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STUDIES ON THE GENOTOXICITY OF TWO ANTIPROTOZOAL AND ANTIBACTERIAL AGENTS IN SOMATIC AND GERM LINE CELLS OF *Drosophila*

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ABSTRACT

Two antiprotozoal and antibacterial compounds, metronidazole and furazolidone, were tested for their genotoxic effects in somatic and male germ line cells of *Drosophila melanogaster* following chronic exposures of 2nd. and 3rd. instar larvae. Larvae carrying the recessive genetic markers *mwh* and *flr*^{3} in a high bioactive background were tested for genotoxic effects in the somatic cells (wing primordia). To study the genotoxic effects of these compounds in the male germ line cells, the sex-linked recessive lethal test was performed using the *Basc* and Oregon-R strains. The results show that metronidazole is only genotoxic at the highest testable concentration both in the somatic and germ line cells whereas furazolidone is genotoxic even at lower concentrations.

RESUMEN

Fueron probados dos compuestos antiprotozoarios y antibacterianos, metronidazol y furazolidona, en sus efectos genotóxicos en células de las líneas germinal y somática de *Drosophila melanogaster* después de exposiciones crónicas de los estadios larvarios 2o. y 3o. Se probaron larvas con antecedentes de elevada bioactivación y portadoras de los marcadores *mwh* y *flr*^{3} para reconocer los efectos genotóxicos en las células somáticas (primordios de las alas). Con el objeto de estudiar los efectos genotóxicos de estos compuestos en las células germinales masculinas, se realizó la prueba de letales recesivos ligados al sexo utilizando las líneas *Basc* y Oregon-R. Los resultados mostraron que el metronidazol es solamente genotóxico en las concentraciones más altas que se pueden probar tanto en las líneas celulares somáticas como en las germinales mientras que la furazolidona es genotóxica aún a las concentraciones más bajas.

INTRODUCTION

Metronidazole (CAS No. 443-48-1) and furazolidone (CAS No. 67-45-8) are antiprotozoal and antibacterial agents used effectively for the treatment of gastrointestinal and vaginal infections. They also have ample use in veterinary and poultry medicines (Strecher 1968, Siegmund 1979). Metronidazole is carcinogenic in mice following oral administration. It increases the incidence of lung tumours and malignant lymphomas as well as mammary fibroadenomas (IARC 1977). There was, however, been no evidence of cancer induction in humans following metronidazole administration (IARC 1987). No data are available on the carcinogenicity of furazolidone in man and experimental animals. A summary of results obtained with respect to the genotoxicity of these compounds in different test systems is given in **table I**. The present communication describes the results obtained on the genotoxicity of metronidazole and furazolidone in somatic and germ line cells of *Drosophila melanogaster* following chronic larval feeding.

MATERIAL AND METHODS

Larvae to be treated for the study of genotoxicity of the two compounds in the somatic cells (wing promordia) of *Drosophila* came from the cross of two high bioactivation strains: *ORR*; *mwh* females and *ORR*; *flr*^{3}/*TM3*, *Ser* males (Frölich and Würgler 1990a, b). The high bioactivation strains carry chromosomes 1 and 2 from a DDT resistant Oregon-R (R) line and have high constitutive levels of cytochromes P-450 (Hällström and Blanck 1985, Frölich and Würgler 1989). The recessive genetic markers *mwh* (multiple wing hairs, 3-0.3) and *flr*^{3} (flare, 3-38.8) are expressed as multiple trichomes and short and thick trichomes, respectively, in an otherwise

