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Modulatory effects of metformin on mutagenicity and epithelial tumor incidence in doxorubicin-treated *Drosophila melanogaster*

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Prof. Dr. Mário Antônio Spanó

“Hardship often prepares an ordinary person for an extraordinary destiny”

(C.S. Lewis)

DEDICATION

I dedicate this work to my beloved wife **Sarah Alves Rodrigues Constante,** for the love, affection and encouragement, and to all my family that always supported me. You are my sources of inspiration.

I dedicate also to my dear supervisors: **Prof. Dr. Júlio César Nepomuceno** (*in memorian*) and **Prof. Dr. Mário Antônio Spanó**. One began, and the other concluded my great dream. To my teachers: My eternal gratitude.

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LIST OF ABBREVIATIONS

A498	Renal cell carcinoma
AGEs	Advanced glycation end products
AMPK	AMP-activated protein kinase
Bcl-2	B-cell lymphoma 2
BH	Balancer heterozygote descendants
C2C12	Mouse myoblasts cells
C57BL/6J	Mice Strain
cAMP	Cyclic adenosine monophosphate
CoA-SH	Intramitochondrial coenzyme A
Cyp6A2	Cytochrome P450-6a2
CYPs	Cytochrome P450 enzymes
DNA	Deoxyribonucleic acid
DXR	Doxorrubicin
FHC	Ferritin heavy chain
Flr^3	Flare-3
flr^3	Flare gene
FOXA1	Forkhead box protein A1
FOXO3	Forkhead transcription factor 3
GSH	Glutathione reduced
HB	High bioactivation cross
HepG2	Liver hepatocellular carcinoma
HO-1	Heme oxygenase-1
MET	Metformin
MH	Marker trans heterozygous descendants
miR-23a	MicroRNA-23a
mM	Milimolar
mTOR	Mammalian target of rapamycin
Mwh	Multiple wing hairs
mwh	Multiple wing hairs gene
NFκB	Nuclear factor κB
NQO1	Quinone oxidoreductase

Nrf2	Nuclear factor-erythroid 2-related factor 2
ORR	Oregon R (R) - insecticide-resistant strain
p53	Tumour suppressor gene
RAGE	Receptor for glycated end product
ROS	Reactive oxygen species
SMART	Somatic Mutation and Recombination Test
SOD	Superoxide dismutase
ST	Standard cross
T2DM	Type 2 diabetes mellitus
TM3,Bd^{s}	Balancer chromosome with inversion *TM3,Bd*^{s}
TM3,Sb1	Balancer Chromosome with inversion *TM3,Sb1*
Trx	Antioxidant thioredoxin
Warts test	Test for Detection of Epithelial Tumor Clones
wts	*wts* tumor suppressor gene
γGCSm	γ-glutamyl cysteine synthetase modifier subunit

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SUMMARY

RESUMO

A metformina (MET) é um fármaco antidiabético utilizado para prevenir a liberação de glicose hepática e aumentar a sensibilidade à insulina nos tecidos. Pacientes diabéticos com câncer têm, em adição, terapia medicamentosa antineoplásica. A doxorrubicina (DXR) é um agente quimioterápico antineoplásico que interfere com enzimas topoisomerase II e gera radicais livres. A MET isolada (2,5; 5,0; 10,0; 25,0 ou 50,0 mM) foi avaliada quanto à mutagenicidade, recombinogenicidade e carcinogenicidade e associado com DXR (0,4 mM) para antimutagenicidade, antirecombinogenicidade e anticarcinogenicidade, utilizando o "Teste para Detecção de Mutação e Recombinação Somática" e o "Teste para Detecção de Clones de Tumores Epiteliais" em *Drosophila melanogaster*. A MET isolada não induziu mutação ou recombinação, mas foram observados efeitos moduladores da MET sobre as lesões de DNA induzidas pela DXR nas concentrações mais elevadas. Na avaliação da carcinogênese, a MET isolada não induziu tumores, mas quando associado com DXR, MET também reduziu os tumores induzidos por DXR nas concentrações mais elevadas. Sendo assim, nas presentes condições experimentais a MET isolada não apresentou efeitos mutagênicos, recombinogênicos e carcinogênicos, mas foi capaz de modular o efeito da DXR na indução de danos ao DNA e tumores em *D. melanogaster*. Acredita-se que este efeito modulador esteja relacionado principalmente aos efeitos antioxidantes, anti-inflamatórios e apoptóticos deste medicamento, embora tais efeitos não tenham sido avaliados diretamente.

Palavras-chave: Gene *wts*, SMART, Teste para detecção de mutação e recombinação somática, Tumor, Warts.

ABSTRACT

Metformin (MET) is an anti-diabetic drug used to prevent hepatic glucose release and increase tissue insulin sensitivity. Diabetic cancer patients are on additional therapy with anticancer drugs. Doxorubicin (DXR) is a cancer chemotherapeutic agent that interferes with the topoisomerase II enzyme and generates free radicals. MET (2.5, 5, 10, 25 or 50 mM) alone was examined for mutagenicity, recombinogenicity and carcinogenicity, and combined with DXR (0.4 mM) for antimutagenicity, antirecombinogenicity and anticarcinogenicity, using the Somatic Mutation and Recombination Test and the Test for Detecting Epithelial Tumor Clones in *Drosophila melanogaster*. MET alone did not induce mutation or recombination. Modulating effects of MET on DXR-induced DNA damage were observed at the highest concentrations. In the evaluation of carcinogenesis, MET alone did not induce tumors. When combined with DXR, MET also reduced the DXR-induced tumors at the highest concentrations. Therefore, in the present experimental conditions, MET alone did not present mutagenic/recombinogenic/carcinogenic effects, but it was able to modulate the effect of DXR in the induction of DNA damage and of tumors in *D. melanogaster*. It is believed that this modulating effect is mainly related to the antioxidant, anti-inflammatory and apoptotic effects of this drug, although such effects have not been directly evaluated.

Keywords: Gene *wts*, SMART, Somatic mutation and recombination test, Tumor, Warts.

1. INTRODUCTION

Metformin (MET) (tradename Glucophage) is an oral anti-diabetic drug of the biguanide family widely prescribed as a first choice medication for type 2 diabetes mellitus (T2DM). It prevents hepatic glucose release and increases tissue insulin sensitivity (MALEK et al., 2015; CHEKI et al., 2016; NISHIHAMA et al., 2016). MET has been widely used in the treatment of polycystic ovary syndrome and gestational T2DM (AMADOR et al., 2012; REECE et al., 2014). Besides, several studies have even suggested that MET may have further application in anticancer and antiaging therapies, mainly in tumors driven by insulin resistance and obesity (KASZNICKI et al., 2014; MARYCZ et al., 2016; TALAULIKAR et al., 2016).

In mammals, MET is absorbed predominately from the small intestine and is excreted unchanged in urine (GRAHAM et al., 2011). The mechanisms of MET action are only partially explored and remain controversial (SONG, 2016). Several potential mechanisms of action have been proposed: suppression of liver glucose production (hepatic gluconeogenesis) by inhibiting mitochondrial glycerophosphate dehydrogenase (MADIRAJU et al., 2014); inhibition of the mitochondrial respiratory chain (complex I) (OWEN et al., 2000); activation of AMP-activated protein kinase (AMPK) (a major cellular regulator of lipid and glucose metabolism) in hepatocytes, through liver kinase B1 (ZHOU et al., 2001); suppression of hepatic glucagon signaling by decreasing production of cyclic adenosine monophosphate (cAMP) (MILLER et al., 2013); and changes in the gut microbiota and their metabolic pathways (LEE and KO, 2014).

MET may exert antineoplastic effects through: AMPK-mediated or AMPK-independent inhibition of mammalian target of rapamycin (mTOR), which is up-regulated in many cancer tissues (HAN et al., 2015); or blocking migration and invasion of tumor cells by inhibition of matrix metalloproteinase-9 activation through a calcium and protein kinase Cα-dependent pathway (HWANG and JEONG, 2010).

Several studies indicate that MET has also antioxidant (HOU et al., 2010; ALGIRE et al., 2012; ASHOUR et al., 2012; NA et al., 2013; YANG et al., 2014b; VILELA et al., 2016), anti-inflammatory (WOO et al., 2014; JIN et al., 2015; CAMERON et al., 2016; ZHOU et al., 2016), and apoptotic effects (FANG

et al., 2014; TAKAHASHI et al., 2014; HAN et al., 2015; SUN et al., 2016). Regarding the mutagenic/clastogenic/recombinogenic potential of MET, literature data are conflicting. Some studies have shown that MET is not genotoxic *in vivo* or *in vitro* (ALEISA et al., 2007; ATTIA et al., 2009; AMADOR et al., 2012; MALEK et al., 2015; SANT'ANNA et al., 2013; CHEKI et al., 2016; ULLAH et al., 2016), non-recombinogenic (SANT'ANNA et al., 2013) and may protect from genomic instability (ATTIA et al., 2009; CHEKI et al., 2016; ULLAH et al., 2016). Nevertheless, MET induced genotoxicity in rodent cells *in vitro* (AMADOR et al., 2012) and in T2DM patients *in vivo* (HARISHANKAR et al., 2015).

