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Fundamental and Molecular Mechanisms of Mutagenesis

Metabolic activation of four drugs in the eye mosaic assay measuring principally mitotic recombination in *Drosophila melanogaster*: differences in strain susceptibility and route of exposure

Rosario Rodriguez-Arnaiz *, Judith Hernández Aranda

Laboratorio de Genética, Facultad de Ciencias, Coyoacán, 04510 México, D.F., Mexico

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Abstract

One mycotoxin and three therapeutic drugs widely used in developing countries were examined for genotoxic activity by means of the w/w + somatic recombination assay. Streptozotocin (SZ), an antibiotic antineoplastic agent, gave a frequency of light spots almost one order of magnitude higher than those obtained with the carcinogen mycotoxin sterigmatocystin (STC), the antiprotozoal and antimicrobial metronidazole (MNZ), and the antifungal griseofulvin (GF). Thus the order of response was SZ > STC > MNZ > GF. Chronic treatment turned out to be the better route of exposure for these genotoxins when compared with surface treatment. The performance of the insecticide-resistant strain Hikone-R was better than that of the wild genotype LS (Leiden Standard). The positive test results obtained with all four chemicals showed that the P450 system of Drosophila is capable of metabolizing these genotoxins into electrophilic intermediates.

Key words: Drosophila melanogaster; Eye mitotic recombination assay; Metabolic activation; Streptozotocin (SZ); Metronidazole (MNZ); Griseofulvin (GF); Sterigmatocystin (STC)

1. Introduction

Metabolic activation of xenobiotics in Drosophila is under strict genetic control, and variability in response to genotoxins is therefore observed with different genotypes (Zijlstra et al., 1984). In a pilot study Vogel et al. (1991) reported the development of new tester strains; calibration studies of these stocks with different reference mutagens suggest the non-existence of just one super-strain for activation of several procarcinogens such as benzo[*a*]pyrene, benz[*a*]anthracene, 9,10-dimethylanthracene, monocrotaline and *N*-nitrosodimethylamine. The relative susceptibility of the various strains against the set of test chemicals was labelled as 'low', 'inter-

* Corresponding author.

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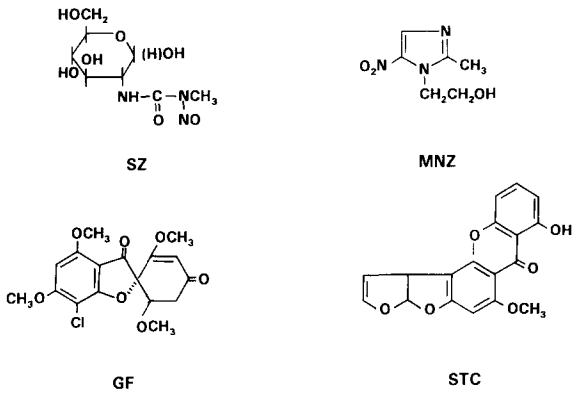

Fig. 1. Structural formulas of streptozotocin (SZ), metronidazole (MNZ), griseofulvin (GF) and sterigmatocystin (STC).

mediate' or 'high'; this ranking system against the background of intra-strain variability has a somewhat arbitrary character, its solely purpose is to illustrate the apparent cross-resistance/susceptibility existing within *Drosophila melanogaster*. In this context the performance of the wild stock Leiden Standard (LS) was 'low' while that of the insecticide-resistant strain Hikone-R was 'high' (Rodriguez-Arnaiz et al., unpublished).

A comparison is made between a standard Leiden wild-type strain and the newer HK strain to evaluate three drugs and one mycotoxin in the w/w+ eye mosaic system of Drosophila. The compounds (see Fig. 1 for structural formulas) are carcinogenic after metabolic activation. The drugs are widely used as therapeutics in the developing countries; they may therefore constitute a hazard to humans raising questions of risk versus benefit.

