Metronidazole Induced DNA Damage in Somatic Cells of *Drosophila melanogaster*

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Abstract: The standard version of the wing somatic mutation and recombination test (SMART) in *Drosophila melanogaster* was employed in order to evaluate the genotoxic potential of metronidazole (MTZ) as a function of exposure concentration. MTZ was administered by chronic feeding of 3-day-old larvae with the parenteral parental solution at 0, 500, 1000 and 2000 μg/ml until pupation. The marker-heterozygous progeny (*mwh+/+flr3*) with phenotypically wild-type wings was analyzed. Non significant differences were found between control and each MTZ concentration tested for single small spots (SSS) frequencies. Large single spots (LSS) and twin spots (TS) were significantly increased with the higher dose. MTZ treatments with 1000 and 2000 μg/ml also significantly increased the frequency of Total spots. These findings suggest that MTZ is genotoxic in the present experimental conditions and induces recombinagenesis and/or gene conversion, two major mechanisms that cause loss of heterocigosity and could play an important role in tumorigenesis and carcinogenesis processes.

Keywords: Drosophila, genotoxicity, Metronidazole (MTZ), loss of heterozygosity, wing spot assay.

INTRODUCTION

Benefit–risk analysis of medicines is a complex process that requires evaluation of a large amount of relevant data, and at the present it is highlighted the value of post-marketing studies and the dynamic balance of perceived benefit and perceived risk. Consequently, it is critical the evaluation and characterization of a product's risk profile for making informed decisions on risk minimization [1].

Nitro-heterocyclic compounds are widely used as therapeutic agents against a variety of protozoan and bacterial infections. Among them, metronidazole (MTZ, 1-2-hydroxyethyl-2-methyl-5-nitroimidazole) possesses direct trichomonacidal and amebacidal activities as well as activity against most obligate anaerobes [2, 3]. The literature on different undesired biological effects of these imidazolic compounds, which are even suspected of being carcinogenic, is controversial [4]. Besides, as the use of MTZ is also permitted during pregnancy provided the indications for its use have been strictly verified [5], it is very important to establish if this antibiotic puts the individual at risk during gestation. Thus, all information on positive or negative effects of this compound on different tests systems is valuable.

In the last 20 years MTZ has been re-evaluated regarding its potential cytotoxicity, genotoxicity, reproductive and developmental toxicity in different animal and vegetal *in vivo*/*in vitro* model systems [6-16].

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The aim of the present work is to report the results obtained with the wing Somatic Mutation and Recombination Test (SMART) in order to contribute to the knowledge of MTZ genotoxic profile. This assay monitors the loss of heterozygosity (LOH) in somatic tissues of *D. melanogaster* larvae and detects several genetic end-points, including point mutation, deletion, unbalanced half-translocation, mitotic recombination and gene conversion [17-22]; it has been applied to a variety of compounds and complex mixtures [21, 22].

MATERIALS AND METHODS

Two *D. melanogaster* strains were used for the SMART wing assay: (i) the multiple wing hairs — *mwh/mwh* and (ii) the flare[3] strain — *flr[3]/In(3LR)TM3, ri p[p] sep l(3)89Aa bx[34e]e Bd[S]*. The standard (ST) cross was performed using females from the *flr[3]* strain and *mwh* males. Ten females were crossed to 15 males for 5 days (ST) and then they were permitted to lay eggs for 8 h in regular media. When larvae were 72 h old, they were transferred to vials containing mashed-potatoes medium prepared with the parenteral solution of the drug diluted in water at concentrations of 500, 1000 and 2000 μg/ml of MTZ. The medium contained 1g of dry instant mashed potato powder per 5 ml of the solution at the concentration to be tested, with the addition of an alcoholic solution of Nipagin (3.6 ml/100 ml of water). The larvae were fed on these media until pupation. Concurrent control series in water plus Nipagin were run. All series were kept at 25 ± 1 °C. The emerging flies were stored in 70% ethanol until the wings were mounted on slides in Faure's solution and examined for spots under a microscope at 400x magnification. Single spots (*mwh* or *flr[3]*) can result from different genotoxic events: mitotic recombination, mutation

1574-8863/13 $58.00+.00

and chromosomal aberration. Twin spots (*mwh* and *flr*^{3}) are produced only by mitotic recombination. For a full description of the test see Graf *et al.* [17, 23, 24]. In the present experiments only the marker-heterozygous progeny (*mwh+/+flr3*) with phenotypically wild-type wings were analyzed.

Statistical evaluation was according Frei and Würgler [25]; Kastenbaum and Bowman tables for the Conditional Binomial Test where applied when frequencies were < 5 and X^2 Test when frequencies were ≥5.

RESULTS

The induction of LOH in the marker-heterozygous flies produces two types of mutant clones: single or twin. Single spots are generated by mitotic recombination, somatic gene mutation, and deletion or chromosome aberrations at *mwh* or *flr*^{3} locus. They are recorded as small (1-2 cells, SSS) or large (>2 cells, LSS) single spots, being the size related to the number of divisions that occurred after the genetic change. Twin spots (TS), manifesting both *flr*^{3} and *mwh* subclones, originate exclusively from mitotic recombination occurring between the proximal marker *fir* and the centromere.

