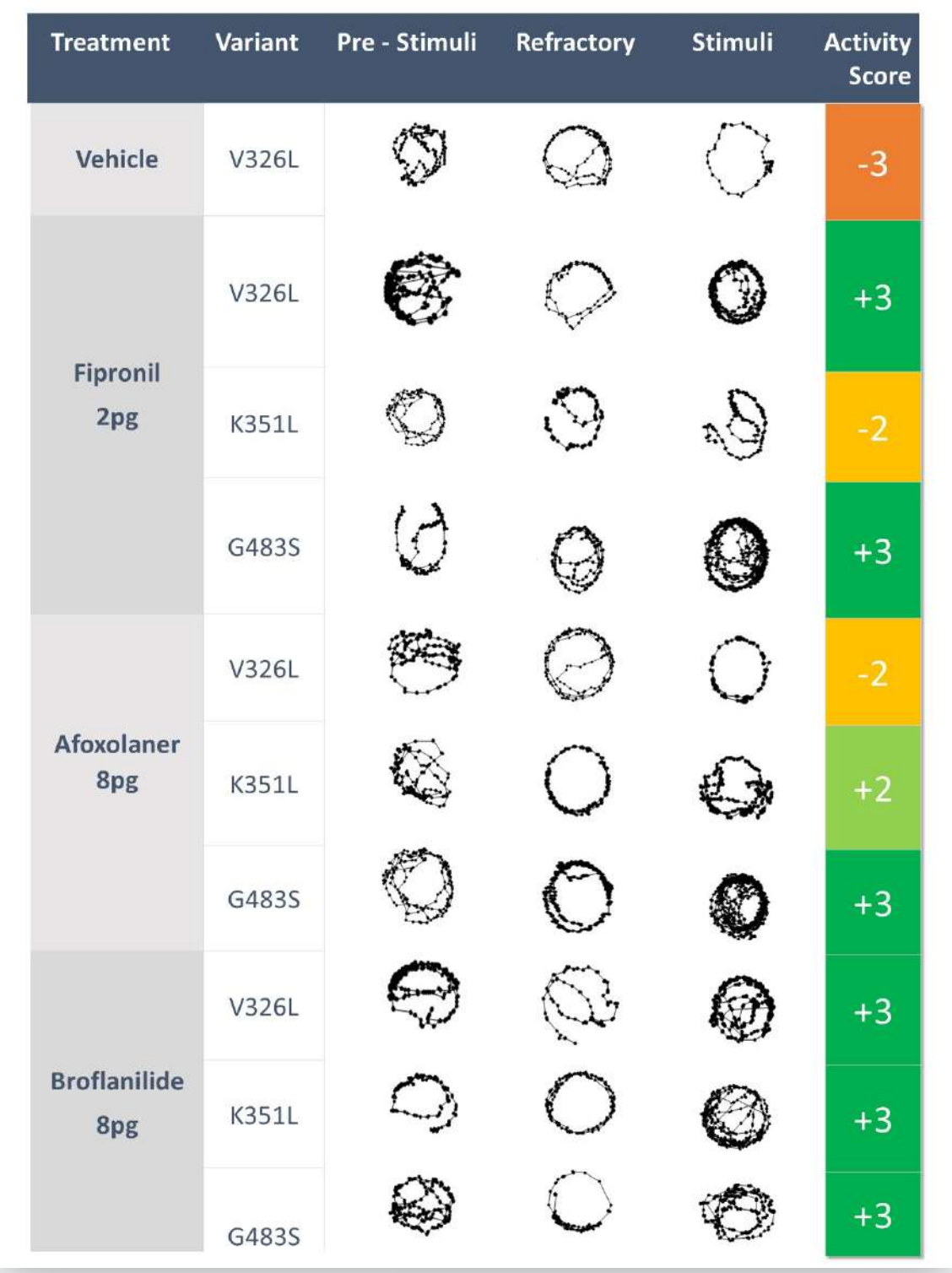

Figure 5: Study results showing tracking path of variants dosed with insecticides and scoring for hyperactivity. The higher hyperactivity in stimuli phase corelates with a positive bioactivity score and vice versa.

RESULTS

Vehicle treated shows a score of -3 on treating the variant V326L. Since there is no bioactivity of inhibiting GABA receptor, the animal returns to a non-hyperactive state. Fipronil treated shows resistance in the case of K351L. Similarly, Afoxolaner treated variants showed resistance in the case of V326L and reduced activity in the case of K351L. Broflanilide treated showed activity in all three variants namely V326L, K351L and G483S.

APPLICATIONS OF TARGET VARIANT MODEL SYSTEM:

- Identify insecticides that survive ever changing resistance
- Possible to screen cocktails of insecticides that survives resistance even if a new single molecule does not work
- Validate biomolecules based candidates
- Identify new modes of insecticidal activity such as feed inhibition
- Validate candidates that have wide range bioactivity at low dose

CONCLUSION:

The changing landscape of insect population presents one of the largest diversity of insect's in the field applications. The change is mainly driven by several factors, but most importantly by insects with a better ability to mutate and survive single target insecticides. Insecticides developed from a Target Variant screen can perform with an excellent track record in the field.

P. Kalaichitra

Sincerely

P. Kalaichitra

S. Sandhiya

Sincerely

S. Sandhiya

BRIEF BACKGROUND

Pentagrit is an AAALAC and CCSEA accredited Pre-Clinical CRO based out of India. With a fully owned facility, Pentagrit develops in house zebrafish models and assays to address the evolving landscape of pharmacological compounds. This could be pharmaceuticals or agrochemicals discovery.

Insect targets can be accurately studied through knock in and gene edited zebrafish models ensuring accurate replication of insect targets.

The hallmark of zebrafish as a model is due to its sensitivity, that is “minor changes in effect due to minor changes in dose”: This enables a precise read out of physiological effect while maintaining high statistical strength. Tools to study zebrafish are globally harmonized ensuring universal replication of zebrafish methods.

Target variant screening system has created a huge upside for our new candidate development

- CEO, Agrochemicals

Contact Info:

kal@pentagrit.com
www.pentagrit.com

Chennai, Tamil Nadu,
India - 600100

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