9.0 Introduction

Genotoxicology is an emergent area of research related to the estimation of potential risk of toxicity of drugs. The fruit fly (D. melanogaster), a commonly occurring organism in nature, is characterized by a short life cycle with distinct developmental stages. It is one of the best genotoxic models because of its easy manipulation and cultivation, production of numerous offsprings and possibility to induce mutations. So it is commonly used as a model organism for many biological processes including toxicity testing. In fact, D. melanogaster has been exploited in testing the toxicity of several drugs [1-4].

*Drosophila* wing somatic mutation and recombination test (SMART; also known as the wing spot test) provides a rapid means to assess the potential of a chemical to induce loss of heterozygosity (LOH) resulting from gene mutation, chromosomal rearrangement, chromosome breakage or chromosome loss. The wing somatic mutation and recombination test has been the test of choice to determine the mutagenic effect of drugs. This test is based on the principle that the loss of heterozygosity and the corresponding expression of the suitable recessive markers, multiple wing hairs and flare-3 can lead to the formation of mutant clone cells in growing up larvae, which are expressed as mutant spots on the wings of adult flies. When a genetic alteration is induced in a mitotically dividing cell of a developing wing disc, it may give rise to a clone(s) of mwh and/or flr3 cells (i.e., a “spot”) visible on the wing surface of the adult fly. The total number of clones induced in a group of chemically treated flies gives the quantitative data reflecting the whole genotoxic activity of a compound. On the other hand, the types of clones can reveal the mutational mechanisms involved in the clone production [5-7]. Therefore, the aim of
the present study was to determine the toxicity effects of CAMPs to *D. melanogaster*. In the case of toxicity testing, numbers of larvae, pupae and hatched adult individuals have been determined together with the phenotype changes in the *Drosophila* organism. Furthermore, the toxicity testing was also done qualitatively by the WING SPOT method. The DNA fragmentation assay allows determination of the amount of DNA that is degraded upon exposure of the fruit flies to genotoxic agents. The flies are exposed for a stipulated time to various concentrations of genotoxic agents followed by isolation of DNA by phenol chloroform method and finally subjecting them to electrophoresis (similar to the principle applied to blood and cell lines). This assay is performed for the comparison of damages observed *in vitro* by the test drugs.

### 9.1 Methodology

#### 9.1.1 Exposure of flies with drugs

Different concentrations of VSL2 & CHX (10µM, 50µM, and 100µM) were each mixed with prepared instant food (Potato flakes, glucose, and yeast). The food was labeled with appropriate concentrations and the duration of exposure. It was then foiled and allowed to set for 2 hours at 25 °C (to avoid fermentation). About 30 flies (Canton) were exposed to each of the concentrations of test drugs. Then, the exposed flies were observed under the microscope for morphological changes (phenotypic changes) in the flies.

#### 9.1.2 DNA fragmentation assay

The flies were exposed to VSL2, CHX (10µM, 50µM, and 100µM) and Benzaldehyde (100µM), transferred to a culture vial and placed in a beaker containing ice in the refrigerator for 30 minutes. After the flies collapsed, they were transferred...
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into a mortar and pestle and crushed by adding 500µl of Solution A (Tris HCL, EDTA, NaCl, SDS). The contents were then transferred into avial and incubated in a water bath set to 70 °C for 30 minutes. DNA was then isolated using the phenol chloroform isoamyl alcohol (PCI) method.

9.1.3 Quantification of DNA using nanodrop

The quality of DNA was checked using nanodrop Spectrophotometer. About 1.5µl of the isolated DNA samples were added to the pedestals of the nanodrop and the results were analyzed. The quality checked DNA samples were subjected to fragmentation assay, to study the damage, by subjecting the samples to 1.5% agarose gel electrophoresis for 45 minutes. The results were documented.

9.1.4 WING SPOT assay

9.1.4.1 Strains

The two D. melanogaster strains used to determine the mutagencity of CAMPs are: the multiple wing hairs strain with the genetic constitution y; mwh j and the flare-3 strain with the genetic constitution, flr3/In (3LR) TM3, Bds. The two wing markers used are the multiple wing hairs (mwh), and the flare-3 (flr3). Mwh is a completely recessive homozygous viable mutation, which is kept in the homozygous condition. It produces multiple trichomes per cell instead of the normally unique trichome. Flr3 is a recessive mutation affecting the shape of wing hairs, producing malformed wing hairs that have the shape of a flare. Canton flies are also used for experiments to determine the genotoxicity of peptide (VSL2).
9.1.4.2 Treatments