Table **1** shows that there are not significant differences in SSS frequencies between control and all MTZ doses tested. LSS induction is significantly increased by 500 µg/ml; 1000 µg/ml MTZ treatment gave inconclusive results for LSS and TS. Treatments with 2000 µg/ml significantly raised the frequency of LSS and TS. Total spots were not affected by the lower MTZ dose (500 µg/ml), but raised significantly with 1000 and 2000 µg/ml. These results indicate that MTZ is genotoxic and suggest a recombinagenic effect of this drug. Fig. (**1**) shows that these genotoxic effects are dose dependant (R= 0.90066 for SSS; R= 0.81057 for LSS; R= 0.97086 for TS; R= 0.98571 for Total; lineal fit).

DISCUSSION

Data previously reported in Drosophila indicate that MTZ gave contradictory results for SLRL test (reported negative by Mohn *et al.* [26] and by Kramers [27], but positive by Tripathy *et al.* [28]). With much higher doses than those we report here, Tripathy *et al.* [28], found inconclusive results regarding LSS and TS, which could be interpreted as due to toxic effects of DL50 applied by the above mentioned authors. Rodriguez-Arnais and Hernandez Aranda [29] using the eye mitotic recombination assay reported that MTZ chronic treatments were the best exposure and concluded that MTZ action was mediated by electrophilic intermediates

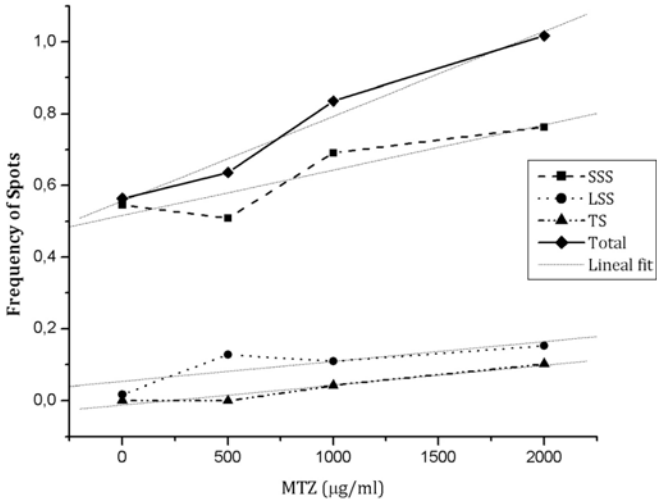

Fig. (1). Dose response of spots induction after MTZ treatments in *D. melanogaster* (SSS: small single spots; LSS: large single spots; TS: twin spots; Total: total spots).

Metronidazole is classified as a Group 2B carcinogen, thus it is considered possibly carcinogenic to humans but with limited evidence of carcinogenicity in humans and insufficient evidence in experimental animals [30]. Brambilla *et al.* [31] reviewed the long-term carcinogenesis assays for 535 pharmaceuticals and reported that MTZ gave positive results in Swiss mice (lung tumors, 0.06% in diet) but equivocal data in Sprague-Dawley weanling female rats (mammary tumors, 0.135% in diet) and equivocal or weakly positive in humans (lung and cervix tumors, cancer at any site).

As mentioned in above, the genotoxic and mutagenic properties of nitroimidazoles are well documented but information regarding the recombinational hazards of their human and veterinary applications is scarce. Mitotic recombination and gene conversion are two major mechanisms that cause LOH, thus they could play an important role in the processes of tumorigenesis and carcinogenesis [32]. The results presented here are in line with those reported previously that found a significant

Table 1. Results Obtained with the Drosophila Wing Spot Test (SMART) in the Marker-Heterozygous After Chronic Treatment of Larvae with MTZ

Treatments	Number of Flies	Spots Per Fly (Number of Spots); (Statistical Diagnosis)*			
		Small Single Spots (1–2 cells) *m=2*	Large Single Spots (>2 cells) *m=5*	Twin Spots *m=5*	Total Spots *m=2*
Control	55	0.545 (30)	0.018 (1)	---	0.564 (31)
MTZ 500 µg/ml	55	0.509 (28) (-)*	0.127 (7) (+)*	---	0.636 (35) (-)*
MTZ 1000 µg/ml	55	0.691 (38) (-)*	0.109 (6) (i)*	0.042 (2) (i)*	0.836 (46) (+)*
MTZ 2000 µg/ml	59	0.763 (45) (i)*	0.152 (9) (+)*	0.102 (6) (+)*	1.017 (60) (+)*

*Positive (+); negative (-); inconclusive (i) according Frei and Würgler [25]. (Kastenbaum and Bowman tables for the Conditional Binomial Test when frequencies are < 5; X^2 Test when frequencies are >5).
m=multiplication factor.

increase in sister chromatid exchange (SCE) frequency following MTZ treatments *in vitro* as well as *in vivo* [33-35]. Consequently, they had to be taken into account when risk-benefit evaluation of MTZ is performed in order to avoid an indiscriminate use of the drug.

CONCLUSIONS

1. MTZ evaluated by the wing spot test, significantly increased the frequency of large, total and twin spots above control values, confirming the genotoxic and mutagenic properties of this nitroimidazole in *D. melnogaster*.
2. The effects reported here are in line with with data in the literature, particularly with those found in sister chromatid exchange (SCE) frequency following *in vitro* and *in vivo* MTZ treatments.
3. The rising in twin spots suggests the induction of recombinagenesis and/or gene conversion with the consequent loss of heterozygosis.
4. This loss of heterozygosity has to be considered in risk-benefit evaluation of MTZ, since it could play an important role in tumorigenesis and carcinogenesis processes.

ACKNOWLEDGEMENTS

This work was supported by grants MDM PIP 057262/2012-14 and UBACyT FX 0136/2011-14.

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Received: July 24, 2012 Revised: May 23, 2013 Accepted: May 24, 2013