Anthracycline (epirubicin) induced mutation studies in *Drosophila melanogaster*.

Parvathi V, Deepa, Mathangi Ravi, and Solomon F.D. Paul. Department of Human Genetics, Sri Ramachandra University, Porur, Chennai 600116; Email: deepa_305@yahoo.com.

The fruit fly, *Drosophila melanogaster*, is a powerful model genetic organism that has been used since the turn of the previous century to evaluate genotoxins. In this study, the antineoplastic drug Epirubicin was scrutinized for its genotoxic effects in *Drosophila melanogaster*. The end points chosen for this study directly evaluate the genotoxic effects of the drug, which can be extrapolated to humans owing to the homology between the two species. The four concentrations of drug used to estimate genotoxicity in this study include 200µg, 300µg, 400µg, and 500µg. The drug-treated flies were analyzed for any somatic mutations after 48 hour exposure, and F1 analysis was performed to check for germ line mutations. The results of fragmentation assay indicate DNA damage as a result of apoptosis revealing DNA instability when exposed to Epirubicin, which confirms the genotoxic activity of the antineoplastic agent. The wing somatic mutation and recombination test, a one-generation test, makes use of the wing imaginal disc cells in larvae. They are based on the principle that the loss of heterozygosity of suitable recessive markers can lead to the formation of mutant clones of cells that are then expressed as spots on the wings of the adult flies. The two markers employed in this study include multiple wing hair (*mwh*), and flare (*flr3*). Three-day-old larvae, trans-heterozygous for these markers, are treated chronically by oral administration with Epirubicin. After eclosion, the wings of the adult flies were scored for the presence of single and twin spots. The results revealed spots/mutations caused due to different genotoxic events: either mitotic recombination or mutation (deletion, point mutation, or translocation).

Introduction

Genotoxicity Testing

Drosophila melanogaster is a eukaryotic organism linked to the development of the Genetic Toxicology (Alentorn, *et al*., 1995). *Drosophila melanogaster* is a well-established insect model for human diseases and toxicological research due to its well-documented genetics and developmental biology (Buschini, *et al*., 2003).

Chemical carcinogenesis is a multi-step process featuring alteration of genome integrity detected as pivotal gene mutations and chromosome damage, malignant transformation of cells, and ultimately development of cancer after exposure to chemical agents. Because of the association between DNA damage and cancer development, the preclinical safety evaluation paradigm for drugs and chemicals consists of assessing their genotoxicity, *i.e*., their potential to cause DNA damage, and carcinogenicity, the latter comprising the ability to produce tumors in animals upon long-term exposure. The standard genotoxicity testing battery consists of (a) a bacterial gene mutation assay, (b) an *in vitro* mammalian mutation and/or chromosome damage assay, and (c) an *in vivo* chromosome damage assay. The test battery allows for relatively simple, accurate, and economical hazard identification associated with exposure to chemicals, namely a potential to cause DNA damage resulting in mutations at the gene and chromosome levels (Henderson, 2004).

Obtaining positive results in *in vitro* genotoxicity tests is not uncommon. Follow-up studies to determine the biological relevance of positive genotoxicity results are costly, time consuming, and utilize animals. More efficient methods, especially for identifying a putative mode of action like an indirect mechanism of genotoxicity (where DNA molecules are not the initial primary targets), would greatly improve the risk assessment for genotoxins.

Anticancer drugs are the most common genotoxic agents studied on *Drosophila*. There are three goals associated with the use of the most commonly-used anticancer agents.

1. DNA damage
2. DNA synthesis inhibition
3. Termination of mitosis

The majority of drugs currently on the market are not specific, which leads to the many common side effects associated with cancer chemotherapy including genotoxicity.

Antineoplastic drugs generally have a narrow therapeutic index and are delivered at doses close to toxicity. Endogenous factors affecting drug response involve genetic predisposition, disease states, and other factors that influence absorption, distribution, activation, and detoxification of the drug. In particular, the pharmacological activity of any genotoxic anticancer drug is strictly dependent on tumour-specific physiological/biochemical conditions, such as a functional respiratory chain and the presence/absence of drug metabolizing enzymes (Hu *et al*., 2004).