Hyperglycemia is commonly observed in a wide variety of diseases, including cancer. Diabetic cancer patients are on additional therapy with anticancer drugs (ALEISA et al., 2007).

Doxorubicin (DXR) (also called Adriamycin ® or 14-hydroxydaunorubicin) is an anthracyline drug first extracted from *Streptomyces peucetius* ATCC 27952 that is used to treat many different types of cancer (MALLA et al., 2010). Nevertheless, its use as an antitumor therapeutic agent is limited due to its cardiotoxic effects (SHETA et al., 2016). DXR may intercalate on DNA and induce formation of DNA adducts at active promoter sites, increasing torsional stress and enhancing nucleosome turnover. Furthermore, it may trap topoisomerase II at breakage sites, causing double strand breaks. Enhanced nucleosome turnover might increase the exposure of DNA to reactive oxygen species (ROS) resulting in DNA damage and cell death (YANG et al., 2014a). Previous studies have demonstrated that MET may have protective effects against DXR-induced cardiotoxicity and clastogenicity (ALEISA et al., 2007; SHETA et al., 2016).

In the present study, the wing Somatic Mutation and Recombination Test (SMART) was used to assess MET mutagenicity and its anti-mutagenic potential against DXR-induced mutagenicity. We also investigated the carcinogenic potential of MET alone and its anti-carcinogenic potential against DXR-induced carcinogenicity using the Test for Detection of Epithelial Tumor Clones (Warts) in *D. melanogaster*.

2. OBJECTIVE

In the present study, the wing Somatic Mutation and Recombination Test (SMART) was used to assess MET mutagenicity and its anti-mutagenic potential against DXR-induced mutagenicity. We also investigated the carcinogenic potential of MET alone and its anti-carcinogenic potential against DXR-induced carcinogenicity using the Test for Detection of Epithelial Tumor Clones (Warts) in *D. melanogaster*.

3. MATERIAL AND METHODS

3.1. Chemical agents

Metformin (N, N′-dimethylbiguanide; CAS 657-24-9) was purchased from Merck, Rio de Janeiro, Brazil. Doxorubicin (DXR; CAS 25316-40-9), commercially known as Adriblastina®, was produced by Actavis Italy, Nerviano, Italy. The solutions were always prepared immediately before use with ultrapure water obtained from a MilliQ system (Millipore; Vimodrome, Milan, Italy). The structural formulas of these substances are shown in Fig. 1.

3.2. Strains and stock

In this study the following strains of *D. Melanogaster* were used: [1] multiple wing hairs (*mwh/mwh*); [2] flare-3 (*flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx34^e and Bd^S*); [3] ORR; flare-3 (*ORR/ORR; flr^3/In(3LR)TM3, ri p^p sep l(3)89Aa bx34^e and Bd^S*); and [4] *wtsTM3*, *Sb*^{1}. These strains were maintained in glass vials filled with a maintenance medium (i.e., banana, sucrose, yeast and methylparaben) under light/dark cycles (12 h:12 h), at 25 ± 1ºC and approximately 60% humidity in a BOD-type chamber (Model: SL224, SOLAB – Equipamentos para Laboratórios Ltda., São Paulo, SP, Brazil).

3.3. Somatic Mutation and Recombination Test – SMART

3.3.1. Crosses and treatments

The SMART assay allows the detection of different genetic end-points, using two different strains of *D. melanogaster* that carry specific genetic markers (*mwh* and *flr*^{3}) on the left arm of chromosome 3 (GRAF et al., 1984).

Two crosses were carried out to produce the experimental larval progeny: (1) Standard cross (ST): *mwh/mwh* males crossed with *flr*^{3}*/In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx34*^{e} *and Bd*^{S} virgin females (GRAF et al., 1984; GRAF et al., 1989); (2) High bioactivation (HB) cross: *mwh/mwh* males crossed with *ORR/ORR; flr*^{3}*/In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx34*^{e} *and Bd*^{S} virgin females (GRAF and VAN SCHAIK, 1992). The two crosses produce two types of flies: marker trans-heterozygous (MH) flies (*mwh* +/+ *flr*^{3}) and balancer-heterozygous (BH) flies (*mwh*+/+*TM3*, *Bd*^{S}).

Detailed information on genetic symbols can be found in Lindsley and Zimm (1992). The ST cross uses strains carrying basal levels of the metabolizing cytochrome P450 enzyme (Cyp6A2) and is used to detect direct-acting genotoxins. The HB cross uses strains with high levels of Cyp6A2 and is used to detect indirect-acting genotoxins that exert their genotoxic activity only when metabolized (GRAF and van SCHAIK, 1992; SANER et al., 1996; REZENDE et al., 2011).

3.3.2. Experimental procedure

Eggs, from both crosses, were collected for 8 h in culture bottles containing a solid agar base (5% w/v agar agar in water) covered with a thick layer of live baker's yeast supplemented with sucrose. Approximately 72 h after the end of the egg-laying stage, larvae were collected and distributed in four sets of vials for each cross with 1.5 g of mashed potato flakes and 5 mL of a solution containing metformin alone (at a final concentration of 2.5, 5, 10, 25 or 50 mM or with DXR at 0.4 mM). Negative (ultrapure water) and positive doxorubicin (DXR 0.4 mM) controls were included.

The larvae were counted before distribution into two series of these vials. The number of hatched flies was used to calculate the survival rates upon exposure. The experiments were conducted at a temperature of 25 ± 1^{o}C at a

relative humidity of 60%. The hatching adult flies were collected from the treatment vials and stored in 70% ethanol. The wings were removed and mounted in Faure's solution on microscope slides and inspected under 400X magnification for the presence of spots.

On marker-heterozygous (MH) wings (*mwh/flr*^{3}) three different categories of spots can be observed: (i) small single spots (1–2 cells in size) and (ii) large single spots (more than two cells), expressing either the multiple wing hairs (mwh) or the flare (*flr*^{3}) phenotype, as well as (iii) twin spots, consisting of both *mwh* and *flr*^{3} sub clones.

On balancer heterozygous (BH) wings (*mwh/TM3*) only *mwh* single spots can be observed, as the inverted *TM3* balancer chromosome does not carry *flr*^{3} or any other suitable marker mutation. While in the MH flies mutant clones can be originated by somatic point mutation, chromosome aberration and/or mitotic recombination, in the BH genotype this last genotoxic event is suppressed due to the presence of multiple inversions in the *TM3* balancer chromosome. Through comparison of these two genotypes, it is possible to quantify the recombinogenic action of the drug tested (FREI et al., 1992).

3.3.3. Statistical Analysis

The frequency of each type of spot (small single, large single or twin) and the total frequency of spots per fly for each treatment were compared pair-wise (i.e., negative control *versus* metformin; positive control (DXR) alone *versus* DXR plus metformin) according to Kastenbaum and Bowman (1970) with p = 0.05 (FREI and WURGLER, 1988, 1995). The data were initially evaluated according to the multiple-decision procedure of Frei and Würgler (1988). Afterwards, to exclude false positive and inconclusive results due to overdispersion of the data, the U-test of Wilcoxon, Mann, and Whitney was applied, resulting in two different diagnoses: positive and negative (FREI and WÜRGLER, 1995).

Based on the clone induction frequency per 10^5 cells, the recombinogenic activity was calculated as follows. Frequency of mutation (FM) = frequency of clones in BH flies/frequency of clones in MH flies. Frequency of recombination (FR) = 1 – frequency of mutation (FM). Frequencies of total spots (FT) = total spots observed in MH flies (considering *mwh* and flr^3 spots)/number

of flies (SANTOS et al., 1999; SINIGAGLIA et al., 2004, 2006).

Based on the control-corrected spot frequencies per 10^5 cells, the percentage of metformin inhibition was calculated as: (DXR alone – metformin plus DXR/DXR alone) – 100 (Abraham, 1994).

