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Genotoxic studies in hypertensive and normotensive rats treated with amiodarone

Mara Ribeiro Almeida[a], Estela de Oliveira Lima[a], Valdo José Dias da Silva[a], Mateus Gandra Campos[a], Lusânia M.G. Antunes[b], Ana Karina Dias Salman[c,e], Francisca L. Dias[d,e,*]

[a] Depto. Ciências Biológicas, Universidade Federal do Triângulo Mineiro, Uberaba, MG, Brazil
[b] Depto. Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, SP, Brazil
[c] Embrapa Rondônia, Porto Velho, RO, Brazil
[d] Instituto de Pesquisa em Patologia Tropical-IPEPATRO, Porto Velho, RO, Brazil
[e] Faculdades Integradas Aparício Carvalho-FIMCA, Porto Velho, RO, Brazil

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ABSTRACT

Amiodarone, a benzofuran derivative, is a very effective antiarrhythmic medication, but has potential to cause side effects. Although its cytotoxicity potential is very well-known, there are few reports about its genotoxicity effects. Since amiodarone has not been investigated in genotoxicity studies, and the spontaneously hypertensive rat (SHR) is a well-characterized model for hypertension, the aim of the present study was to perform cytogenetic analysis on chromosome aberrations in bone marrow cells of SHRs and normotensive Wistar-Kyoto rats (WKYs) that received oral amiodarone treatment for 4 weeks. Amiodarone activity was also monitored using electrocardiograms. The presence of bradycardia in amiodarone-treated rats confirmed that this drug was really active. Metaphase analysis on bone marrow cells showed that there were significant differences in total chromosomal damage and percentage abnormal metaphase between WKY and SHR negative controls. In the SHR negative control, the frequencies of basal chromosomal aberrations and abnormal metaphases were significantly higher ($p < 0.05$). There were high numbers of chromosomal aberrations in all amiodarone-treated groups, compared with negative controls. In amiodarone-treated groups, the most frequent chromosomal aberration was chromatid breaks. More chromosomal aberrations were found in WKYs that received amiodarone, with a statistically significant difference in comparison with negative controls ($p < 0.05$). However, in SHR rats there was no significant difference between the amiodarone and negative groups regarding chromosomal damage induction. These results showed that treatment with amiodarone was genotoxic in WKYs, but not in SHRs. Further studies are needed to confirm whether amiodarone is genotoxic or efficient and harmless, among humans undergoing therapy.

1. Introduction

Arterial hypertension is an important risk factor for many cardiovascular disorders, including myocardial infarction, heart failure and coronary diseases [1]. Under hypertensive conditions, many functional organs can suffer irreparable lesions [2]. Essential hypertension is a common trait caused by many factors, and it gives rise to increased risk of cardiovascular, cerebrovascular and renal disease [3]. Although it has long been accepted that essential hypertension has a genetic basis, recent experimental evidence has suggested that oxidative stress plays an important role in the development of high blood pressure in some animal models [3]. Oxidative modification of DNA, protein and lipids by reactive oxygen species plays a role in aging and disease progression, including in relation to cardiovascular, neurodegenerative and inflammatory diseases and cancer [4]. Overproduction of reactive oxygen species may contribute towards the pathogenesis of hypertension by affecting vascular contraction and causing organ damage.

Achieving effective control over established hypertension has not been an easy goal. Although numerous experimental and clinical studies have proven the efficiency of treatment with antihypertensive drugs, some patients requiring antihypertensive therapies need long-term administration of drugs, which may cause severe adverse reactions [1]. According to Brambilla and Martelli [5], full data as required by the guidelines for testing pharmaceutical

Abbreviations: SHR, spontaneously hypertensive rat; WKY, normotensive Wistar-Kyoto rat.

* Corresponding author at: Faculdades Integradas Aparício Carvalho-FIMCA, Rua das Araras, no. 241, Jardim Eldorado, Porto Velho, RO, CEP 78912-640, Brazil. Tel.: +55 69 3217 8922/3219 3156.
E-mail address: franluzdias@yahoo.com.br (F.L. Dias).

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products was only available for 42 of the 164 antihypertensives on the market. Few data concerning the genotoxic and carcinogenic evaluation of antihypertensive drugs are available.