Streptozotocin (SZ), originally discovered as an antibiotic (Goodman et al., 1990), is used in humans in the treatment of pancreatic carcinoma and malignant carcinoid tumors, as well as against other human malignancies, and in animals for the induction of experimental diabetes. This compound has a methylnitrosourea attached to the 2 carbon of glucose (Herr et al., 1967). During decomposition of SZ highly reactive carbonium ions are formed which cause alkylation of DNA bases, forming O^6-alkylguanine residues (Bennet and Pegg, 1981; Le Doux et al., 1986).

Metronidazole (MNZ) has an extremely broad spectrum of antiprotozoal and antimicrobial activity; it is clinically effective in trichomoniasis, amebiasis and giardiasis and other infections caused by anaerobic bacteria. The nitro group of metronidazole accepts electrons from electron-transporting proteins, the source of electrons for the reduction may be a number of endogenous reduced substrates such as NADPH. The reduced forms of the drug lead to the formation of cytotoxic products that may react with DNA (Goodman et al., 1990). In mammalian cells the principal reductases seem to be cytochrome P450 dependent (Binderup, 1991). In *Drosophila melanogaster* MNZ gave inconclusive results in the sex-linked recessive lethal test (Lee et al., 1983). In *Saccharomyces cereviseae* it produces mitotic recombination and gene conversion, and the drug is carcinogenic in rats and mice (Binderup, 1991).

Griseofulvin (GF) is fungistatic for various species of Microsporum, Epidermophyton and Tricophyton. GF produces multinucleate cells as it inhibits fungal mitosis (Goodman et al., 1990). The antibiotic fungicide interacts with cellular structures involved in chromosome segregation, probably binding to a microtubule-associated protein (Roobol et al., 1977). It induces changes in mammalian and plant cells in vitro (Larizza et al., 1974; Lo Schiavo et al., 1980; De Carli and Larizza, 1988) and also has aneugenic activity in female mouse germ cells (Marchetti et al., 1992; Tiveron et al., 1992).

Sterigmatocystin (STC) is a carcinogenic mycotoxin produced by fungal species of Aspergillus, Bipolaris and Penicillium genera (Schroeder and Kelton, 1975). It induces hepatocellular carcinomas after oral or intraperitoneal administration as well as squamous cell carcinomas after repeated application to the skin (Dickens et al., 1966). Because of its structural homology with aflatoxin B1, STC is of interest as a model compound in studies of the mechanism of carcinogenesis (Black et al., 1992). The DNA lesion induced by STC has been isolated and characterized (Essigmann et al., 1979).

The aim of this study was the analysis of the ability of these compounds to induce X-chromosome recombination in somatic cells of

Drosophila melanogaster, using the w/w + eye mosaic test (Vogel and Zijlstra, 1987). In the study two tester strains, one with 'low' and another with 'high' bioactivation performance, and two routes of exposure (Vogel and Nivard, 1993) were used.

2. Materials and methods

2.1. Chemicals and treatments

The test compounds were obtained from Sigma Chemicals, Saint Louis, MO, USA.

The two routes of exposure used were: chronic and surface treatment.

For chronic treatments 25 pairs of flies were permitted to lay eggs for 3 days on food medium supplemented by the test substance, which was first dissolved in a mixture of 3 parts ethanol: 1 part Tween 80, yielding in the medium a final concentration of 3% ethanol and 1% Tween 80.

For surface treatment flies were kept in bottles for 48 h, the parental flies were then discarded and 6 h later 1 ml of the test substance was dropped and uniformly distributed on the surface of the food.

2.2. Drosophila strains

Two Drosophila stocks were used: one wild-type strain, namely Leiden Standard (LS), and one insecticide-resistant strain, Hikone-R (HK), chosen on the basis of their similar spontaneous frequencies of light spots. White females were mated with yellow or wild males, depending on the strain (Vogel et al., 1991). The cytochrome P450 contents in both stocks are very similar (Zijlstra et al., 1984).

2.3. Somatic assay

The w/w + assay (Vogel and Zijlstra, 1987; Vogel et al., 1991) was used. Newly hatched females were transferred to fresh medium and scored 1–5 days later. The scoring of females was carried out in a liquid consisting of 90 parts ethanol, 1 part Tween 80 and 9 parts water. The eyes of the females were inspected under a dissecting microscope at a magnification of 120 × and with artificial illumination of an optical fiber.