3.4. Test for the detection of epithelial tumor clones in *Drosophila melanogaster* (*Warts*)

3.4.1. Crosses and treatments

To obtain *wts* +/+ *mwh* heterozygotic larvae, virgin females *wts*TM3, Sb^1 were crossed with *mwh/mwh* males (Nepomuceno, 2015). The eggs of the descendants were collected as in the SMART assay. After approximately 72 h, third-instar larvae from this cross were treated. The larvae were placed in glass flasks containing 1.5 g of instant mashed potatoes (Hikari® brand, São Paulo, Brazil) culture medium, and 5 mL of a solution containing metformin (2.5; 5; 10; 25 or 50 mM). The concentrations were chosen based on the survival rates of a dose-response test performed by Slack et al. (2012). Negative (reverse osmosis water) and positive (Doxorubicin 0.4 mM) controls were included.

The larvae were distributed into two series of vials, and they were allowed to feed on the above medium for approximately 48 h. Only adult flies without the chromosome balancer (TM3, Sb^1), characterized by the absence of truncated bristles, were used. The hatched flies were stored in 70% ethanol.

3.4.2. Analysis of the flies

Adult flies of the *wts +/+ mwh* genotype, which have wild hairs (long and thin), were analyzed for tumor (*wart*) presence. The flies were observed using a stereoscopic magnifying glass and only tumors that were large enough to unmistakably classify were recorded. Each fly, immersed in glycerin ($C_3H_8O_3$), was analyzed using a thin brush. The tumor frequency was calculated as the number of tumors/number of *wts +/+ mwh* flies (NEPOMUCENO, 2015).

3.4.3. Statistical Analysis

The carcinogenic and the anticarcinogenic potentials of metformin were validated by the Mann, Whitney and Wilcoxon nonparametric *U* test, using α=0.05 level of significance.

4. RESULTS

The objective of the present study was to evaluate the mutagenic, recombinogenic and carcinogenic effect of metformin, as well as its possible modulatory effects on DNA induced damage induced by Doxorubicin (DXR). In this study, third-instar larvae (72 + 4h) from both tests, Somatic Mutation and Recombination Test (SMART) and Epithelial Tumor Clone Detection Test (WTS) were treated for approximately 48 h. Each treatment was done in duplicate. The data were collected after verification that there were no significant differences between the replicates.

The concentrations used of metformin alone or in combination with DXR were selected based on survival assay. The survival rates are presented in Table 1. According to the survival rate, it was possible to observe that none of the concentrations tested, including the controls, showed toxic effect. All rates were higher than 85% ($p > 0.05$). Similar results were observed by Slack et al. (2012). There was no significant reduction in the number of individuals treated with concentrations of 1, 2.5, 5, 10, 25 or 50 mM of metformin. On the other hand, there was a significant decrease in the survival of individuals treated with 100 mM.

4.1. SMART

The Somatic Mutation and Recombination Test (SMART) in wing somatic cells of *D. melanogaster* was used to assess the mutagenic and recombinogenic potential of metformin and its possible effects on modulating the damage induced by doxorubicin (DXR). The results for the marker transheterozygous (MH) and balancer heterozygous (BH) descendants, derived from the Standard Cross (ST), treated with different concentrations of metformin alone or in combination with DXR are shown in Table 2.

The DXR treatment, as expected, induced positive results for all categories of spots when compared to the negative control ($p < 0.05$). While the

positive control (DXR) significantly increased the number of spots in all classes of stains (single, large, twin and total) when compared to the negative control (ultrapure water). The statistically significant increases of twin spots indicate the recombinogenic activity of DXR.

As shown in Table 1, none of the five metformin concentrations (2.5, 5, 10, 25 or 50 mM) evaluated, significantly increased the total number of mutant spots ($p > 0.05$) in MH descendants of ST cross, when compared to the negative control.

Metformin suppressed the DNA damage induced by DXR without interfering in the recombinogenic activity of DXR (Figure 2). The simultaneous administration of DXR (0.4 mM) with metformin (2.5; 5; 10; 25 or 50 mM) presented a statistically significant reduction for the following categories of spots: small single, large single and total of spots when compared with DXR alone ($p < 0.05$). On the other hand, the same result was not observed for twin spots.

The simultaneous treatment with metformin (2.5, 5; 10; 25 or 50) and DXR was ineffective in the inhibition of all spot classes when compared to DXR alone ($p > 0.05$). Nevertheless, metformin (25 and 50 mM) reduced significantly the total frequency of spots (42.23% and 42.55% respectively).

Based on the clone induction frequency per 10^5 cells, we compared the number of observed spots in the MH and BH flies and quantified the contribution (%) of mutation and recombination to the total number of observed spots (FREI et al., 1992; GRAF et al., 1992; ABRAHAM, 1994). This procedure enabled us to quantify the contribution of mutagenic and recombinogenic events to the final genotoxicity observed (FREI et al., 1992; GRAF et al., 1992).

In the BH individuals of the ST cross, DXR (0.4 mM) induced a significant increase in the mutant spot frequency relative to the negative control. The wings of the BH flies resulting from the simultaneous application of metformin 25 or 50 mM with DXR (0.4 mM) were also scored. We found that the induced spots were mainly due to recombination, but metformin does not affect the recombinogenic activity of DXR (Figure 2).

The results of the HB cross of the SMART assay are depicted in Table 3. The results obtained with the MH individuals treated with metformin alone were negative at all tested concentrations. DXR statistically increased, as

expected, all categories of spots when compared to the negative control. Once more, the recombinogenic activity was the major response to DXR-induced DNA damage (94.60%).

When administered with DXR, only the highest concentration of metformin (50 mM) was found to statistically inhibit DXR-induced DNA damage (33.15%). Although the total number of spots diminished, there was an increase of recombination contribution to the total number of spots. It was again observed that metformin exhibits only antimutagenic activity and does not interfere with the recombinogenicity (Figure 2)

4.2. WTS

The epithelial tumor detection test in *D. Melanogaster* was used in order to estimate the contribution of somatic recombination in the process of cancer cell development. The present study evaluated the carcinogenic potential of metformin (2.5; 5; 10; 25 or 50 mM) as well as its protective effects in the presence of DXR (0.4 mM). A total of 200 flies of both sexes were analyzed in each group. The presence of epithelial tumor in different appendages was counted and the total frequency of epithelial tumor and the tumor rate in the appendices of the flies were compared pair-wise.

The results for tumor frequencies are shown in Table 4. In all concentrations evaluated, metformin was not able to induce a significant increase ($p > 0.05$) in the total tumor frequency of epithelial tumor, when compared to the negative control. However, DXR (0.4 mM) statistically increased the frequency of tumors in all appendices. For all treated series, the highest frequencies of tumors are located in the wing and in the body of the flies.

In order to evaluate the anticarcinogenic effect of metformin, the same simultaneous treatments performed in SMART assay were repeated. Metformin associated with DXR (0.4), regardless of the concentration (2.5; 5; 10; 25 or 50), was able to reduce the total tumor frequency. However, the reduction was not dose-dependent.

5. DISCUSSION

In the present study, the mutagenic and/or recombinogenic activities of MET alone, as well as its modulatory effects against DXR-induced DNA damage were evaluated using the ST and HB crosses of the Somatic Mutation and Recombination Test (SMART) in *D. melanogaster.* Similar results were obtained in both crosses. MET alone did not alter the frequency of spontaneous mutant spots in this test system, but displayed a modulating effect on DNA damage induced by DXR.

The reference mutagen (DXR) significantly increased the frequency of all mutant spot categories. Previous studies using the *Drosophila* wing SMART demonstrated that the main genetic contribution of DXR is its ability to induce recombination (COSTA et al., 2011; ORSOLIN et al., 2015, 2016). While MET was able to significantly reduce the total frequency of mutant spots in the two highest concentrations of the ST crosses (25 and 50 mM) and in the highest concentration of the HB crosses (50 mM), this drug was not able to alter the recombination pattern of DXR.

The differences in the results observed with DXR in both crosses are related to the basal and high levels of metabolic enzymes of cytochrome P450 complex present in the ST and HB crosses, respectively. Cytochrome P450 enzymes (CYPs) form one of the enzyme families involved in the metabolism of xenobiotics, including drug compounds. CYPs comprise many isoforms which catalyze a wide variety of reactions leading to different metabolites (OLSEN et al., 2015). The first cytochrome P450 cloned from *Drosophila* was the phase I enzyme cytochrome P450 6A2, which presents a peak of expression in the third larval and pupal stage. CYP6A2 mRNA was found to be present in the insecticide-resistant strain [OR(R)] at higher levels than in the insecticide-sensitive (flr^3) strain (SANER et al., 1996), which may explain the different frequencies of mutations observed with DXR in the ST and HB crosses.