Broadly classifying anticancer based upon their genotoxic ability as:

Direct-acting genotoxins (where DNA is the initial primary target) includes the DNA crosslinking agents, mitomycin C (MMC) and cisplatin (CIS), and an alkylating agent, methyl methanesulfonate (MMS).

Indirect-acting genotoxins includes hydroxyurea (HU), a ribonucleotide reductase inhibitor, taxol (TXL), a microtubule inhibitor, etoposide (ETOP), a DNA topoisomerase II inhibitor, doxorubicin and its analogs (antitumor antibiotics) are microtubule inhibitor and DNA topoisomerase II inhibitor (Lehmanna *et al*., 2003).

The toxicity of most of these drugs is associated with their enzymatic conversion to toxic metabolites (Hu *et al*., 2004).

Many chemicals/drugs are investigated for their genotoxic properties through the wing spot test (SMART) in *Drosophila melanogaster*, which is the gold standard for genotoxicity assessment. Almost 300 chemicals have been evaluated in the wing spot test. These include various antineoplastic drugs, small alkylating agents, bulky adduct-forming compounds, crosslinking agents, clastogenic intercalating and nonintercalating topoisomerase inhibitors, antimetabolites that disturb nucleotide pools, DNA synthesis inhibitors, and nucleoside analogs. The genotoxic effects of these representative compounds are, in general, strong and dose related.

The wing spot test is also well suited for testing complex mixtures, such as airborne aerosol extracts, plant extracts, beverages such as coffee, herbal teas and wine, as well as tannic acid. Although both caffeine and tannic acid were determined to be genotoxic in the wing SMART, they also both showed antigenotoxic activity in combination with several known strong mutagens. Thus, the SMART can also be used to assess the effects of nongenotoxic chemicals, which may act as modulators when combined with genotoxins. Such approaches identified the protective effects of chlorophyllin, ascorbic acid, novo-biocin, antipyretic analgesics, sodium thiosulfate, epigallocatechin, and tannic acid.

The applicability of the SMART to studies of antigenotoxic effects is reinforced by the demonstration that some modulators that decrease the incidence of mutational effects are equally able to increase the occurrence of mitotic recombination. This means that modulating agents must be

evaluated not only in terms of their action on mutagenic events (point and chromosomal mutations) but also in relation to their effects on mitotic recombination.

The broad spectrum of genetic end points monitored as LOH in somatic cells—including point mutations, deletions, unbalanced half-translocations, mitotic recombination, chromosome loss, and nondisjunction—makes the wing SMART a most versatile *in vivo* test. It is also technically simple, quick and inexpensive to do, and allows flexibility in the choice of both route of administration of the test chemical and time of exposure. In addition, it allows analysis of an extensive sample size, because microscopic inspection covers approx 50,000 cells per fly. Moreover, statistical procedures applicable to the SMART are well established, and different statistical tests can be applied according to the peculiarities that specific sets of data may show (Siddique *et al*., 2005).

Doxorubicin and Its Analogs – Anti Tumor Antibiotics

The genotoxic effects of the anthracycline doxorubicin and two of its analogues, epirubicin and pirarubicin, were studied using the wing Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster*. These compounds are classified as topoisomerase II poisons and act by stabilizing topoisomerase II-cleaved DNA complex. By using SMART test it was possible to estimate the quantitative and qualitative genotoxic effects of these compounds, comparing the wing spot frequencies in marker- and balancer-heterozygous flies. On exposure to doxorubicin and its analogs, it was found that all the three compounds induce high frequency of spots related to homologous recombination, which is the major event responsible for their genetic toxicity. Pirarubicin was the most genotoxic anthracycline, inducing ~21 times more genetic lesions than doxorubicin, probably due to the presence of a second sugar ring in the amino sugar moiety in its chemical structure. Although the only difference between epirubicin and doxorubicin is the steric position of the amino sugar 4'-OH in the molecule, epirubicin is approximately 1.6 times as genotoxic as doxorubicin.

Genotoxic Potential Attributed to the Mechanism of Action

This experimental study indicates that all three anthracyclines analyzed were capable of damaging the DNA of *D. melanogaster* somatic cells. About 91–100% of the genetic toxicity observed for all drugs was associated with homologous mitotic recombination.

