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Research Article

Recombinogenic activity of Pantoprazole® in somatic cells of *Drosophila melanogaster*

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Abstract

Pantoprazole® is one of the leading proton pump inhibitors (PPIs) used in the treatment of a variety of diseases related to the upper gastrointestinal tract. However, studies have shown an increased risk of developing gastric cancer, intestinal metaplasia and hyperplasia of endocrine cells with prolonged use. In the present study, the somatic mutation and recombination test (SMART) was employed to determine the mutagenic effects of Pantoprazole on *Drosophila melanogaster*. Repeated treatments with Pantoprazole were performed on 72-hour larvae of the standard (ST) and high bioactivation (HB) crosses at concentrations of 2.5, 5.0, and 10.0 μM. In addition, doxorubicin (DXR) was administered at 0.4 mM, as a positive control. When administered to ST descendants, total number of spots were statistically significant at 2.5 and 5.0 μM concentrations. For HB descendants, a significant increase in the total number of spots was observed among the marked transheterozygous (MH) flies. Through analysis of balancer heterozygous (BH) descendants, recombinogenic effects were observed at all concentrations in descendants of the HB cross. In view of these experimental conditions and results, it was concluded that Pantoprazole is associated with recombinogenic effects in *Drosophila melanogaster*.

Keywords: *Drosophila melanogaster*, mutagenicity, proton pump inhibitors, Pantoprazole, recombinogenicity, SMART.

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Introduction

Pantoprazole [5 - (difluoromethoxy -2 - [[(3,4-dimethoxy-2-pyridinyl) methyl] sulfinyl]-1H-benzimidazol] is a weakly basic "prodrug" which accumulates in highly acidic environments and becomes rapidly activated in cationic sulfonamide (Raffin *et al.*, 2007; Vishvakarma and Singh, 2011). According to Stupnicki *et al.* (2004), it has a low potential for metabolic interaction with cytochrome P450 (CYP450) oxidation systems and is, for this reason, especially suitable for patients treated with other medications. Mathews *et al.* (2010) found that Pantoprazole is completely metabolized by the hepatic cytochrome P450 system, and more than 80% of the inactive metabolites are eliminated via renal excretion.

Yeo *et al.* (2008) tested the effects of several drugs used in the treatment of gastric cell tumors. They demonstrated that the cytotoxic effect of Pantoprazole triggers mitochondria-dependent apoptosis in the cells of the tumor. The long-term use of Pantoprazole, however, may result in hypergastrinemia, possible hyperplasia of the cells of the enteric nervous system, carcinoid tumors of the stomach, liver cell adenoma and other carcinomas as well as thyroid neoplasms (Pantoloc, 2003).

Genetic toxicology is an important field that studies the genotoxic/mutagenic properties of agents (chemical, physical and biological) to which organisms are exposed, using various assays to assess the damage that these may cause to DNA in the presence or absence of mass metabolic systems. These assays include the SMART (Somatic Mutation and Recombination Test) developed by Graf *et al.* (1984). The use of SMART on *Drosophila melanogaster* wings can detect a wide spectrum of genetic abnormalities, such as mutation, deletion and recombination (Graf *et al.*, 1984). The test is based on the fact that during early embryonic development of *D. melanogaster*, groups of cells composing imaginal discs proliferate mitotically until during metamorphosis they become differentiated into body structures of the adult fly. If there is a genetic alteration in an imaginal wing disc, a clone of mutant cells will be formed and detected as a spot on the wings of the mutant adult fly

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(Guzmán-Rincón and Graf, 1995). The analysis of these spots determines the phenotypic expression of the marker genes *flr*^{3} or *mwh*, responsible for changes in the shape of wing hairs or trichomes (Graf *et al.*, 1984).

The worldwide growing consumption of Pantoprazole and easy access to this drug, which no longer requires a medical prescription, have generated increased interest in assessing possible genotoxic effects associated with its use. Hence, the objective of the present study was to evaluate genotoxic effects of Pantoprazole by applying the *Drosophila melanogaster* wing spot test. Differences in the levels of cytochrome P450 on Pantoprazole genotoxic activity was evaluated by way of standard (ST) and high-bioactivation (HB) crosses of *Drosophila*. An HB cross is characterized by an increased cytochrome P450-dependent bioactivation capacity for promutagens when compared with an ST cross.