Although the OR(R) lineage has higher levels of cytochrome P450 enzymes than the flr^3 strain, similar results were found in both crosses (ST and HB) when third-stage larvae were treated with MET alone, demonstrating that MET is not metabolized by CYPs. Likewise, and for that reason, no significant differences were observed in the percentages of inhibition of the mutant spot frequencies between individuals from ST and HB crosses treated

simultaneously with MET plus DXR. Thus, similar percentages of inhibition (37.33% and 34.59%) were found for individuals treated with MET 50 mM + DXR in the ST and HB crosses. This result is in line with literature data that MET is excreted unchanged in the urine and there is no evidence that it is metabolized (GRAHAM et al., 2011).

Previous studies have shown that MET (100, 500 or 2500 mg/kg) was neither genotoxic nor cytotoxic for normal and diabetic rats, and may protect from genomic instability induced by reactive oxygen species produced in T2DM (ATTIA et al., 2009). The clastogenic property of MET (concentrations ranging from 6.25 µg/ml to 1600 µg/ml) and of the analgesic drug Celecoxib (ranging from 2.34 µg/ml to 600 µg/ml) alone and in combination was assayed in human peripheral blood mononuclear cells (lymphocytes and monocytes) using single cell gel electrophoresis (Comet Assay). MET produced non-significant DNA damage, while Celecoxib produced significant DNA damage. The DNA damage or DNA tail protrusions by combinations of both drugs were less than what was observed with Celecoxib alone (ULLAH et al., 2016).

MET also was not clastogenic, but significantly decreased the frequency of X-radiation-induced chromosomal aberrations (dicentrics, acentric fragments, rings, micronuclei, and nucleoplasmic bridges) and the apoptotic incidence when human lymphocytes were pre-treated with MET (10 and 50 µM) for 2 h and irradiated with 6MV X-rays (CHEKI et al., 2016). Nevertheless, when mutagenic effect was determined by performing bacterial reverse mutation assay (Ames test) using *Salmonella typhimurium* mutated strains TA-100 and TA-98 with and without metabolic activation, MET and Celecoxib had no mutagenic effects, but their combined concentration exhibited mutagenic potential at much higher doses (ULLAH et al., 2016).

The carcinogenic potential of MET alone and its anti-carcinogenic potential against DXR-induced carcinogenicity were evaluated by the test for detection of epithelial tumor clones (Warts) in *D. melanogaster*. As expected, MET alone, at the same concentrations used in this study to assess its mutagenic/recombinogenic effects, did not induce tumor formation. On the other hand, MET displayed modulatory effects on the DXR-induced tumors at the four highest concentrations (5-50 mM). The significant reduction of tumors by MET

may have occurred by the same mechanisms involved in modulating the damage induced by DXR.

Although there is a lack of data in the scientific literature regarding to the use of metiformin and the increased or reduced risk of cancer, Franciosi et al. (2013) concluded, in a systematic review, that MET might be associated with a significant reduction in the risk of cancer and cancer-related mortality.

There is a large amount of evidence indicating that DNA damage is a major primary cause of cancer. DNA damage gives rise to mutations and epimutations that can cause progression to cancer. The importance of DNA damage and repair to the induction of carcinogenesis became evident when it was recognized that almost all carcinogens also are mutagens. Thus, all the effects of carcinogenic chemicals on tumor induction can be explained by the DNA damage that they cause and by the errors introduced into DNA during the repair of this damage (LODISH et al., 2000; BERNSTEIN et al., 2013). Algire et al. (2012) considered the possibility that cancer risk reduction by MET could be attributed at least in part to inhibition of mutagenesis. Thus, it is likely that the modulatory mechanisms exerted by MET on the reduction of mutant spots induced by DXR (antimutagenic effect) protected the DNA from a mutation and, consequently, also protected it from the onset of tumors.

The mechanisms by which MET reduces DNA damage and tumor formation induced by DXR were not directly evaluated in the present study. The hypothesis about the effect of MET when associated with DXR is that it has different characteristics capable of modulating the damage induced by this chemotherapeutic agent.

One of the characteristics of MET, which may be related to the modulation of DNA damage and/or DXR-induced tumors, is its antioxidant capacity. Previous studies demonstrated that MET significantly reduces intracellular ROS levels by increasing the expression of the antioxidant thioredoxin (Trx) through the AMP-activated protein kinase (AMPK) pathway. MET-regulated Trx at the transcriptional level and forkhead transcription factor 3 (FOXO3) were involved in this process (HOU et al., 2010); it attenuates paraquat-induced elevations in reactive oxygen species (ROS), and related DNA damage and mutations in AMPK$\alpha^{+/+}$ and AMPK$\alpha^{-/-}$ mouse embryonic fibroblasts (ALGIRE et al., 2012); reduced oxidative stress-related accumulation

of DNA damage on intestinal stem cells derived from *Drosophila* midgut (NA et al., 2013).

Besides, the effects of MET on AMPK and on nuclear factor-erythroid 2-related factor 2 (Nrf2) signaling, which plays a crucial role in protecting cells from oxidative damage, were tested in C2C12 mouse myoblasts cells *in vitro* and in Male C57BL/6J mice *in vivo*. MET activated AMPK and Nrf2 signaling and induced the expression of antioxidant genes NQO1 and γGCSm in C2C12 cells, and activated Nrf2 signaling and induced the expression of antioxidant genes such as HO1 and SOD, and resulted in increased GSH level in mouse liver and skeletal muscle tissues, demonstrating that MET activated Nrf2 signaling and enhanced the tissue antioxidant capacity (YANG et al., 2014b).

Clinical use of DXR is limited by its cardiotoxic side effects. Recent studies demonstrate that MET successfully prevents DXR-induced cardiotoxicity *in vivo* by inhibiting DXR-induced oxidative stress, energy starvation, and depletion of intramitochondrial coenzyme A (CoA-SH) (ASHOUR et al., 2012); or through its modulation of ferritin heavy chain (FHC) (ASENSIO-LÓPEZ et al., 2013; 2014).

However, it is possible that the modulatory effect exerted by MET may also be related to its anti-inflammatory effect. Inflammatory mediators are implicated in the production of ROS (KIM and CHOI, 2012). Recent studies have shown that MET protects against acute inflammatory responses by inhibiting ROS generation, fluid extravasation, and neutrophil migration *(*PANDEY and KUMAR, 2016*).* MET inhibits advanced glycation end (AGEs) products-induced inflammatory response in murine macrophages partly through AMPK activation and RAGE/NFκB (involved in AGEs-induced macrophage inflammatory activation) pathway suppression (JIN et al., 2015; ZHOU et al., 2016).

MET may also reduce the frequency of DXR-induced mutant spots and/or tumors by regulating the expression of different genes involved in the apoptosis process. Takahashi et al. (2014) demonstrated that MET may suppress Ishikawa endometrial cancer cell growth through the induction of cell cycle arrest and concomitant caspase-dependent apoptosis and enhanced autophagic flux. On the other hand, Sun et al. (2016) identified a novel signaling pathway that involves AMPK, p53, miR-23a, and FOXA1 in MET-caused

apoptosis in human hepatocellular carcinoma HepG2 cells; and Fang et al. (2014) demonstrated that MET inhibited A498 cell proliferation in a time- and dose-dependent manner, as well as induced the activation of AMP-activated protein kinase and promoted A498 cell apoptosis mediated by the downregulation of B-cell lymphoma 2 and concurrent upregulation of Bcl-2-associated X protein.

6. CONCLUSION

The results observed in our study allow us to conclude that, under the experimental conditions, MET has no mutagenic, recombinogenic or carcinogenic effects, but modulates the effects of DXR in the induction of DNA damage and tumors in *D. melanogaster*. Based on literature data, we may suggest that the modulatory effects of MET may be explained by its anti-oxidant and anti-inflammatory capacities, besides apoptotic induction.