Table 1
Induction of light spots by streptozotocin (SZ) in w/w + female larvae

Concentration [mM]	Stock [a]	Treatment [b]	Eyes tested	Spots per 100 eyes			Activity
				S	L	T	
Control	LS	CH	350	8.28	1.71	10.0	
0.1			596	26.51	2.51	29.02	+
0.5			540	60.92	8.33	69.25	+
1.0			80	256.25	93.75	350.00	+
Control		SUR	520	5.57	1.73	7.3	
0.1			408	8.82	0.49	9.3	
0.5			552	16.66	4.71	21.37	+
1.0			418	26.31	5.02	31.33	+
Control	HK	CH	440	7.95	3.18	11.13	
0.1			562	23.20	8.00	31.31	+
0.5			386	53.10	13.98	67.09	+
1.0			90	222.22	102.22	324.44	+
Control		SUR	650	4.77	1.38	6.15	
0.1			578	9.34	3.29	12.63	+
0.5			496	9.67	4.03	13.70	+
1.0			484	17.97	8.06	26.03	+

[a] LS, Leiden Standard, HK, Hikone-R.
[b] CH, chronic; SUR, surface.

Classification of mosaic light spots was done on the basis of small spots (2–4 ommatidia affected), large spots (> 4 ommatidia affected) and total spots. Spots separated from each other by at least four non-mutated ommatidia are considered independent events (Vogel and Nivard, 1993).

Two separate experiments were run for each compound and treatment. Different doses were tested. The number of eyes scored varied between 400–800 for treated series and between 400 and 1000 for concurrent controls.

2.4. Statistical analysis

Statistical evaluation of the data was done by the chi-square for proportions (Frei and Würgler, 1988).

3. Results and discussion

The w/w + eye mosaic assay detects genetic alterations in the DNA of somatic cells of Drosophila treated larvae. The expression of white is mainly due to loss of heterozygosity which is the basic principle of the w/w + system (Vogel and Nivard, 1993). Genetic alterations causing this event can be due to deletions, chromosome loss, reciprocal and non-reciprocal (gene conversion) mitotic recombination. From a quantitative point of view reciprocal mitotic recombination between the two X-chromosomes seems to be the major event measured by the test (Vogel and Szakmary, 1990).

We have used two different strains of this system because it has been demonstrated that cytochrome P450 isoenzymes have a crucial role

Table 2
Induction of light spots by metronidazole (MNZ) in w/w + female larvae of two different genotypes

Concentration [mM]	Stock [a]	Treatment [b]	Eyes tested	Spots per 100 eyes			Activity
				S	L	T	
Control	LS	CH	1088	6.25	2.11	8.36	
0.1			598	8.52	2.00	10.52	
0.5			608	8.71	2.63	11.34	
1.0			364	10.16	2.74	12.90	+
5.0			634	16.08	3.15	19.24	+
10.0			450	12.0	1.77	13.77	+
20.0			500	8.4	2.20	10.60	
Control		SUR	758	8.57	1.31	9.88	
1.0			266	9.39	2.25	11.64	
5.0			266	12.03	2.63	14.66	
10.0			252	14.68	4.76	19.44	+
Control	HK	CH	656	6.10	2.28	8.38	
0.1			802	7.48	4.36	11.84	$+^{w}$
0.5			706	10.20	3.68	13.88	+
1.0			778	13.23	2.95	16.19	+
5.0			778	14.52	6.29	20.82	+
10.0			448	15.84	3.57	19.41	+
20.0			290	16.90	1.72	18.62	
Control		SUR	626	5.59	2.55	8.14	
1.0			560	6.25	3.39	9.64	
5.0			432	5.32	3.00	8.32	
10.0			494	8.50	1.82	10.32	

[a] LS, Leiden Standard; HK, Hikone-R.
[b] CH, chronic; SUR, surface.