The high recombinational activity of these drugs can be attributed to their mechanism of action.

- The block of DNA replication due to the attachment of topoisomerase II in both strands, leading to non-homologous recombination repair.
- The unfavorable DNA topology related to the inhibition of topoisomerase II function after replication, leading to the occurrence of homologous and non-homologous recombination events.

Doxorubicin and pirarubicin were identified as inducers of both mutagenic and recombinagenic events, whereas epirubicin was considered as a pure recombinagenic compound.

Genotoxic Potential Attributed to the Chemical Structure

Epirubicin or 4'epidoxorubicin differs from doxorubicin in the steric position of the amino sugar 4'OH group. This unique difference confers a genotoxic activity ~56% when compared to doxorubicin. Pirarubicin or 4'-tetrahydropyranyl-doxorubicin is a disaccharide analogue to doxorubicin, which presented a superior genotoxic as substitutions at the 4'-position enhances the biological activity of anthracyclines (Ziegelbauerb and Aubrechta, 2009).

Materials and Methods

Canton-S flies were bred for two generations on corn meal agar. Drug exposure studies were performed in Carolina Formula 4 instant medium. Various concentrations of epirubicin (control, 200µg, 300µg, 400µg, and 500µg) were each mixed with 3g of instant food. Test and duplicates were set up for each concentration. The food was labeled with appropriate drug concentrations, and foiled, and allowed to set for two hours. For phenotypic change analysis, 100 male and 100 female flies were exposed to each of the drug concentrations and analyzed microscopically after 48 hours of exposure. F1 generation analysis was performed on emergence, microscopically. 100 flies, both males and females, were exposed to each of the four concentrations. DNA isolation was performed after 48 hours of exposure, which was followed by fragmentation assay. 100 trans-heterozygous larvae of the *mwh*/*flr3* cross were exposed to each of the four concentrations and adult flies were allowed to emerge. The emerged flies of the *mwh*/*flr3* cross were etherized using 500µl ether. The wings of the flies were dissected and placed on a glass slide. The wings were observed microscopically for any mutations.

Results

The results are tabulated in Table 1, Images 1, 2, 3, 4, 5, 6, 7, 8, 9.

Discussion

The present experimental study indicates that the anthracycline used (epirubicin) was capable of damaging the DNA of *D. melanogaster*. The various concentrations of epirubicin used (200µg, 300µg, 400µg, 500µg) were found to be genotoxic as it induced toxic DNA damage in both the somatic cells and germ cells of the exposed flies.

Epirubicin being classified as topoisomerase II poisons act by stabilizing a topoisomerase II-cleaved DNA complex and enhancing DNA double strand breaks. The genetic toxicity observed for the drug is associated with homologous mitotic recombination as a result of which significant increments are observed in the different end points chosen.

All four concentrations used in this study demonstrated 100% viability. Phenotypic changes revealed somatic mutations, which were observed in both the parental and F1 generations. With increase in the concentration of the drug, more somatic mutations were observed, indicating dose dependent expression of mutations. Abdominal curling and change in the abdominal color was observed in most concentrations and could be attributed to the feeding of the flies on the drug, which causes mutations in the gut cells, which was observed only in the parental generation suggesting that the mutation could be somatic. The color change in the thorax was seen in both the parents and the F1 generation, indicating germ line mutations that were carried to the F1 offspring and expressed. The change in the eye color though not consistent in all concentrations indicates the toxicity of the drug in the omatidium.

DNA fragmentation assay was performed with the adult flies exposed to epirubicin after 48 hours of feeding. The results of the fragmentation assay confirm the genotoxic effects of the drug at the DNA level/molecular level. Series of fragments of size ranging from 3,000 to 1,000 base pairs and significant dense shearing below 1,000 base pairs pose a threat to the stability of DNA when exposed to epirubicin.

Table 1.