TABLE I. SUMMARY OF RESULTS ON THE GENOTOXICITY OF METRONIDAZOLE AND FURAZOLIDONE IN DIFFERENT TEST SYSTEMS

Test organisms	Test type	Compound tested	Result*	Reference
Klebsiella pneumoniae	Mutation	Metronidazole	+	Voogd *et al.* (1974)
Salmonella typhimurium	Mutation	Metronidazole	+	McCann *et al.* (1975)
				Dayan *et al.* (1982)
				Xu *et al.* (1984)
			-	Dobias (1980)
				Cantelli-Forti *et al.* (1983)
		Furazolidone	+/w	Byeon *et al.* (1976)
				Ebringer and Bencova (1980)
			-	Ohta *et al.* (1980)
Escherichia coli	Mutation	Metronidazole	+	Chessin *et al.* (1978)
				Mohn *et al.* (1979)
		Furazolidone	+	Klemencic and Wang (1978)
				Lu *et al.* (1979)
	DNA repair	Furazolidone	+	Ebringer and Bencova (1980)
Bacillus subtilis	Rec-Assay	Furazolidone	+	Ohta *et al.* (1980)
Saccharomyces cerevisiae	Gene conversion	Metronidazole	-	Mohn *et al.* (1979)
Neurospora crassa	Mutation	Metronidazole	-	Mohn *et al.* (1979)
				Onge *et al.* (1980)
Drosophila melanogaster	SLRL test	Metronidazole	-	Mohn *et al.* (1979)
				Kramers (1982)
			+	Present study
		Furazolidone	+	Blijleven *et al.* (1977)
				Kramers (1982)
				Present study
	Wing spot test	Metronidazole	+	Present study
		Furazolidone	+	Present study
Mouse	Sperm	Metronidazole	+	Shulman and Gray (1978)
	abnormality		-	Pylkkanen and Lahdetie (1984)
	Micronucleus	Metronidazole	w	Molina *et al.* (1978)
			-	Hartley-Asp (1979)
Rat hepatocyte	UDS	Metronidazole	-	Probst *et al.* (1981)
		Furazolidone	+	Probst *et al.* (1981)
	Micronucleus	Metronidazole	-	Trozs *et al.* (1978)
Chinese hamster (V79 cells)	Clastogenicity	Metronidazole	+	Korbelik and Horvat (1980)
	HPRT	Metronidazole	-	Dayan *et al.* (1982)
			w	Olive (1981)
	SCE	Metronidazole	-	Geard *et al.* (1981)
(K1 cells)	SCE	Metronidazole	-	Mahood and Wilsons (1983)
	Mutation	Furazolidone	+	Gao *et al.* (1989)
Human lymphocytes	SCE	Metronidazole	w	Wulf (1980)
			-	Prosser and Priseman (1980)
(*in vitro*)	Clastogenicity	Furazolidone	-	Tonamura and Sasaki (1973)
(*in vivo*)	Clastogenicity	Metronidazole	-	Mitelman *et al.* (1980)

* + = positive, - = negative, w = weak

phenotypically normal adult wing. The details of the genetic symbols are referred in Frölich and Würgler (1989) and Lindsley and Zimm (1992). Since there are a few drawbacks to this cross (Frölich and Würgler 1991), viz., irregular whorling in the pattern of wing hairs, low egg production, delay in development of larvae, etc., the use of *ORR*; flr^3 females with standard *mwh* males has been recommended by Graf and Singer (1992), because the progeny of this cross develop normally, exhibit high bioactivation without whorling of the wing hairs. Further, use of flr^3 females assures better fertility and, therefore, large number of larvae are obtained for treatment.

The wing mosaic assay, used presently for the detection of genotoxicity of compounds in the wing primordial cells, is a fast, one-generation test and is capable of assaying a wide spectrum of genetic events ranging from mutation to deletion and somatic recombination. The test involves the exposure of a population of wing primordial cells, transheterozygous for the marker mutations *mwh* and flr^3, to a chemical mutagen, so that the genetic alterations induced are expressed in the form of mosaic spots on the adult wing. In the present experiments, larvae of 2nd. and 3rd. instar, respectively, were exposed to the LD_{50} and lower doses for the rest of the larval life. The experiments were repeated, and since the spot

frequencies did not differ significantly in the repeated experiments, the data were pooled.

Oregon-R, the wild type strain, and *Basc* homozygous strain were used in the sex-linked recessive lethal test to study the genotoxicity of the compounds in male germ line cells.

Metronidazole with 95% purity, obtained from Cipla Ltd., Bombay, and furazolidone with 97% purity, obtained from Eskayef Ltd., Bangalore, were used in the present experiments. Both compounds were dissolved in distilled water to obtain the desired concentration for the treatment of larvae.

For details of the methodology for larval exposures, collecting the data, etc., refer to Tripathy *et al.* (1994) and Frei and Würgler (1988). Since metronidazole was less toxic to larvae, the LD_{50}, where 50% of the treated larvae survived to adult stage, was 100 mM. In contrast, since furazolidone was highly toxic to the larvae, the maximum concentration tested was 10 mM.