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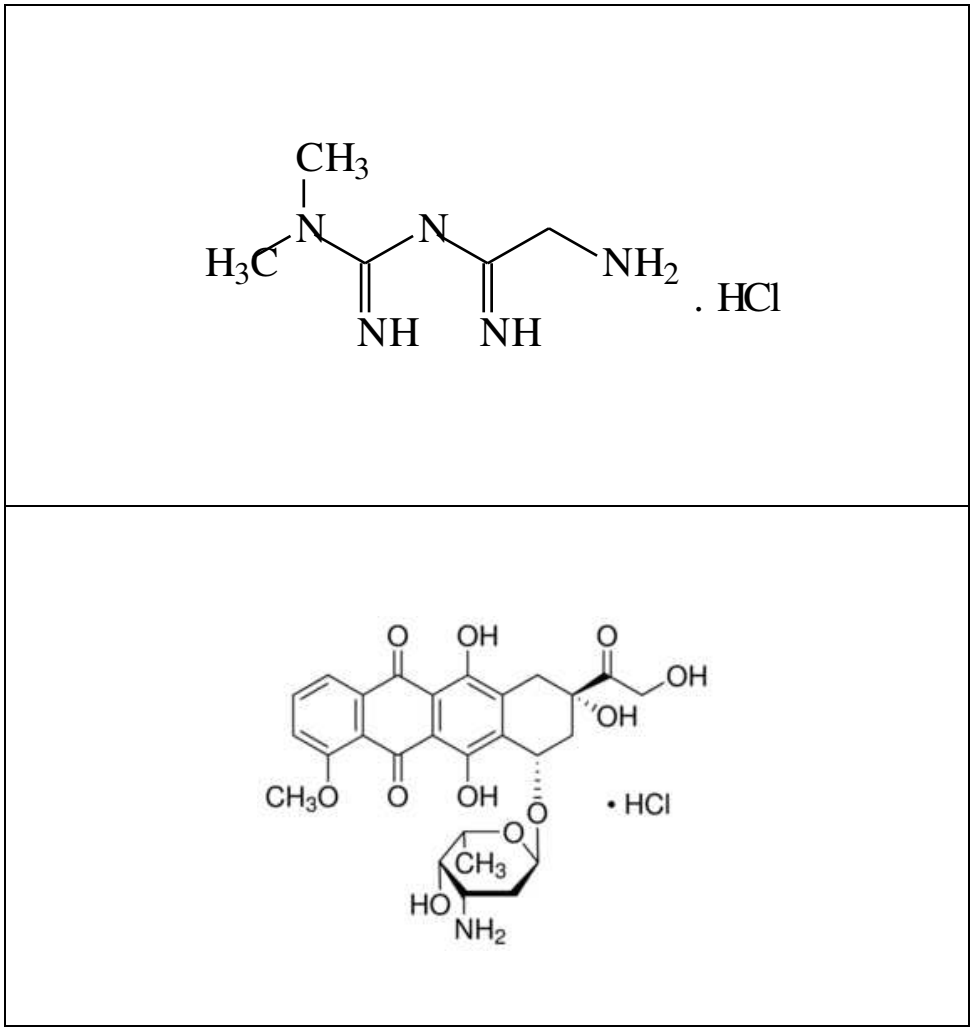
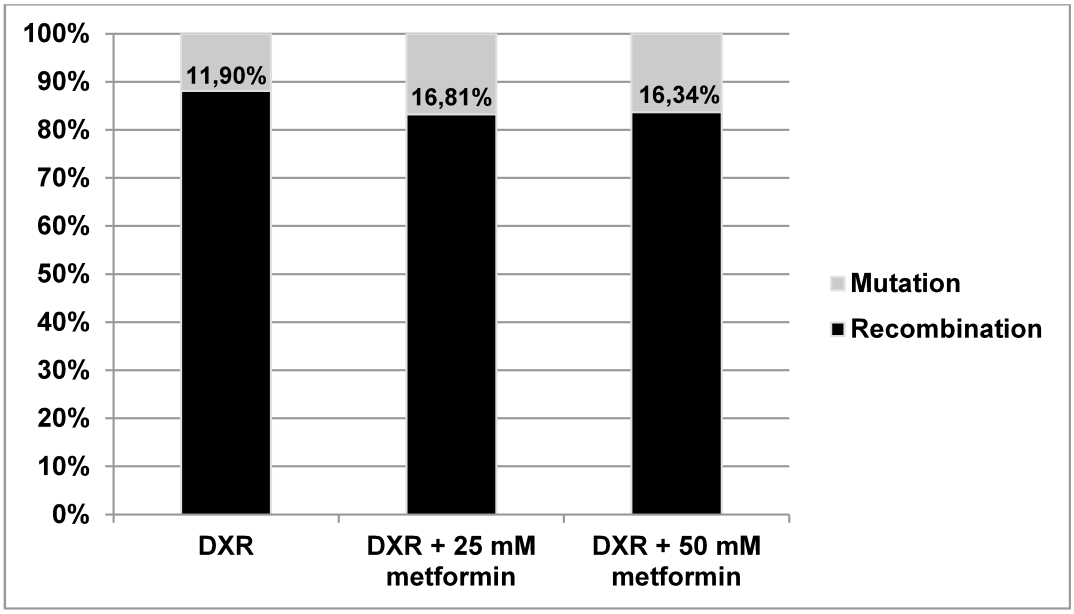


Figure 1. Structural formulas of the substances used in the present study: A. Metformin (MET); B. Doxorubicin (DXR) (Oliveira et al., 2017, in Food and Chemical Toxicology 106, 283-291).

A. Standard cross



B. High Bioactivation cross

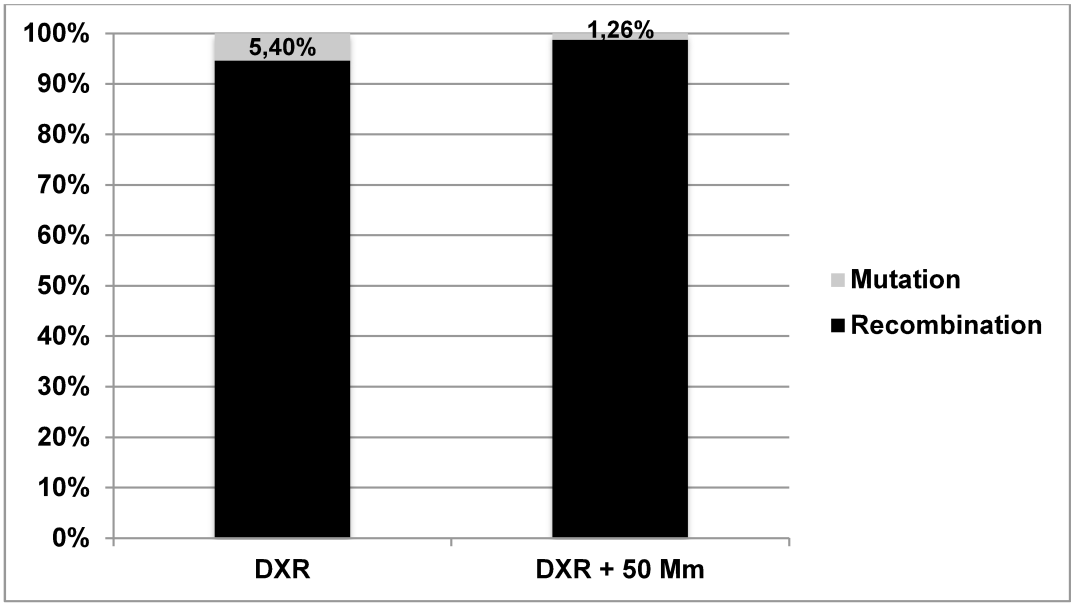


Figure 2. Contribution of recombination and mutation (in percentage) to total mwh wing spot induction observed in MH individuals from the ST and HB crosses treated with DXR alone and in combination with different concentrations of metformin (Oliveira et al., 2017, in Food and Chemical Toxicology 106, 283-291).

Table 1. Survival rates upon exposure to different concentrations of metformin (MET) in combination with doxorubicin (DXR) relative to control group (ultrapure water) in the wing Somatic Mutation and Recombination Test (ST and HB crosses) and in the Test for Detection of Epithelial Tumor Clones (warts) in *D. melanogaster*.

Treatments		**ST cross**		**HB cross**		**Warts cross**	
DXR (mM)	**MET (mM)**	**Survival (%)**	***p*-value**	**Survival (%)**	***p*-value**	**Survival (%)**	***p*-value**
0	0	100	>0.05	100	>0.05	94	>0.05
0	2.5	100	>0.05	100	>0.05	97	>0.05
0	5	97	>0.05	100	>0.05	100	>0.05
0	10	100	>0.05	97	>0.05	96	>0.05
0	25	100	>0.05	100	>0.05	100	>0.05
0	50	100	>0.05	90	>0.05	92	>0.05
0,4	0	97	>0.05	94	>0.05	95	>0.05
0,4	2.5	100	>0.05	100	>0.05	98	>0.05
0,4	5	100	>0.05	100	>0.05	100	>0.05
0,4	10	100	>0.05	97	>0.05	100	>0.05
0,4	25	90	>0.05	97	>0.05	94	>0.05
0,4	50	87	>0.05	87	>0.05	86	>0.05

Statistical comparisons of survival rates were made by using Chi-square test for ratios for independent samples (Ref.: Oliveira et al., 2017, in Food and Chemical Toxicology 106, 283-291).

Table 2. Sumary of results obtained with the Somatic Mutation and Recombination Test (SMART) in the marker-heterozygous (MH) and balancer trans-heterozygous progeny of the Standard (ST) cross after chronic treatment of larvae with metformin (MET), doxorubicin (DXR, positive control), and ultrapure water (negative control).