Amiodarone, a benzofuran derivative, was initially developed in the 1960s to treat angina pectoris. This drug is considered to be an important antiarrhythmic agent for maintaining the sinus rhythm and it is widely used for patients with paroxysmal atrial fibrillation [6,7]. As a type III antiarrhythmic agent, the main action mechanism of amiodarone is through blocking the myocardial potassium channels, but it also possesses some beta-blocking properties. Thus, chronic administration of amiodarone accumulates in high concentrations in the liver, lungs and adipose tissue. Amiodarone undergoes almost exclusively hepatic biotransformation into its major pharmacologically active metabolite, N-desethylamiodarone, which can accumulate in the lungs and liver in concentrations up to four times greater than those of the parent compound [2,8].

By cytogenetic assaying, the changes induced in cells following exposure to genotoxic agents can be analyzed and quantified. Chromosomal aberrations are changes in chromosome structure, visible under an optical microscope, that represent a significant fraction of the abundant variety of changes that occur to chromosomal DNA under the action of genotoxic agents [9]. Amiodarone has not yet been investigated in genotoxicity studies, but it has been found that the arterial blood pressure of spontaneously hypertensive rats (SHRs), a well-characterized model for hypertension, was reduced by using chronic administration of amiodarone [2]. Therefore, the aim of the present study was to perform a cytogenetic analysis on chromosome aberrations in bone marrow cells from SHRs and normotensive Wistar-Kyoto rats (WKYs) that received oral amiodarone treatment. Amiodarone activity was also monitored using electrocardiograms.

2. Materials and methods

2.1. Chemical agents

The molecular formula of amiodarone is $C_{25}H_{29}I_2NO_3$ and its full chemical name is 2-butyl-3-[4-(3-diethylamino-1-oxapropyl)-3,5-diiodobenzoyl]-benzofuran (CAS No. 1951-25-3). It was supplied by Indústria de Medicamentos Pharma Nostra (Rio de Janeiro, Brazil) and was dissolved in drinking water (2 mg/ml). Cyclophosphamide (CAS No. 50-18-0) was obtained in the form of Cytoxan® (English) and was dissolved in water. All other chemicals were of the highest purity commercially available.

2.2. Animals and treatments

The animal protocols were approved by the Ethics Committee for Animal Use of the Federal University of the Triângulo Mineiro, Brazil (protocol number 02). Sixteen-week-old female SHRs and WKY weighing 250–300 g were obtained from the Physiology Laboratory of the same university. The animals were acclimatized to laboratory conditions, with a room temperature of 23 °C, relative humidity of 70%, and light/dark photoperiods of 12 h each. The animals had free access to standard rat chow (Nuvilab CR1, NUVITAL S.A., Curitiba, PR, Brazil) and water was available *ad libitum*.

The female SHRs ($n = 21$) and age-matched WKYs ($n = 21$) were divided into three groups with seven animals in each group. The rats in the amiodarone groups were offered a solution of this drug for 4 weeks. The rats drank approximately 30 ml of this solution per day, thus receiving around 60 mg of amiodarone daily. This represented a dose of approximately 200 mg/kg per day. This dose of amiodarone was defined according to data in the literature. It has been shown that this dose of amiodarone does not present neurotoxicity to rodents [10].

The negative control groups received only tap water over the same period. The positive control groups also received only tap water over this period, but were injected intraperitoneally with cyclophosphamide (30 mg/kg of body weight) 24 h before sacrificing. On the last day of treatment, under sodium pentobarbital anesthesia (40 mg/kg, intraperitoneally), an electrocardiogram was recorded on all animals in each group. The animals were then sacrificed by ether inhalation after the last day of treatment.