To obtain transheterozygous individuals, virgin flr3 females were mated to mwh males, as previously described [8]. Eggs from this cross were collected during 8-h periods in culture bottles containing standard medium (Corn flour, agar, sodium chloride and yeast). The resulting 3-day-old larvae were then transferred to plastic vials with 4.5 g of Drosophila instant medium (Potato flakes, glucose, yeast) prepared with the different concentrations of VSL2 & CHX (10µM, 50µM, 100µM). The food medium was dehydrated and then rehydrated with 10 mL of the different concentrations of the tested compounds. The larvae were exposed to this medium until pupation. Treatment vials were maintained in a culture room at 25°C, 75% humidity and a light/dark daily cycle of 12/12 h. The flies that survived were collected from the treatment vials and were stored in 70% ethanol. Afterwards, their wings were removed and mounted in a sterile clean glass slide using a mountant. The wings were scored at 40x magnification for the presence of spots.

Single mwh spots result from point mutation, recombination or small deletion of the wild type allele. On the other hand, single flr3 spots arise from small deletion of the wild type allele. Twin spots, consisting of both mwh and flr3 sub clones originate exclusively from mitotic recombinations between flr3 and the centromere [9]. A view of the aspect of mutant clones is observed in figure 9.1. In each series, 70 wings (35 individuals) were examined. The scoring of flies and data evaluation was performed following the standard procedures for the wing-spot assay [10,11]. The differences between the frequencies of each type of spot in treated and negative control groups were compared using one way ANOVA.
9.2 Results

9.2.1 Morphological changes

Male and female flies were exposed to VSL2, Ampicillin, Silver nitrate & CHX and the phenotypic changes were observed microscopically. The silver nitrate and CHX treated flies showed discoloration of thorax and enlarged abdomen, whereas the peptide treated flies showed no morphological changes. The observations are summarized in table: 9.1

9.2.2 DNA fragmentation assay

Fragmentation assay was performed on the DNA from control and drugs exposed flies to study the damage and the results obtained were documented. Mild shearing was observed in all concentrations (50 and 100µM) of VSL2 & CHX treated samples,
after 24 hours of exposure. Fragmented DNA was observed in the Benzaldehyde treated samples, which served as the positive control (figure: 9.2).

9.2.3 WING SPOT assay

Table: 9.2 shows that the frequency of distribution of mutant spots on the wings exposed to VSL2, CHX (10, 50,100µM) were minimal, when compared to the control wings. Twin spots that serve as preliminary indications of mitotic recombination of drugs were not seen in CAMPs exposed flies. The absence of statistically significant number of Single flr or mwh spots (both small and large clones) in the flies indicated that there were no occurrences of point mutations (in flr+ or mwh+), chromosomal alterations (e.g., a deletion of flr+ or mwh+) or mitotic recombinations in flies. This clearly showed that the VSL2 (10, 50 &100µM) was not genotoxic under the present experimental conditions at all the three concentrations used. However, the frequencies of spontaneous spots (2 hair & 3 hair) were much lesser than those observed in the control group treated with EMS. The distribution of mwh and flr mutation in the wings is represented in figure 9.3 a&b.
### Table: 9.1 Phenotypic analysis of flies exposed to drugs

<table>
<thead>
<tr>
<th>Sample name &amp; concentration</th>
<th>% of viability</th>
<th>Phenotypic changes observed</th>
<th>Images</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100%</td>
<td>No changes observed</td>
<td><img src="Male" alt="Male" />, <img src="Female" alt="Female" /></td>
</tr>
<tr>
<td>Ampicillin (20µg)</td>
<td>75%</td>
<td>Brackish brown thorax (Male) and enlarged female and stout abdomen</td>
<td><img src="Female" alt="Female" />, <img src="Male" alt="Male" /></td>
</tr>
<tr>
<td>VSL2 (100µM)</td>
<td>100%</td>
<td>No morphological changes were observed</td>
<td><img src="Male" alt="Male" />, <img src="Female" alt="Female" /></td>
</tr>
</tbody>
</table>
### Genotoxicity of CAMPs - *D.melanogaster*

#### Antimicrobial activity of cationic synthetic peptides

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect on Flies</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver nitrate (100µM)</td>
<td>Almost all flies were dead</td>
<td>Discoloration of thorax (both male and female) was observed</td>
</tr>
<tr>
<td>CHX (100µM)</td>
<td>75%</td>
<td>Enlarged abdomen was observed in both female and male</td>
</tr>
</tbody>
</table>
Figure: 9.2 DNA fragmentation assay