RESULTS

The results of the experiments testing the genetic toxicity of the two antibacterial and antiprotozoal agents in the somatic cells of *Drosophila* are shown in the **table II**. The frequencies of induction of small single spots alone were significantly positive at the highest tested dose (100 mM) of metronidazole following exposures of 2nd. and 3rd. instar larvae. The frequencies of induction of such spots at 50 mM and 25 mM as well as those of large single and twin spots at all three concentrations of this compound were inconclusive.

The exposures of both 2nd. and 3rd. instar larvae to the three doses (10 mM, 5 mM and 2.5 mM) of furazolidone led to a significant increase in the frequencies of small single spots. The frequencies of large, single and twin spots were, however, inconclusive.

A summary of the data obtained in the sex-linked recessive lethal test is given in the **table III**. Following larval exposures to different concentrations of metronidazole, the induced lethal frequencies were different from the control only for the exposures of 2nd. instar larvae to the highest dose (100 mM). On the other hand, the frequency of lethal induction was positive at all three test doses of furazolidone following exposures of both 2nd. and 3rd. instar larvae.

DISCUSSION

In the wing mosaic assay single spots with *mwh* or *flr*^{3} phenotype originate following the induction of mutations in the corresponding wild type loci (Graf *et al.* 1984). Induction of segmental aneuploidy through chromosome breakage (Haynie and Bryant 1977) might also lead to the formation of single spots. Single spots with *mwh* phenotype may also stem from the induction of mitotic recombination in the region between the *mwh* and the *flr*^{3} loci on chromosome 3. In the present experiments with metronidazole the frequency of small single spots was positive only at the highest concentration of the compound following exposures of larvae of both instars.

TABLE II. SUMMARY OF DATA OBTAINED IN THE WING SPOT TEST

Chemical	Larval age (h)	Duration of treatment (h)	Conc. (mM)	Wings' tested	Spots per wing (No. of spots) Stat. Diagn.*			
					Small singles (1-2 cells) [m = 2.0]	Large singles (> 2 cells) [m = 5.0]	Twins [m = 5.0]	Total [m = 2.0]
Metronidazole	72	48	0	80	0.19 (15)	0.01 (1)	0.00 (0)	0.20 (16)
			25	80	0.23 (18)i	0.01 (1)i	0.00 (0)i	0.24 (19)i
			50	80	0.29 (23)i	0.03 (2)i	0.01 (1)i	0.33 (26)i
			100	80	0.36 (29)+	0.04 (3)i	0.00 (0)i	0.40 (32)+
	48	72	0	80	0.21 (17)	0.03 (2)	0.00 (0)	0.24 (1)
			25	80	0.29 (23)i	0.03 (2)i	0.00 (0)i	0.31 (25)i
			50	80	0.34 (27)i	0.03 (2)i	0.00 (0)i	0.36 (29)i
			100	80	0.40 (32)+	0.04 (3)i	0.01 (1)i	0.45 (36)+
Furazolidone	72	48	0	80	0.20 (16)	0.03 (2)	0.00 (0)	0.23 (18)
			2.5	80	0.41 (33)+	0.06 (5)i	0.00 (0)i	0.48 (38)+
			5.0	80	0.43 (34)+	0.04 (3)i	0.01 (1)i	0.48 (38)+
			10.0	80	0.48 (38)+	0.06 (5)i	0.03 (2)i	0.56 (45)+
	48	72	0	80	0.23 (18)	0.01 (1)	0.01 (1)	0.25 (20)
			2.5	80	0.43 (34)+	0.05 (4)i	0.03 (2)i	0.50 (40)+
			5.0	80	0.49 (39)+	0.05 (4)i	0.04 (3)i	0.58 (46)+
			10.0	80	0.53 (42)+	0.08 (6)i	0.03 (2)i	0.63 (50)+

* Statistical diagnoses according to Frei and Würgler (1988): + = positive, i = inconclusive, m = multiplication factor.
Probability level: $\alpha = \beta = 0.05$, one sided statistical tests