2.3. Electrocardiogram

The electrocardiogram was recorded to evaluate the effects of the treatment (amiodarone vs. tap water). On the last day of the treatment, each animal underwent acute electrocardiogram studies under sodium pentobarbital anesthesia (40 mg/kg, intraperitoneally). The recordings was made using a bioelectric amplifier (model 8811A, Hewlett Packard, Whaltham, MA, USA) and were continuously sampled (at 1000 Hz) using a personal computer (Pentium 133 MHz) equipped with a 12-bit analog digital interface (CAD12/36, Lynx Tecnologia Eletrônica, São Paulo, SP), for 4 min for each animal. Electrodes were placed under the skin for recording the conventional bipolar limb leads (I, II and III), the unipolar limb leads (aVR, aVL and aVF), and the unipolar precordial (chest) leads (VA, immediately to the right of the sternum in the fourth intercostal space; VB, just to the left of the sternum in the fourth intercostal space; and VC, in the fifth intercostal space at the midaxillary line). The following electrocardiographic parameters were examined: (1) RR interval, defined as the interval between the apex of adjacent R waves; (2) QT interval, defined as the interval between the Q wave and the T wave apex; (3) P wavelength in D2, D3, aVF and VB; (4) PR interval, defined as the interval between the apex of the P wave and the Q wave; and (5) QRS length for each derivation. These parameters were calculated using the Aqdados software and tabulated in Excel 95 (Microsoft Corp). Furthermore, other parameters such as the presence of cardiac arrhythmias, atrioventricular and intraventricular blockages and other alterations were also analyzed.

2.4. In vivo chromosome aberration assay

All the animals were injected intraperitoneally with 0.5 ml 0.16% colchicine (Sigma–Aldrich) per 100 g of body weight, 90 min before they were sacrificed. Immediately after the animals had been sacrificed, the femurs were freed from adherent tissues and were dissected out. Bone marrow cells were collected from the femurs, subjected to hypotonic shock (KCl 0.075 M) and fixed three times using methanol-acetic acid, in accordance with the technique of Ford and Hamerton [11], with modifications.

The cells were spread on glass slides and then air-dried at room temperature to conserve them. Finally, they were stained with a 4% dilution of Giemsa reagent in Sorenson's phosphate buffer, at pH 6.8 for 10 min. After coding of the slides, 100 well-spread metaphases containing 42 ± 2 chromosomes were examined per animal to score for different types of aberrations. The slides were analyzed at a magnification of 1000× using an optical microscope (Nikon Eclipse E 400). Chromosome aberrations were identified according to criteria described by Savage [12].

2.5. Statistical analysis

All data were subjected to statistical analysis using the SigmaStat for Windows, version 2.03, software package. Data were presented as means ± standard deviations or median and interquartile range. The Kolmogorov–Smirnov's and Levene's tests were performed to verify the normality of distribution and homogeneity of the variance of the data, respectively. Data with non-normal distribution and/or non-homogenous variance were submitted to transformation procedures. A two-way ANOVA followed by Tukey's multiple comparison tests was performed to evaluate the effects of treatment and the arterial pressure state on ECG data. For all other

Table 1
Mean values (±S.E.M.) of ECG parameters measured in anesthetized WKY and SHR lines according to each treatment group.

	WKY			SHR		
	Negative control	Amiodarone	Positive control	Negative control	Amiodarone	Positive control
RR interval (ms)	144 ± 4.8	205 ± 7.3*	149 ± 4	146 ± 5.6	193 ± 5.2*	160 ± 4.4
P wave length (ms)	25.7 ± 2.4	25.4 ± 3.4	21.5 ± 4	24.1 ± 2.3	25.3 ± 2.5	18.2 ± 3.5
PR interval (ms)	32 ± 4.4	36.8 ± 4	33.9 ± 3.3	32 ± 3.5	31.9 ± 5.6	33.3 ± 5.5
QRS length (ms)	27.4 ± 2.9	32.3 ± 4.7	28.5 ± 6.7	28.6 ± 3	28.2 ± 1.4	20.5 ± 6
QT interval (ms)	37.4 ± 3.4	44 ± 4	48.3 ± 7.8	42.9 ± 3.1	43 ± 2.2	36.3 ± 6

* There is a statistically significant difference between amiodarone ($p < 0.05$) and negative and positive control groups. Positive control: animals treated with cyclophosphamide.

Table 2
Data on total chromosomal aberrations, percentage of abnormal metaphases and mitotic index (MI) in bone marrow of Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) induced by the treatment with amiodarone, and respective controls.