END POINTS → / CONCENTRATION ↓	PHENOTYPIC CHANGES	F1 GENERATION	DNA FRAGMENTATION ASSAY	SMART
200 µg	Abdomen curling observed in males and females.	Flies appeared small, round and swollen. Curling was observed in both sexes (100%). Wings appeared small and rounded. Orange discoloration in thorax.	Single intact band at 1kb. A single fragment at 3,000 base pairs. A series of 2 fragments between 2,000 and 1,000 base pairs.	mwh/flr3 mutation. Single spots
300 µg	Orange discoloration in thorax and eyes.	Color change in thorax.	Single intact band at 1kb. A single fragment at 3,000 base pairs. A series of 2 fragments between 2,000 and 1,000 base pairs. Significant dense shearing was observed below 1,000 base pairs.	mwh/flr3 mutation. Single spots
400 µg	Orange discoloration in eyes, thorax and abdomen.100% abdominal curling in males.	Deep orange discoloration in thorax (100%).	Single intact band at 1kb. Series of 2 fragments between 2,000 and 1,000 base pairs- in pellet. A single fragment was observed at 1,000 base pair- in supernatant. Significant shearing was observed below 1,000 base pairs.	mwh/flr3 mutation. Single spots
500 µg	Orange discoloration in eyes, thorax and abdomen.100% abdominal curling in males.	Deep orange discoloration in thorax (100%).	Single intact band at 1kb. Series of 2 fragments was observed below 1,000 base pairs- in pellet. A single fragment was observed at 1,000 base pairs- in supernatant. Significant shearing was observed below 1,000 base pairs.	mwh/flr3 mutation. Single spots

The wing spot test (somatic mutation and recombination test – SMART) in *D. melanogaster* has been shown to be an efficient short term bioassay for the detection of genotoxic or antigenotoxic activity of pure compounds or complex mixtures. It is capable of activating enzymatically, promutagens and procarcinogens, for a quantitative determination of the recombinagenic potential of genotoxic agents.

In this study, SMART was used to investigate the genotoxicity of epirubicin, because of their beneficial effects in treatment for cancers. With SMART test it was possible to estimate the quantitative and qualitative genotoxic effects of these compounds, by comparing the wing spot

frequencies in marker- and balancer-heterozygous flies. The results obtained indicate that epirubicin induced a high frequency of spots (both single spots and *mwh*/*flr3* spots/mutations) related to homologous recombination, chromosomal alterations (mainly deletions), point mutations, or mitotic recombination, which is the major event responsible for their genetic toxicity.

Figure 1. Exposure to epirubicin: 200 µg. Abdominal curling (both sexes) (Left: male; Right, male and female).

Figure 2. Exposure to epirubicin: 300 µg. Orange discoloration in eye and thorax in both sexes (Left: female; Right, male).

Figure 3. Exposure to epirubicin: 300 µg. Orange discoloration in eye, thorax, and abdomen.

Figure 4. Exposure to epirubicin: 500 µg. Orange discoloration in eye, thorax, and abdomen in both sexes (Left, male; Right, female).

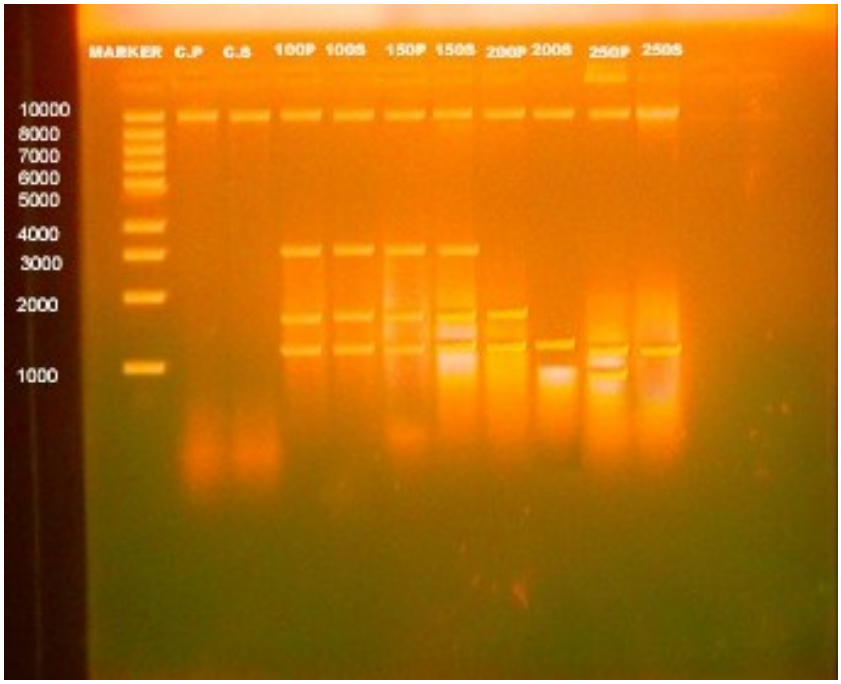

Figure 5. Agarose gel electrophoresis showing DNA fragmentation.