Benz-Benzaldehyde, UT-Untreated, L-Ladder
Figure: 9.3 WING SPOT Assay (Distribution of 2 hair and 3 hair in exposed wings)
### Table: 9.2 WING-SPOT test data obtained after the treatment of larvae with different agents

<table>
<thead>
<tr>
<th>Group</th>
<th>VSL2 10µM</th>
<th>VSL2 50µM</th>
<th>VSL2 100µM</th>
<th>CHX 10µM</th>
<th>CHX 50µM</th>
<th>CHX 100µM</th>
<th>EMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>-5.178*</td>
<td>1.508</td>
<td>.014</td>
<td>-9.75</td>
<td>4.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSL2 10µM</td>
<td>-5.836*</td>
<td>1.473</td>
<td>.002</td>
<td>-10.31</td>
<td>-1.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSL2 50µM</td>
<td>-5.109*</td>
<td>1.527</td>
<td>.000</td>
<td>-19.73</td>
<td>-10.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSL2 100µM</td>
<td>-9.259*</td>
<td>1.655</td>
<td>.000</td>
<td>-14.28</td>
<td>-4.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHX 10µM</td>
<td>-4.693*</td>
<td>1.531</td>
<td>.046</td>
<td>-9.34</td>
<td>-0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHX 50µM</td>
<td>-3.765</td>
<td>1.655</td>
<td>.309</td>
<td>-8.79</td>
<td>1.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHX 100µM</td>
<td>-7.431*</td>
<td>1.621</td>
<td>.000</td>
<td>-12.35</td>
<td>-2.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMS</td>
<td>-56.470</td>
<td>1.490</td>
<td>.000</td>
<td>60.99</td>
<td>51.95</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table indicates the frequency of distribution of mutant spots on the wings exposed to VSL2, CHX & EMS and their statistical evaluation.

The mean difference is significant at the .05 level.*
9.3 Discussion

*D. melanogaster* is a suitable biosystem to detect *in vivo* genotoxicity of various agents and wing rest is a qualitative method to determine the mutagenic effect as well. The acute toxic effects of CAMPs on *D. melanogaster* were investigated for detecting the genotoxic potential of peptides. *D. melanogaster* is used for testing the mutagenic effect of drugs, both for mutagenicity [12] and genotoxicity [13]. The wing test is an easy method to detect the *in vivo* genotoxicity. In this context, it must be remembered that the quantification of the recombinagenic activity of a compound is of primary importance for genotoxicity screening [14]. *Drosophila* is considered as a good model system, since over 60% of human disease genes have fly homologues, indicating that the fly response to physiological insults is comparable to humans [15]. This would reinforce the usefulness of the *Drosophila* model as a first tier *in vivo* test for drug toxicity.

The present study indicated that CAMPs (VSL2) did not induce any amount of damage to the DNA at the given concentrations. Canton flies (both males and females) were exposed to varying concentrations of VSL2 and analyzed for phenotypic changes and the quality of the DNA obtained from the exposed flies was checked. Canton strains exposed to different concentrations of VSL2 & CHX did not cause any phenotypic changes, when compared to the positive controls. Abnormalities such as discoloration of thorax, elongation of abdomen and curling of abdomen were not observed in CAMPs treated samples at 24 hours as well as 48 hours of exposure, when analyzed under the stereo zoom microscope. Fragmentation assay of DNA obtained from flies exposed to VSL2, CHX (10, 50 & 100µM) for 24 hours showed mild shearing at higher concentrations. Significant fragments of DNA were observed in the positive control (benzaldehyde) lane at 150bp.
Wing spot assay is an appropriate tool to study cellular events, including mutagenesis, somatic mutation and recombination effects [16]. Through the use of the mwh and the flare markers, point mutations induced in the mwh+ gene can be evaluated [17,18]. The number and size of the mwh clones and a significant number of Single flr or mwh spots (both small and large clones) would allow a quantitative evaluation of the effectiveness of the environmental or genetic “treatments” to induce the loss of the mwh+Y chromosome [12]. Based on the earlier reports, the mwh and flare system is an adequate tool to detect in vivo, the effects of environmentally and genetically induced chromosome loss in higher eukaryotic organisms. Hence, it was used in the present study to ascertain the genotoxicity of CAMPs.

9.4 Conclusion

The in vivo genotoxicity and mutagenicity of the peptides (VSL2) were assessed using D.melanogaster models. The results confirmed that the peptide was not mutagenic and genotoxic in vivo.

9.5 References