Material and Methods

Chemical compounds

Pantoprazole®, Lot No. 73078 (CAS 102625-70-7; Total impurity: ≤ 0.69%; Density: 0.88 g/mL), obtained from the University Pharmacy of the University Center of Patos de Minas (UNIPAM), Patos de Minas, Brazil, was prepared in three concentrations (2.5, 5.0 and 10.0 μM), based on research previously published by Masubuchi and Okazaki (1999) on primary cultured hepatocytes from female Sprague-Dawley rats. Doxorubicin hydrochloride (DXR) known by the trade name "Doxolen" (Lot No. 83520) was produced by Eurofarma Laboratories São Paulo, Brazil and distributed by Zodiac Pharmaceuticals SA, Sao Paulo, Brazil. In the present research, Doxolen was used at a concentration of 0.4 mM.

Somatic Mutation And Recombination Test (SMART) in somatic cells of *Drosophila melanogaster*

Strain stock crosses and treatment

For testing with SMART (Graf *et al.*, 1984), mutant strains of *D. melanogaster* were provided by Dr. Urich Graf of the Institute of Toxicology, University of Zurich, Schwerzenbach, Switzerland. Three mutant strains of *Drosophila melanogaster* with genetic markers were used in the study: *multiple wing hairs* (*mwh, 3-0.33*), *flare-3* (*flr*^{3}*, 3-38.8*) and *ORR; flare-3* (*ORR; flr*^{3}). Stocks of these strains were kept in a BOD incubator 411D New Ethics (Nova Ética Indústria Comércio e Serviços Ltda, São Paulo, Brazil) at a temperature of about 25 °C ± 2 and 60% humidity in 250 mL flasks containing a medium prepared with 820 mL of water, 11 g agar, 156 g of banana, 1 g of nipagin (Fagron do Brasil Farmacêutica, São Paulo, Brazil) and 25 g of biological yeast *Saccharomyces cerevisiae*.

Two types of crosses were performed: (1) a Standard Cross (ST), in which virgin females *flr*^{3}*/ In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} Bd^s were crossed with *mwh/ mwh* males, and (2) a High Bioactivation Cross (HB), with virgin females *ORR; flr*^{3}*/ In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *Bd*^{s} crossed with *mwh/mwh* males. In both crosses, two types of offspring were obtained: trans-marker heterozygous (MH) with the (*mwh +/+ flr*^{3}) genotype and wings phenotypically of the wild type; and heterozygous balancer (BH) with the (*mwh +/+ Bd*^{s} *TM3*) genotype and wings phenotypically serrated. The larvae, of both genotypes from these crosses were treated with three concentrations of the chemical agent to be tested.

Eggs were collected for a period of 8 h in flasks containing solid agar (4% agar in water) and a layer of yeast (*S. cerevisiae*) supplemented with sugar. After 72 ± 4 h, the third instar larvae were washed with reverse osmosis water and collected in a fine mesh steel sieve. Groups of approximately 100 larvae were transferred to glass tubes (2.5 cm in diameter and 8.0 cm in height) containing 1.5 g of a culture medium of instant mashed potatoes (HIKARI®, São Paulo Brazil) and 5.0 mL of each of three concentrations of the agent to be tested. The emerging adult flies were collected and stored in 70% ethanol.

The 72-hour old larvae from both crosses (ST and HB) were transferred to 2.5 cm x 8.0 cm high glass tubes containing 1.5 g of instant mashed potatoes with three concentrations of Pantoprazole: 2.5 μM, 5.0 μM or 10.0 μM. The larvae subsequently continued on to develop through the pupal stage (48 h). Reverse osmosis water was used as a solvent and negative control and doxorubicin (DXR, 0.4 mM) as a positive control. DXR was used as positive control, because in SMART assays with *D. melanogaster* it was classified as a strong mutagen, inducing all types of wing spots (Orsolin *et al.*, 2012).

Preparation and microscopic analysis of the wings

The wings of adult flies preserved in 70% ethanol were removed with entomological forceps under a stereomicroscope. They were soaked in Faure solution (30 g of gum arabic, 20 mL of glycerol, 1.5 g chloral hydrate and 50 mL distilled water), and stretched on slides. The slides were dried for approximately 2 h on a hot plate (40 °C). Finally, a cover slip was applied and the wings were coded. Wing spot analysis was performed using a light optical microscope at 400X magnification (40x). The number, types (single or twin), position and size of the spots were calculated and recorded. Approximately 48,000 cells were analyzed per fly.

Statistical analysis

Statistical analysis of the experiment was performed using a chi square test as described by Frei and Würgler (1988). The non-parametric *U* test of Mann-Whitney and a Wilcoxon test were used to exclude false positive results.

For the analysis of anti-mutagenicity, the frequencies of each type of spot were compared in pairs, using the *U* test (Frei and Würgler, 1995) at a significance level of $\alpha = 0.05$.