Groups	*n*	Chromosome aberrations		Total	% Abnormal metaphases	MI (%)
		Chromatid-type	Chromosome-type			
WKY						
Negative control						
Total	6	5	0	5	5	
Median (interquartile range)		1.00 (1.00)	0.00 (0.00)	1.00 (1.00)	1.00 (1.00)	2.15 (0.50)
Amiodarone						
Total	7	19	2	21	20	
Median (interquartile range)		3.00 (2.75)[a]	0.00 (0.75)	3.00 (3.75)[a]	3.00 (3.50)	1.70 (1.20)[a]
Cylophosphamide						
Total	7	258	54	312	81	
Median (interquartile range)		36.00 (29.00)	2.00 (7.00)	37.00 (35.25)	13.00 (5.00)	1.30 (0.60)
SHR						
Negative control						
Total	7	29	5	34	31	
Median (interquartile range)		4.00 (5.50)[b]	0.00 (0.75)	4.00 (6.25)[b]	4.00 (5.50)[b]	2.50 (0.85)
Amiodarone						
Total	6	37	5	42	37	
Median (interquartile range)		6.50 (1.00)[b]	0.50 (1.00)	7.00 (3.00)[b]	6.50 (2.00)[b]	1.60 (0.50)
Cyclophosphamide						
Total	5	351	41	392	115	
Median (interquartile range)		76.00 (59.75)	3.00 (17.00)	79.00 (76.75)	23.00 (21.00)	0.80 (0.45)

One hundred metaphases per animal were analyzed; a thousand cells per animal were analyzed for mitotic index. Data were analysed by the Mann–Whitney non-parametric test.

[a] ($p < 0.05$) Amiodarone treated groups versus negative control.

[b] ($p < 0.05$) versus Wistar-Kyoto groups.

data, the Mann–Whitney non-parametric test was performed. Differences were considered significant when $p < 0.05$.

3. Results

The electrocardiographic parameters are shown in Table 1. The RR interval for the rats of both strains was larger in the groups treated with amiodarone than in the controls. The P wavelength, PR interval, QRS length and QT interval did not differ between treatments, for both strains.

The cytogenetic analysis on the bone marrow cells from WKYs and SHRs treated with amiodarone or the positive control cyclophosphamide consisted of investigating chromatid and chromosome gaps and chromatid and chromosome breaks. Gaps were not included in the total number of chromosomal aberrations or in the percentage of abnormal metaphases. The results obtained from this study are shown in Table 2. The WKYs and SHRs treated with amiodarone presented a significant decrease or a trend of decrease, respectively, in the mitotic index (evaluated as the percentage of dividing cells), compared with the negative control groups ($p > 0.05$), thus demonstrating mild cytotoxicity. In addition, in both WKYs and SHRs treated with cyclophosphamide, there was a statistically significant difference in the mean mitotic index, in relation to the respective negative control ($p < 0.05$).

The data showed that there were significant differences in the total chromosomal damage and the percentage of abnormal metaphases between the WKY and SHR negative controls (Table 2). In the SHR negative control, the frequencies of basal chromosomal aberrations and abnormal metaphases were significantly higher ($p < 0.05$). In all amiodarone-treated groups, we found a high number of chromosomal aberrations, compared with the negative control groups. In the amiodarone-treated groups, the most frequent chromosomal aberration was chromatid breaks. There were more chromosomal aberrations in WKYs that received amiodarone, and a statistically significant difference was observed in relation to the negative control. However, in SHRs, there was no significant difference between the amiodarone and negative groups regarding the induction of chromosomal damage.

Administration of a single dose of cyclophosphamide, used as a positive control, produced significantly increased numbers of chromosomal aberrations and abnormal metaphases in WKYs and SHRs, with a marked increase in abnormal metaphases in SHR animals (Table 2).

4. Discussion and conclusion

The bradycardia observed in the amiodarone-treated rats showed that this drug really was active. The animals with amiodarone treatment had a significantly larger RR interval than in the negative and positive control groups. This is in accordance with previous findings with experimental animals and it demonstrates that amiodarone had the expected action [3].

Genotoxicity studies *in vivo* are an important and mandatory component of safety-assessment programs that create a baseline of reporting requirements for evaluating the safety of pharmaceutical products. Genotoxic data on antiarrhythmic drugs are scarce in the literature. This study was carried out to investigate the effects of amiodarone-induced chromosomal aberrations in WKYs and SHRs.