Thus, with the above end points, we are able to conclude the toxicity of epirubicin on both somatic and germ cells. Epirubicin being the synthetic analog of doxorubicin is widely used in the treatment of a variety of malignancies and is known for its reduced cardiotoxicity. The toxicity of epirubicin has also been demonstrated in cultured mammalian cells through SCEs and was found to exhibit dose dependent behavior.

The only reason behind the effective genotoxicity of epirubicin in comparison with doxorubicin arises at the differences in the steric position of the amino sugar 4'-OH in the molecule

(which enhances lipid permeability), thus making it approximately 1.6 times more genotoxic than doxorubicin.

In spite of the similarity concerning therapeutical effectiveness, epirubicin showed increased genotoxic effects expressed as loss of heterozygosity (LOH) in somatic cells of *D. melanogaster* especially in terms of homologous recombination.

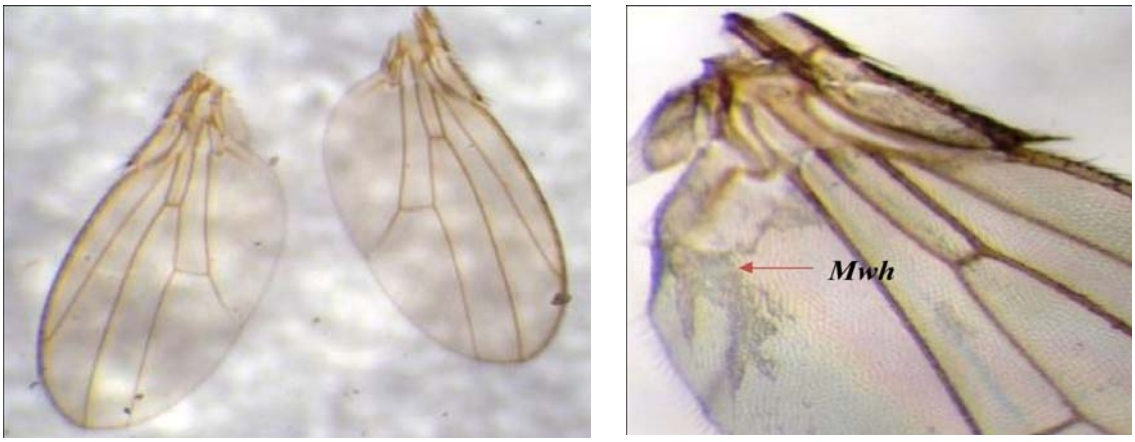

Figure 6. SMART analysis. Control (Left); Exposure to epirubicin: 200 µg (Right). Wing showing *mwh* pattern.

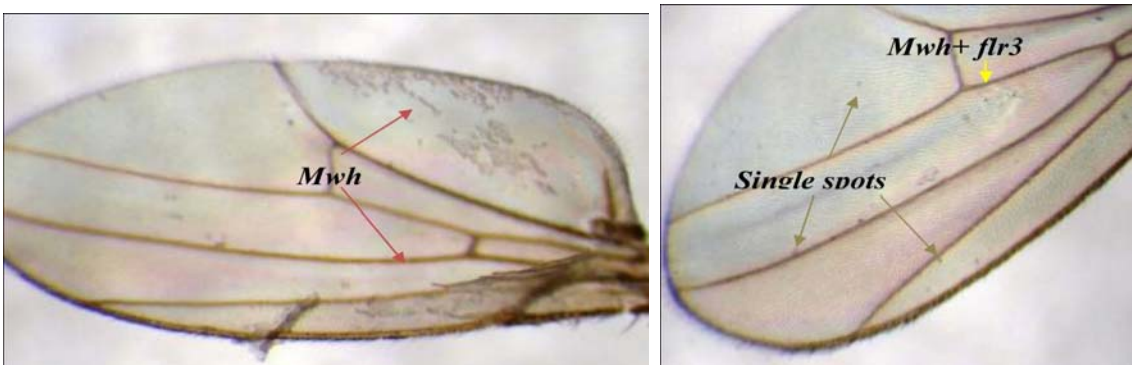

Figure 7. Exposure to epirubicin: 300 µg (SMART analysis).
Left: *mwh* pattern; Right: mwh + *flr3* pattern along with single spots.