TABLE III. SUMMARY OF DATA OBTAINED IN THE SEX-LINKED RECESSIVE LETHAL TEST

Chemical	Larval age (h)	Duration of treatment (h)	Conc. (mM)	Males tested	X chromosomes				Lethals per male		
					Total	Lethal	%	Concl.*	0	1	2
Metronidazole	72	48	0	54	1086	3	0.28		51	3	0
			25	57	1168	4	0.34	NS	53	4	0
			50	62	1296	6	0.46	NS	56	6	0
			100	68	1429	7	0.49	NS	61	7	0
	48	72	0	49	1024	3	0.29		46	3	0
			25	56	1208	7	0.58	NS	49	7	0
			50	72	1463	9	0.62	NS	63	9	0
			100	58	1272	12	0.94	+	46	12	0
Furazolidone	72	48	0	52	1083	2	0.18		50	2	0
			2.5	62	1278	11	0.86	+	52	9	1
			5.0	53	1127	15	1.33	+	40	11	2
			10.0	48	987	14	1.41	+	35	12	1
	48	72	0	68	1392	3	0.22		65	3	0
			2.5	61	1228	13	1.06	+	49	11	1
			5.0	64	1417	16	1.13	+	48	16	0
			10.0	56	1163	18	1.55	+	40	14	2

* Conclusion on the basis of Kastenbaum and Bowman (1970), NS = no significant, + = positive, level of significance $p < 0.05$

On the contrary, the frequency of small singles was positive at all the test doses of furazolidone after exposures of both 2nd. and 3rd. instar larvae. With both compounds, there was no difference in the frequency of small single spots between the wings of individuals which were treated either from the 2nd. or 3rd. instar. This indicates that possibly the compounds act late in larval life, thereby leading to the induction of only smaller clones. In the present experiments larvae of two different stages were treated with the compound. Although according to Graf (1995) the optimal age for larval treatment is 72 h, younger larvae may be used as the size of the spots increases when the genetic change is induced early in the larval life. It is concluded that irrespective of the mechanism(s) involved, both compounds are genotoxic in the wing disc cells of *Drosophila* but metronidazole is less toxic.

The *mwh/ /flr*^{3} twin spots are induced on the adult wings due to a mitotic recombination event in the chromosome region between the *flr*^{3} locus and the centromere (Becker 1976). In the present experiments, since the frequency of twin spots was not significantly higher at any of the test doses or duration of treatment with both compounds, it is concluded that the compounds may be non-recombinogenic or weakly so in the wing primordial cells of *Drosophila*. In the present experiments the frequencies of induction of large singles and twin spots were inconclusive at different test doses. The statistical conclusion depends on the number of wings screened (Frei and Würgler 1995). Larger number of wings (i.e., approximately 110 wings for each concentration) are needed to resolve the inconclusive diagnoses.

The sex-linked recessive lethal test is effective in detecting mutations and deletions of small chromosome parts with equal efficiency (Lee *et al.* 1983). With metronidazole, the lethal frequencies were different from the control only when 2nd. instar larvae were exposed to the highest concentration. Earlier reports of Mohn *et al.* (1979) and Kramers (1982) showed this compound to be non-mutagenic in a test following adult feeding and injection. Following larval exposures to furazolidone, the lethal frequencies were significantly higher than the control at all the concentrations tested. Since this compound is already known to be mutagenic and clastogenic in different test systems, the induction of sex-linked recessive lethals was expected in the present experiments. Our data agree with the findings of Blijleven *et al.* (1977) and Kramers (1982) who also reported this compound mutagenic in the male germ line cells of *Drosophila* following adult feeding and injection.

One, two and one males, respectively, at 10 mM, 5 mM and 2.5 mM of furazolidone following exposures of 3rd. instar larvae and 2 and 1 males, respectively, following exposures of 2nd. instar larvae to 10 mM and 2.5 mM, respectively, yielded two lethal chromosomes per male. This indicated the possible induction of clusters (Clark 1982, Kramers 1982). Clusters are a group of lethals originating from a common mutational event when a genetic change is induced in the premeiotic germ cells of *Drosophila*. The data were subjected to calculations on the basis of the formula for Poisson distribution followed by chi-square goodness of fit (Szabad *et al.* 1983) to determine if such lethals represented clusters. Since the expected and observed values did not differ significantly ($p > 0.05$), it was concluded that the lethals represented independent mutational events in the X chromosomes of the treated male larvae.

The sex-linked recessive lethal test is regarded as the best validated genotoxicity test in *Drosophila*. But this test is time consuming and expensive as it involves 2-3 fly generations. This assay is being currently replaced by more efficient wing and eye mosaic assays which involve the somatic cells and only one fly generation. These assays are thus quicker and cheaper. In the present experiments also the data obtained in the wing spot test compare well with the data obtained in the sex-linked recessive lethal test.

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