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis[a]				Spots with mwh clone[c]	Frequency of clone formation/10^5 cells per cell division[d]		Recombination (%)	Inhibition[e] (↓) (%)
DXR (mM)	MET (mM)		Small single spots (1-2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
mwh/flr[3]											
0	0	60	0.67 (40)	0.18 (11)	0.00 (00)	0.85 (51)	51	1.74			
0	2.5	60	0.32 (19) -	0.07 (04) -	0.00 (00) i	0.38 (23) -	23	0.79	-0.96		
0	5.0	60	0.43 (26) -	0.03 (02) -	0.00 (00) i	0.47 (28) -	28	0.96	-0.79		
0	10	60	0.33 (20) -	0.02 (00) -	0.00 (00) i	0.35 (21) -	21	0.72	-1.02		
0	25	60	0.60 (36) -	0.08 (00) -	0.00 (00) i	0.68 (41) -	41	1.40	-0.34		
0	50	60	0.67 (40) -	0.02 (00) -	0.00 (00) i	0.68 (41) -	41	1.40	-0.34		
0.4	0	60	2.60 (156) +	4.17 (250) +	0.17 (10) +	6.93 (416) +	413	14.11	12.36	88.10	
0.4	2.5	60	1.87 (112)	3.27 (12)	0.07 (04) i	5.20 (312)	310	10.59	8.85		↓ 28.39
0.4	5.0	60	1.90 (114)	3.32 (196)	0.03 (02) *	5.25 (315)	311	10.62	8.88		↓ 28.15
0.4	10	60	1.70 (102)	3.78 (199)	0.13 (08) i	5.62 (337)	336	11.48	9.73		↓ 21.27
0.4	25	60	1.52 (91) *	2.73 (164) *	0.10 (06) i	4.35 (261) *	260	8.88	7.14	83.19	↓ 42.23
0.4	50	60	1.52 (91) *	2.68 (161) *	0.15 (09) i	4.35 (261) *	259	8.85	7.10	83.66	↓ 42.55
mwh/TM3											
0	0	60	0.15 (09)	0.00 (00)	[f]	0.15 (09)	09	0.31			
0.4	0	60	0.72 (43) +	0.15 (09) i		0.87 (52) +	52	1.78	1.47		
0.4	25	60	0.62 (37) -	0.12 (07) i		0.73 (44) -	44	1.50	1.20		
0.4	50	60	0.72 (43) -	0.00 (00) i		0.72 (43) -	43	1.47	1.16		

Marker-trans-heterozygous flies (*mwh/flr3*) and balancer-heterozygous flies (*mwh/TM3*) were evaluated;
*P ≤ 0.05 vs. URE only;
[a]Statistical diagnoses according to Frei and Würgler (1988; 1995).U-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; P ≤0.05 vs. untreated control;
[b]Including rare *flr3* single spots;
[c]Considering mwh clones from mwh single and twin spots;
[d]Frequency of clone formation: clones/flies/48,800 cells (without size correction);
[e]Calculated as{[DXR alone – (DXR + Metformin) /DXR]} x 100, according to Abraham (1994);
[f]Balancer chromosome *TM3* does not carry the *flr3* mutation and recombination is suppressed, due to the multiply inverted region in these chromosome;
(Oliveira et al., 2017, in Food and Chemical Toxicology 106, 283-291).

Table 3. Sumary of results obtained with the Somatic Mutation and Recombination Test (SMART) in the marker-heterozygous (MH) and balancer trans-heterozygous progeny of the High-bioactivation (HB) cross after chronic treatment of larvae with metformin (MET), doxorubicin (DXR, positive control), and ultrapure water (negative control).

Genotypes and treatments		Number of flies	Spots per fly (number of spots) statistical diagnosis[a]				Spots with mwh clone[c]	Frequency of clone formation/10^5 cells per cell division[d]		Recombination (%)	Inhibition[e] (↓) or Induction[e] (↑) (%)
DXR (mM)	MET (mM)		Small single spots (1-2 cells)[b]	Large single spots (>2 cells)[b]	Twin spots	Total spots		Observed	Control Corrected		
mwh/flr3											
0	0	60	1.52 (91)	0.05 (03)	0.00 (00)	1.57 (94)	94	3.21			
0	2.5	60	0.87 (52) -	0.15 (09) i	0.00 (00) i	1.02 (61) -	60	2.05	-1.16		
0	5.0	60	1.15 (69) -	0.12 (07) i	0.00 (00) i	1.27 (76) -	76	2.60	-0.61		
0	10	60	1.27 (76) -	0.08 (05) i	0.02 (01) i	1.37 (82) -	82	2.80	-0.41		
0	25	60	1.05 (63) -	0.03 (02) i	0.00 (00) i	1.08 (65) -	65	2.22	-0.99		
0	50	60	1.17 (70) -	0.07 (04) i	0.00 (00) i	1.23 (74) -	74	2.53	-0.68		
0.4	0	60	2.60 (156) +	8.10 (486) +	2.08 (125) +	12.78 (767) +	685	23.39	20.18	94.60	
0.4	2.5	60	2.00 (120)	9.00 (540)	1.37 (82) *	12.37 (742)	711	24.28	21.07		↑ 4.41
0.4	5.0	60	2.23 (134)	7.55 (453)	1.75 (105)	11.53 (692)	623	21.28	18.07		↓ 10.45
0.4	10	60	1.98 (119)	9.17 (550)	1.82 (109)	12.97 (778)	734	25.07	21.86		↑ 8.32
0.4	25	60	2.63 (158) *	6.45 (387) *	1.65 (99)	10.73 (644)	619	21.14	17.93		↓ 11.15
0.4	50	60	1.60 (96) *	5.52 (331) *	1.67 (100)	8.78 (527) *	489	16.70	13.49	98.74	↓ 33.15
mwh/TM3											
0	0	60	0.43 (09)	0.00 (00)	[f]	0.45 (27)	27	0.92			
0.4	0	60	0.80 (48) +	0.15 (09) i		0.98 (59) +	59	2.02	1.09		
0.4	50	60	0.72 (26) *	0.00 (00) i		0.53 (32) *	32	1.09	0.17		

Marker-trans-heterozygous flies (*mwh/flr3*) and balancer-heterozygous flies (*mwh/TM3*) were evaluated;
*P ≤ 0.05 vs. URE only;
[a]Statistical diagnoses according to Frei and Würgler (1988; 1995).U-test, two sided; probability levels: -, negative; +, positive; i, inconclusive; P ≤0.05 vs. untreated control;
[b]Including rare *flr3* single spots;
[c]Considering *mwh* clones from *mwh* single and twin spots;
[d]Frequency of clone formation: clones/flies/48,800 cells (without size correction).
[e]Calculated as{[DXR alone – (DXR + Metformin)]/DXR} x 100, according to Abraham (1994);
[f]Balancer chromosome *TM3* does not carry the *flr3* mutation and recombination is suppressed, due to the multiply inverted region in these chromosome;
(Oliveira et al., 2017, in Food and Chemical Toxicology 106, 283-291).

Table 4. Tumor clone frequency observed in *D. melanogaster* heterozygote for the *wts* tumor suppressor gene, after chronic treatment of larvae with metformin (MET), doxorubicin (DXR, positive control), and ultrapure water (negative control).

Treatments			Frequency of tumors analyzed (total of tumors)							
MET (mM)	DXR (mM)	Number of flies	Eye	Head	Wing	Body	Leg	Halter	Frequency of tumor/fly (Total)	Reduction (%)
0	0	200	0.01 (03)	0.04 (09)	0.06 (13)	0.07 (15)	0.03 (07)	0.01 (02)	0.24 (49)	
0	0.4	200	0.64 (128)*	0.52 (104)*	2.73 (546)*	1.25 (250)*	0.98 (196)*	0.38 (76)*	6.49 (1300)*	
2.5	0	200	0.01 (03)	0.04 (08)	0.03 (07)	0.08 (16)	0.02 (05)	0.00 (00)	0.19 (39)	
5	0	200	0.01 (02)	0.02 (04)	0.02 (05)	0.06 (12)	0.05 (11)	0.01 (03)	0.18 (37)	
10	0	200	0.02 (04)	0.02 (04)	0.03 (07)	0.08 (16)	0.03 (07)	0.00 (01)	0.19 (39)	
25	0	200	0.01 (02)	0.02 (04)	0.04 (09)	0.04 (09)	0.05 (11)	0.01 (02)	0.18 (37)	
50	0	200	0.00 (01)	0.02 (05)	0.07 (15)	0.05 (10)	0.02 (05)	0.00 (01)	0.18 (37)	
2.5	0.4	200	0.60 (121)	0.55 (110)	2.54 (508)	1.31 (263)	0.78 (156)	0.31 (62)	6.10 (1220)	
5	0.4	200	0.34 (69)	0.31 (62)	1.65 (331)	0.82 (164)	0.72 (145)	0.22 (44)	4.07 (815)**	37.30
10	0.4	200	0.41 (82)	0.39 (78)	1.63 (327)	0.91 (183)	0.67 (134)	0.20 (40)	4.22 (844)**	35.07
25	0.4	200	0.23 (47)	0.16 (32)	0.90 (180)	0.44 (88)	0.39 (78)	0.09 (18)	2.21 (443)**	65.92
50	0.4	200	0.10 (20)	0.13 (26)	0.53 (106)	0.22 (45)	0.20 (40)	0.07 (14)	1.25 (251)**	80.69

Statistical diagnosis according to the Mann–Whitney Test; Level of significance $p \leq 0.05$;

* different from negative control (ultrapure water);

** different from the positive control (DXR, 0.4 mM);

(Oliveira et al., 2017, in Food and Chemical Toxicology 106, 283-291).