Results and Discussion

All of the compounds were tested in two different experiments. The data were pooled after verifying that the two independent experiments were in agreement with acceptable reproducibility. No significant decreases in the survival rates of larvae submitted to treatments were observed when compared to the negative control. The maximum concentration used in our study corresponded to plasma levels found in patients treated with single oral dose of 40 mg of Pantoprazole (Kamdi and Palkar, 2013). The maximum values found by the authors were seen at 2 h 56 min after exposure.

Table 1 shows the results of mutant spot frequencies observed in the BH and MH descendants of the Standard Cross (ST), treated with Pantoprazole in three different concentrations (2.5, 5.0 or 10.0 μM). The positive control (DXR 0.4 mM) and negative control (reverse osmosis water) are also presented. Table 2 shows the results of mutant spot frequencies observed in the BH and MH descendants of the high bioactivation cross (HB), for the same concentrations of Pantoprazole, as well as the positive and negative controls.

When compared to the negative control, Pantoprazole caused a significant increase in the frequency of small, simple spots at all concentrations. The total number of spots, however, was only statistically significant at 2.5 and 5.0 μM. Results for the HB cross in terms of the potential mutagenic properties of Pantoprazole are presented in Table 2. The total number of mutant spots among the MH descendants, compared to the negative control, was significantly increased in all concentrations.

The analysis of flies with the BH genotype (*mwh/TM3*) was carried out for the purpose of calculating the portion of recombinogenic and mutagenic events. It is possible to separate mutational events from recombinational events, because recombinational events are eliminated in flies with this genotype. A comparison of clone-induction frequencies obtained for Pantoprazole in both genotypes indicated that in ST flies, 49.76% of mutant clones produced by Pantoprazole were due to mutation and 50.24% due to recombination at the 2.5 μM concentration. Clone-induction frequencies for Pantoprazole (5.0 μM) indicated that 52.85% of the mutant clones produced were due to mutation and 47.15% to recombination. However, the very same analysis showed that in HB flies, 31.30% of spots induced by Pantoprazole (2.5 μM) were due to mutation and 68.70% to recombination; 37.56% of spots induced by Pantoprazole (5.0 μM) were due to mutation and 62.44% to recombination; 35.48% mutant clones produced by Pantoprazole (10 μM) were due to mutation and 64.52 to recombination. Thus, our results indicate that recombinogenicity was the major genotoxic effect of Pantoprazole in HB flies.

Many compounds are converted to highly reactive metabolites by oxidative enzymes, principally cytochrome P450. Thus, by introducing one or more hydroxyl groups on a substrate, a pre-carcinogen can become a carcinogen (Gregory, 1986). The genetic control of xenobiotic metabolism in *Drosophila* is complex, and multiple forms of P450 as well as other enzymes (*e.g.*, amine oxidases) are known to be involved in the activation of certain promutagens (Frölich and Würgler, 1989). According to our findings, Pantoprazole has a clear recombinogenic potential in *Drosophila*, and the stock differences demonstrate a strong dependence on levels of metabolic activation (HB flies) as the increased cytochrome P450-dependent biocativation capacity present in these HB larvae leads to significantly increased recombinogenicity. Therefore, the metabolic pathway in the induction of recombinogenicity most probably involves cytochrome P450-dependent enzyme activity. In accordance with these findings, Mathews *et al.* (2010) showed that Pantoprazole is completely metabolized by the hepatic cytochrome P450 system.

Although homologous recombination is an important pathway in DNA repair, there is growing evidence that deleterious genomic rearrangements may result from homologous recombination, which means that homologous recombination events may play a causative role in carcinogenesis (Arossi *et al.*, 2009). The transformation of normal cells into cancer cells is a multistep process, and mitotic recombination can be a mechanism involved in such transformation (Nowell, 1976; Barrett, 1993). In heterozygous cells bearing a mutant and normal alleles for a tumor suppressor gene, somatic recombination may turn out to be a promoter of neoplasms by inducing homozygosis of the mutant tumor suppressor allele (Maher *et al.*, 1993; Sengstag, 1994).