Table 3
Induction of light spots by griseofulvin (GF) in w/w + female larvae of two different genotypes

Concentration [mM]	Stock [a]	Treatment [b]	Eyes tested	Spots per 100 eyes			Activity
				S	L	T	
Control	LS	CH	300	8.33	1.00	9.33	
0.1			536	11.94	3.54	15.48	+
0.25			484	15.49	4.98	20.45	+
Control		SUR	370	7.02	2.43	9.45	
0.05			490	7.95	2.05	9.99	
0.10			446	8.74	2.01	10.76	
0.25			444	9.45	1.80	11.25	
Control	HK	CH	704	8.52	2.13	10.65	
0.1			656	12.50	2.59	15.09	+[w]
0.25			286	14.33	2.79	17.13	+
0.50			542	13.28	5.16	18.45	+
Control		SUR	430	5.81	1.86	7.67	
0.05			578	8.47	1.04	9.51	
0.10			488	8.81	1.02	9.83	
0.25			514	7.97	2.33	10.31	

[a] LS, Leiden Standard; HK, Hikone-R.
[b] CH, chronic; SUR, surface.

in the oxidation of a wide variety of xenobiotics including procarcinogens in various species of animals (Guengerich, 1990).

SZ, MNZ, GF and STC were readily picked up in the present study (Tables 1–4).

SZ gave a clear dose-response effect, with an activity about one order of magnitude higher after chronic exposure compared to surface treatment. SZ induces alkylation of DNA followed by DNA excision repair which induces DNA strand breaks and activation of the nuclear enzyme poly-(ADP-ribose) synthetase (Yamamoto et al., 1981).

MNZ was positive in LS at higher doses after both chronic and surface treatments although under the former condition higher doses were toxic and delayed larval development. In HK-R the antiprotozoal chemical was clearly positive at all concentrations at chronic treatment but negative after surface exposure. The reduced MNZ causes a loss of the helical structure of DNA, strand breakage and impairment of its function. The two principal metabolites of metronidazole result from

Table 4
Induction of light spots by sterigmatocystin (STC) in w/w + female larvae of two different genotypes

Concentration [mM]	Stock [a]	Treatment [b]	Eyes tested	Spots per 100 eyes			Activity
				S	L	T	
Control	LS	CH	400	8.00	3.50	11.50	
2.5			480	10.00	2.08	12.08	
5.0			480	7.91	2.29	10.20	
10.0			400	9.25	2.50	11.75	
Control	HK	CH	400	9.25	4.50	13.75	
2.5			400	12.75	6.00	18.75	
5.0			400	13.25	6.50	19.75	+[w]
10.0			400	21.50	12.75	34.35	+

[a] LS, Leiden Standard; HK, Hikone-R.
[b] CH, chronic.

oxidation of its side chains (Goodman et al., 1990). The essential reductases seem to be cytochrome P450 and its flavin-containing reductase NADPH cytochrome *c* reductase (Binderup, 1991).

The aneugen and spindle poison griseofulvin was very cytotoxic to the flies, causing irregularities of the eye structure, and reduction in the size of the eye, which made it impossible to test the substance at higher doses. GF was active in both strains after chronic exposure. The compound is known to be oxidized by cytochrome P450 (De Matteis et al., 1991).

STC was only tested in chronic treatment, the trivial reason being the limited amount of chemical available. Interestingly it was only active in the HK genotype suggesting a requirement for bioactivation not present in the LS genotype. In *Saccharomyces cereviseae* sterigmatocystin has been shown to be converted to a mutagenic metabolite (STC epoxide) by cytochrome CYP2B1 (Black et al., 1992).

The results of this study confirm earlier findings that chronic exposure should be used for stable promutagens, to assure continous feeding during all three instar larvae stages (Vogel and Nivard, 1993). Due to the lack of sufficient quantities of the carcinogens reaching the 24 h old larvae, surface treatment was less effective.

In conclusion, this study demonstrates the ability of drug metabolizing enzymes of *Drosophila melanogaster* to activate chemical carcinogens mediated by cytochrome P450 isoenzymes. It is further concluded that the w/w + system represents an attractive short-term test system that does not require the addition of exogenous metabolic activation to obtain a positive response by pro-genotoxins, the usefulness of the test for environmental compounds is its ability to detect them at very low doses.

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