Attachment 1

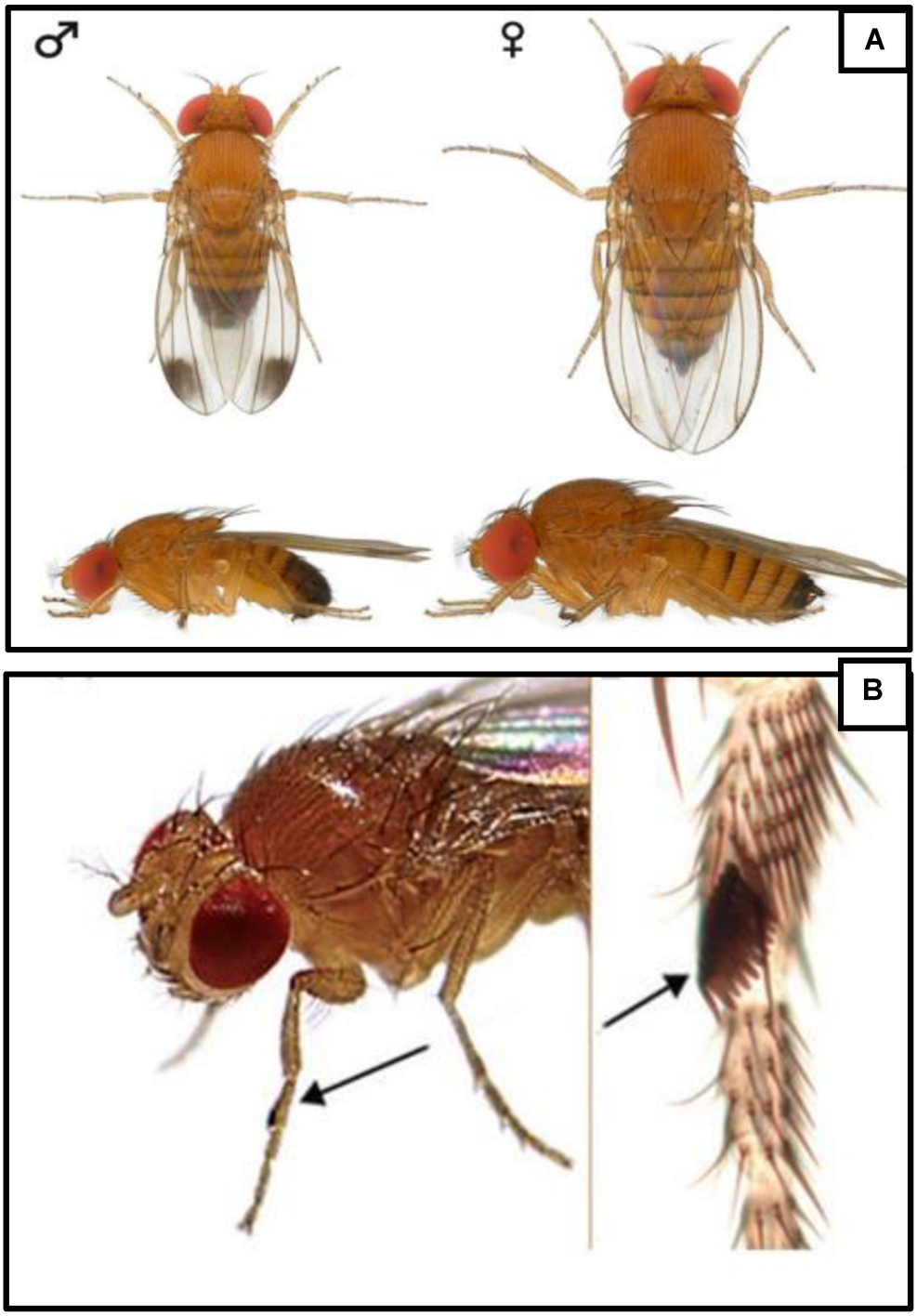


Figure 3. Representation of *Drosophila melanogaster*. A. The male (left) is smaller and has a sexual comb, and the female (right) is larger and has no sexual comb. B. Male sexual comb (represented by the arrow).

Source: <http://dica.madeira.gov.pt/index.php/producao-vegetal/pragas-e-doencas/1110-drosophila-suzukii-a-drosofila-de-asa-manchada>. Acess: January 14, 2017.

Appendice 1

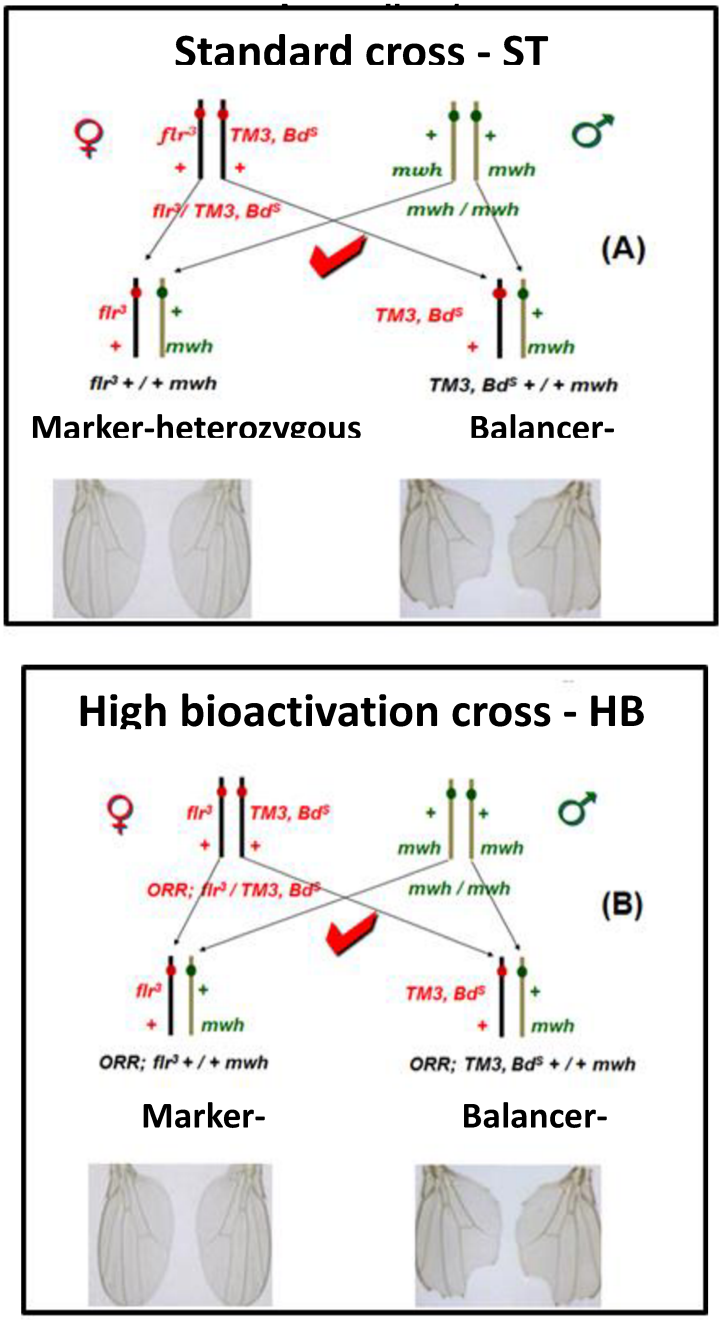


Figure 4. Somatic Mutation and Recombination Test: Schematic representation of the standard (ST) cross (A) and high bioactivation (HB) cross (B).