Kuipers (2006) has stated that prolonged use of proton pump inhibitors (PPIs) may be related to the development of gastric cancer. It is also known that treatment with PPIs does not protect against this type of cancer, but the increased risk of cancer due to prolonged use remains unknown. Thomson *et al.* (2010) have reported that among patients diagnosed as negative for *H. pylori* and without pre-existing gastritis, PPIs did not cause chronic gastritis. In contrast, people infected by *H. pylori* were found to have chronic, persistent gastritis, atrophy and metaplasia, which may progress to gastric atrophy, intestinal metaplasia and gastric cancer. PPIs used in the treatment of this infection may also cause an acceleration of the progression or development of gastritis (Thomson *et al.*, 2010). Nonetheless, until now there is no evidence that PPIs increase the risk of gastric cancer. Persistent, predominant gastritis and atrophic gastritis of the gastric body mucosa, however, are considered important risk factors for the development of

Table 1 - Frequency of mutants spots observed in the marked trans-heterozygotes descendants (MH) of *Drosophila melanogaster* derived from the standard (ST) cross treated with different Pantoprazole concentrations (2.5, 5.0 and 10.0 µM), positive control (DXR 0.4 mM) and negative control (reverse osmosis water).

Genotypes and concentrations (mM)	Number of flies (*N*)	Spots per fly (No. of spots); statistical diagnosis[a]												Spots with mwh[c] clone (*n*)	Mean clone + class[c,d] (*i*)		Frequency of clone formation/10^5 cells per cell division			
		Small single (1-2 cells)[b] *m* = 2			Large single (> 2 cells)[b] m = 5			Twin *m* = 5			Total spots m = 2						Observed[d,e] *n/NC*		Control corrected[d] (2^{i-2}) X (*n/NC*)	
mwh/flr^3																				
Negative control	50	0.36	(18)		0.06	(3)		0.00	(0)		0.42	(21)		20	1.75		0.82		0.69	
DXR 0.4 mM	50	4.90	(245)	+	5.92	(296)	+	3.30	(165)	+	14.12	(706)	+	674	3.29	{3.34}	27.62	{26.80}	67.72	{67.90}
Pantoprazole 2.5 µM	50	0.90	(45)	+	0.08	(4)	i	0.02	(1)	i	1.00	(50)	+	50	1.56	{1.43}	2.05	{1.23}	1.51	{0.83}
Pantoprazole 5.0 µM	50	0.82	(41)	+	0.08	(4)	i	0.04	(2)	i	0.94	(47)	+	47	1.62	{1.52}	1.93	{1.11}	1.48	{0.79}
Pantoprazole 10.0 µM	50	0.66	(33)	+	0.02	(1)	i	0.00	(0)	i	0.68	(34)	i	34	1.44	{1.00}	1.39	{0.57}	0.95	{0.29}
mwh/TM3																				
Negative control	20	0.35	(7)		0.00	(0)					0.35	(7)		7	1.71		0.72		0.59	
DXR 0.4 mM	20	0.75	(15)	i	0.10	(2)	i				0.85	(17)	+	17	1.71	{1.70}	1.74	{1.02}	1.42	{0.83}
Pantoprazole 2.5 µM	20	0.45	(9)	i	0.05	(1)	i				0.50	(10)	i	10	1.50	{1.00}	1.02	{0.31}	0.72	{0.15}
Pantoprazole 5.0 µM	20	0.45	(9)	i	0.05	(1)	i				0.50	(10)	i	10	1.70	{1.67}	1.02	{0.31}	0.83	{0.24}

m, multiplication factor for significantly negative results. Level of significance a = b = 0.05.
[a]Statistical diagnostic according to Frei and Würgler (1988): (+) positive (compared to the negative control); (-) negative; (i) inconclusive.
[b]Including rare single *flr*^{3}spots.
[c]Considering the *mwh* clones for the single spots and *mwh* for the twin spots.
[d]Frequency of clone formation: clones/flies/48,800 cells (without size correction).

Table 2 - Frequency of mutants spots observed in the marked trans-heterozygotes descendants (MH) of *Drosophila melanogaster* derived from the high bioactivation cross (HB) treated with different Pantoprazole concentrations (2.5, 5.0 and 10.0 µM), positive control (DXR 0.4 mM) and negative control (reverse osmosis water).