Cytogenetic characterization and classification of different types of chromosomal aberrations have an important role, and many cancers are associated with specific types of aberrations. Chromosomal aberrations and micronuclei are measurements of genomic instability [13,14] and bone marrow cells are considered to be indicator cells because of their high sensitivity to clastogenic agents [15].

Female rats of both strains were chosen because, according to Alcântara et al. [16], it is well known there is sexual dimorphism regarding blood pressure levels in SHRs, with higher values observed in males. Although the mechanisms responsible for gender differences in blood pressure control are not clear, there is significant evidence that androgens play an important role in

gender-associated differences in blood pressure regulation. The amiodarone administration route was through drinking water because this is similar to human exposure.

There was a possibility that the agents tested in the present study might have induced a cell cycle delay in the animals' bone marrow cells, when sacrificed after 4 weeks of treatment with amiodarone daily or 24 h after receiving cyclophosphamide intraperitoneally, which might have influenced the total number of chromosomal aberrations. In this case, the recorded frequency of chromosomal damage would depend on the number of cells undergoing mitosis and their availability for scoring. The administration of amiodarone *per se* induced a significant change in the mitotic index in WKY strain, without shows any significant difference in the SHR positive control group.

Cyclophosphamide, bleomycin, doxorubicin and cisplatin are potent drugs used against many forms of cancer. These chemotherapeutic agents are used in genotoxic assays as positive controls [17]. The animals were treated with cyclophosphamide intraperitoneally because this method of administration allows high exposure of bone marrow cells to the agent tested. As expected, the genotoxicity of this drug was confirmed in both strains.

The percentage of abnormal metaphases in the SHR negative controls was statistically greater than the percentage in the WKY negative controls. Although the exact mechanism underlying these cytogenetic results in SHRs is still not fully understood, it has been suggested that free radical generation plays an important role in SHRs. Several studies have suggested that oxidative stress is involved in hypertension [18]. According to Schupp et al. [19], hypertensive patients that present elevated levels of ANG II (the active peptide of the renin–angiotensin system, which regulates blood pressure and cardiovascular homeostasis) are known to present genomic damage. There is evidence that ANG II is an activator of NAD(P)H oxidase, leading to the formation of free radicals, which are known to participate in the induction of DNA damage [19].

In the SHRs, microvascular endothelium is exposed to enhanced oxidative stress due to increased xanthine oxidase and NADPH oxidase activity and/or reduction in superoxide dismutase activity [20]. It has been well documented that free radicals induce genetic instability that leads to delayed biological effects (including cell death), chromosome aberrations and gene mutation. However, the principal cause of genetic instability is still poorly understood [21]. In the present study, all groups of the SHR strain had higher total numbers of chromosomal aberrations than the WKY strain.

The results demonstrated that amiodarone induced chromosomal aberrations in both strains of rats, which did not reach statistically significant differences in SHR. WKYs seem to be more sensitive to amiodarone-induced chromosomal damage than SHRs were. It is not a simple task to explain these results, but our data are in line with the literature. Queluz et al. [22] also observed that WKYs treated with amiodarone were more sensitive than were SHRs treated with amiodarone, with lower body weight gain and lower percentages of macrophages, whereas most macrophages in the lungs of treated SHRs were normal.

Two events may explain the results obtained with amiodarone in WKYs. One is the evidence from i*n vitro* and *in vivo* studies that has suggested that this drug can generate free radicals that are related to pathogenesis of amiodarone toxicity and are also involved in generating more than 30 different lesions of DNA bases [23,24]. Recent investigations have also demonstrated that treatment with amiodarone induces alterations in gene expression profiles, apoptosis and transcriptional remodeling [25]. The toxicity of amiodarone has been assessed by means of DNA microarrays. The results suggested that genes down regulated by amiodarone were involved in all stages of cell cycle control [26]. These mechanisms of amiodarone-induced cytotoxicity could also contribute towards the increased chromosomal damage observed in the animals.

In conclusion, more research is needed in order to elucidate the increase in the basal frequency of chromosomal damage in SHRs. The results reported in this study showed that the treatment with amiodarone was genotoxic in WKYs, but not in SHRs. Further studies are needed to confirm whether amiodarone is genotoxic or efficient and harmless, among humans undergoing therapy.

Conflict of interest statement

There are no financial or personal interests that might be viewed.

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