Appendice 2

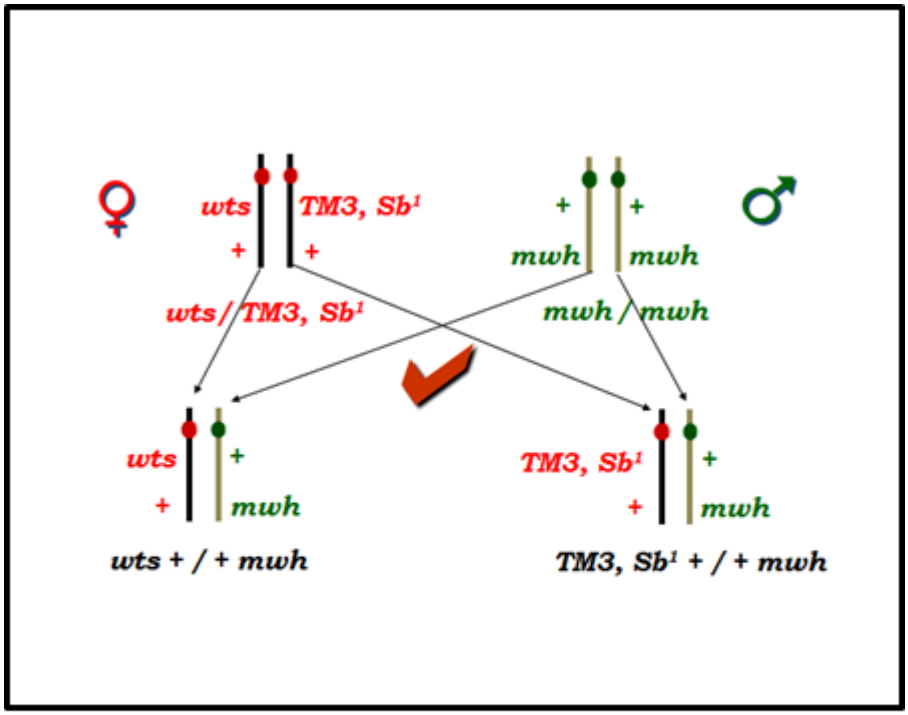


Figure 5. Schematic representation of the cross used in the Test for Detection of Epithelial Tumor Clones in *Drosophila melanogaster*.

Appendice 3

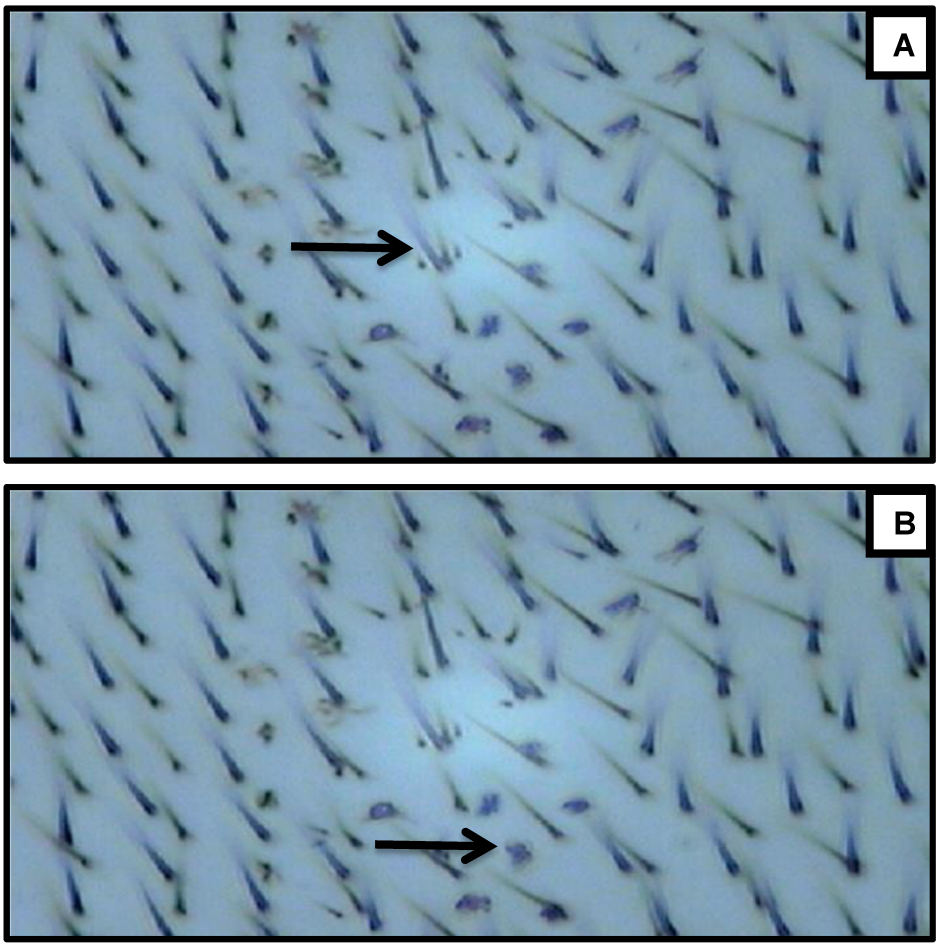


Figure 6: Photomicrographs showing multiple wing hais (mwh) (A) and flare (flr) hairs (B), obtained by light microscopy (magnification of 400x).

Appendice 4

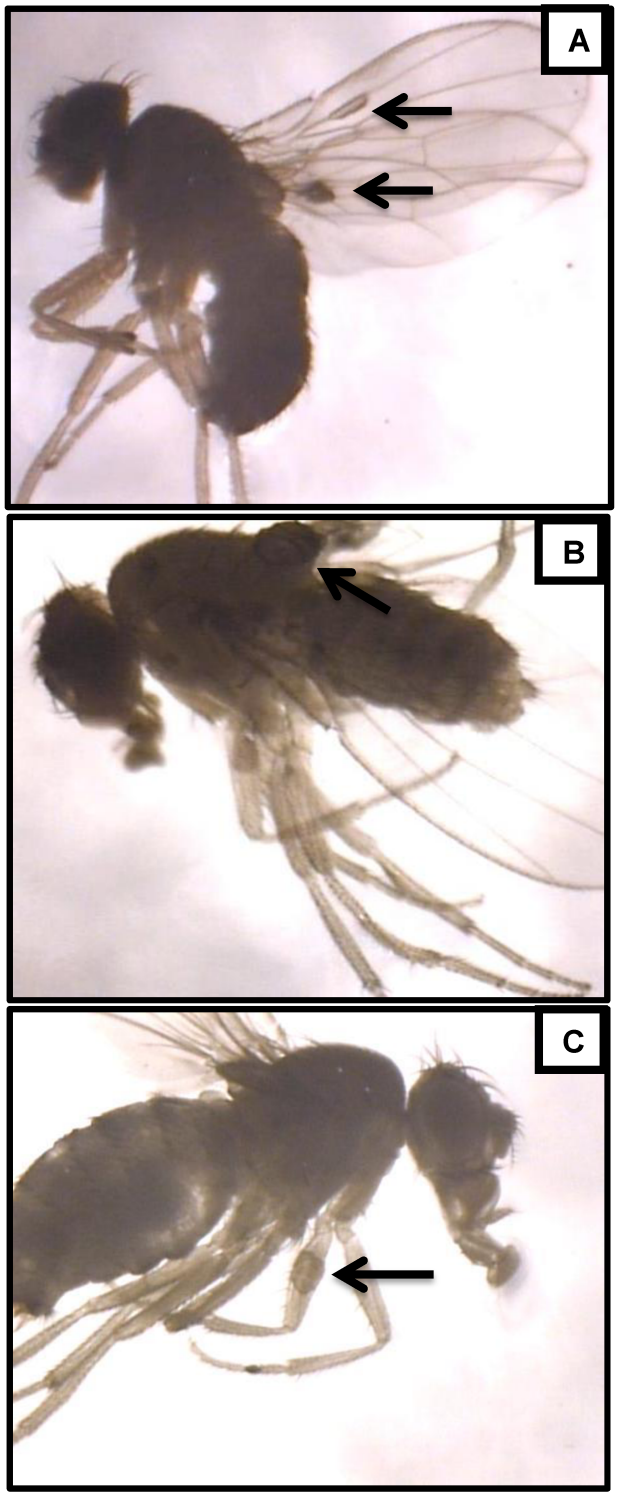


Figure 7: Expression of tumors in the different segments of *Drosophila melanogaster* (indicated by the arrows). A. Tumor on the wing. B. Tumor in the thorax. C. Tumor in the leg.

Appendice 5

Food and Chemical Toxicology 106 (2017) 283–291

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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Modulatory effects of metformin on mutagenicity and epithelial tumor incidence in doxorubicin-treated *Drosophila melanogaster*

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