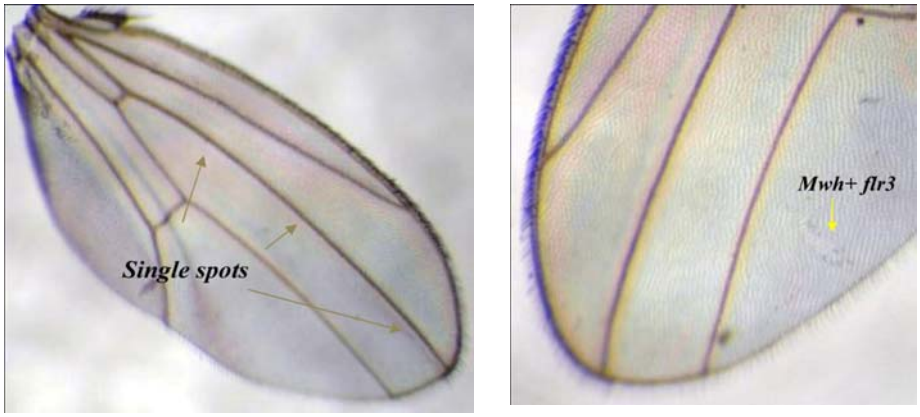

Figure 8. Exposure to epirubicin: 400 µg (SMART analysis).
Left: single spots; Right, *mwh* + *flr3*.

Figure 9. Exposure to epirubicin: 500 µg (*mwh* + *flr3* pattern) SMART analysis.

References: Alentorn, M.B., N. Xamena, A. Creus, and R. Marcos 1995, Mutation Research 341: 161-167; Buschini, A., P. Polil, and C. Rossi 2003, Mutagenesis 18(1): 25–36; Henderson, Daryl S., 2004, Drosophila *Cytogenetics Protocols*. Humana Press, Totowa, New Jersey; Hu, T., D.P. Gibson, G.J. Carr, S.M. Torontali, J.P. Tiesman, and J.G. Chaney 2004, Mutat. Res. 549(1-2): 5-27; Lehmanna, M., A. Franco, and H. Rodrigues 2003, Mutation Research 539: 167–175; Siddique, H.R., D.K. Chowdhuri, Saxena, and A. Dhawan 2005, Mutagenesis 20(4): 285-290; Ziegelbauerb, H.E., and J. Aubrechta 2009, Toxicology Letters 186: 36–44.

Testing gene function in fly head formation using transgenic RNAi.

Jo, Soo Hyun[1], Beth Krauss[1,#], and Francesca Pignoni[2,#]. [1]Upper School, Manlius Pebble Hill, Dewitt, NY; [2]Department of Ophthalmology, Upstate Medical University, Syracuse, NY; [#]co-corresponding authors; bkrauss@mph.net (315-446-2452); pignonif@upstate.edu (315-464-8122).

Abstract and Introduction

In this project, we investigated the potential role of 31 genes in eye/head formation in *D. melanogaster*. We tested the function of each gene by using RNAi as a means of knocking-down a single gene at the time within the progenitor tissue that gives rise to the adult head (eye-antennal imaginal disc). The eye/head phenotype was evaluated first at the adult stage and then during development in the larval stage. The range of phenotypes observed included eyeless, reduced eye, bar eye and rough eye, headless, reduced head, and enlarged head. Specific phenotypic categories were also analyzed at the level of imaginal discs in order to understand the nature of the defect better. The mutant phenotypes induced by RNAi-mediated silencing of *Ccn* or *KCNQ* (reduced eye), garz (disorganized neurons), alien or Trn (reduced head), and syx1A, *dock*, *Gdi*, or *drosha* (headless) suggest that these genes play significant roles in the development of the head and/or eye.