Treatments	Number of flies (*N*)	Spots per fly (No. of spots); statistical diagnosis[a]												Spots with mwh[c] clones (*n*)	Mean clone size class[c,d] (*i*)		Frequency of clone formation/10^5 cells per cell division			
		Small single (1-2 cells)[b] *m* = 2			Large single (> 2 cells)[b] *m* = 5			Twin *m* = 5			Total spots *m* = 2						Observed[d] *n/NC*		Control corrected[d] (2^{i-2}) X (*n/NC*)	
mwh/flr^3																				
Negative control	50	1.26	(63)		0.12	(6)		0.02	(1)		1.40	(70)		70	1.57		2.87		2.13	
DXR 0.4 mM	50	2.74	(137)	+	1.28	(64)	+	0.42	(21)	+	4.44	(222)	+	216	2.72	{3.27}	8.85	{5.98}	14.56	{14.40}
Pantoprazole 2.5 µM	50	2.26	(113)	+	0.14	(7)	i	0.00	(0)	i	2.40	(120)	+	120	1.48	{1.34}	4.92	{2.05}	3.42	{1.30}
Pantoprazole 5.0 µM	50	1.90	(95)	+	0.10	(5)	i	0.02	(1)	i	2.02	(101)	+	100	1.43	{1.10}	4.10	{1.23}	2.76	{0.66}
Pantoprazole 10.0 µM	50	1.96	(98)	+	0.14	(7)	i	0.02	(1)	i	2.12	(106)	+	106	1.51	{1.39}	4.34	{1.48}	3.09	{0.97}
mwh/TM3																				
Negative control	20	0.95	(19)		0.05	(1)					1.00	(20)		20	1.25		2.05		1.22	
DXR 0.4 mM	20	1.10	(22)	i	0.05	(1)	i				1.15	(23)	-	23	1.35	{2.00}	2.36	{0.31}	1.50	{0.31}
Pantoprazole 2.5 µM	20	0.75	(15)	-	0.00	(0)	i				0.75	(15)	-	15	1.13	{1.60}	1.54	-{0.51}	0.84	-{0.39}
Pantoprazole 5.0 µM	20	0.70	(14)	-	0.05	(1)	i				0.75	(15)	-	15	1.47	{0.60}	1.54	-{0.51}	1.06	-{0.19}
Pantoprazole 10.0 µM	20	0.70	(14)	-	0.05	(1)	i				0.75	(15)	-	15	1.20	{1.40}	1.54	-{0.51}	0.88	-{0.34}

m, multiplication factor for significantly negative results. Level of significance a = b = 0.05. Níveis de significância a = b = 0.05.
[a]Statistical diagnostic according to Frei and Würgler (1988): (+) positive (compared to the negative control); (-) negative; (i) inconclusive.
[b]Including rare single *flr*^{3}spots.
[c]Considering the *mwh* clones for the single spots and *mwh* for the twin spots.
[d]Frequency of clone formation: clones/flies/48,800 cells (without size correction).

gastric cancer, and, clearly, more studies are needed to reach a definitive conclusion (Kuipers, 2006).

By means of *in vivo* experiments Chen *et al.* (2012) showed that Pantoprazole pretreatment could enhance the anti-tumor effects of adriamycin on xenografted tumor in nude mice and also improve the apoptotic index in xenografted tumor tissues. According to the authors, Pantoprazole pretreatment enhances the cytotoxic effects of anti-tumor drugs on human gastric adenocarcinoma cells (SGC7901) and reverts multidrug resistance of SGC7901/ADR cells by down-regulating the V-ATPases/mTOR/HIF-1α/P-gp and MRP1 signaling pathway. Shen *et al.* (2013) also showed that pantoprazole inhibits the proliferation and induced apoptosis of SGC7901 human gastric cancer cells. Finally, according to Patel *et al.* (2013), the use of Pantoprazole to enhance the distribution and cytotoxicity of anticancer drugs in solid tumors might be a novel treatment strategy to improve their therapeutic indices.

It is worthy of note that tests for mutagenic evaluation are generally limited to such specific effects and that not every change in genetic material is a mutation. For this reason, SMART is an important tool for mutagenic assessment. It provides an evaluation of mutational events, as well as recombinogenic events, as shown in the present study. It is known that substances that cause DNA damage also induce recombination, which generates more DNA damage (Hoffman, 1994). Recombination can promote loss of heterozygosity in somatic cells and germ cells which, in turn, may influence cancer progression (Happle, 1999).

Brambilla *et al.* (2010) reviewed the genotoxic and carcinogenic effects of 71 gastrointestinal drugs, demonstrating that Pantoprazole was found to be genotoxic and carcinogenic in several assays, in addition to causing chromosomal damage. These results are consistent with those presented herein, where Pantoprazole caused an increase in the frequency of mutant spots for somatic cells, revealing its genotoxic characteristics. The genotoxicity of a particular substance is, thus, not exclusively caused by its association with mutation but also with recombination events, which may cause chromosomal damage. At current, the published studies are conflicting in their results, warranting further examination by means of additional assays and test organisms.

In conclusion, the present study indicates that Pantoprazole possesses recombinogenic activity in the *Drosophila* wing spot test. Nonetheless, although there was an increase in mutant spots in the ST descendants, the increase in recombinogenic activity was observed only in the high bioactivation (HB) descendants, this suggesting the interaction of their constituents (Pantoprazole) with cytochrome P450.

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