Original Research

Metformin's performance in *in vitro* and *in vivo* genetic toxicology studies

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Abstract

Metformin is a hypoglycemiant drug prescribed for the treatment and control of the type 2 diabetes mellitus. Recently, the potential efficacy of this antidiabetic drug as an anticancer agent has been demonstrated in various mammalian cancer cells. This report evaluates the mutagenic as well as the recombinogenic potentials of the metformin drug in therapeutically relevant plasma concentrations (12.5 μM, 25.0 μM or 50.0 μM). Since the loss of heterozygosity is a process associated with carcinogenesis, the recombinogenic potential of such a drug was evaluated by the homozygotization assay using a heterozygous diploid strain of *Aspergillus nidulans.* The homozigotization indices (HI) for the genetic markers from the metformin-treated diploids were not statistically different from the negative control (non-treated diploids). For the first time, this indicated a lack of recombinogenic activity of the antidiabetic drug. The mutagenic potential of the metformin drug was evaluated by the chromosome aberrations and the micronuclei tests in human lymphocytes cultures. The metformin drug did not show any significant increase either in the numerical or in the structural chromosome aberrations and did not affect significantly the mitotic index when compared to the negative control. In the *in vitro* micronucleus test, the drug did not increase the number of micronuclei or nuclear buds when compared with the negative control. The data in this study suggest that the metformin drug is not a secondary cancer inducer, since it has neither showed recombinogenic nor mutagenic activities when used in pharmacological concentrations.

Keywords: Genotoxicity, homozygotization assay, mutagenicity, chromosome aberrations, micronucleus assay, *Aspergillus nidulans*

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Introduction

Metformin, a biguanide family member, is one of the most commonly prescribed drugs worldwide and is the preferred initial treatment for the type 2 diabetes mellitus. The reduction of blood glucose induced by the metformin drug is mainly due to its actions on the liver and muscle, which have an insulin-sensitizing effect. In hepatocytes, the drug inhibits gluconeogenesis and glycogenolysis and stimulates glycogenesis, whereas in insulin-dependent peripheral tissues, especially in skeletal muscle, the metformin drug increases the glucose uptake, causing rapid reduction in the plasma glucose.[1,2]

Previous *in vivo* and *in vitro* studies have demonstrated that such a drug causes improvement in antioxidant activities in various tissues, in addition to limiting lipid peroxidation. The metformin drug's antioxidant properties probably result either from a drug direct effect on reactive oxygen species or from an indirect action on the superoxide anions produced by hyperglycemia.[3] Attia *et al*.[4] evaluated the effect of metformin on the oxidative stress induced by diabetes using rats' bone marrow cells and the biochemical markers lipid peroxidation and reduced glutathione. The authors demonstrated that the free radical generation by hyperglycemia was considerably inhibited by the treatment with the drug. On the other hand, Onaran *et al*.[5]

demonstrated that pharmacological concentrations of metformin were unable to impair the DNA fragmentation induced by the pro-oxidant agent cumene hydroperoxide in human lymphocytes *in vitro.* Despite these contradictory results, epidemiological studies have revealed that diabetic patients treated with the metformin drug have reduced risk of developing various types of cancer.[6,7] In fact, there is increasing evidence of the potential efficacy of this antidiabetic drug as an anticancer drug.[8,9]

The metformin probably displays its anticancer activity through inhibition of the mTOR translational pathway through the AMPK-dependent or independent mechanisms, leading to the G1 arrest in the cell-cycle and to the subsequent cell apoptosis through the mitochondrion-dependent pathway. The AMP-activated protein kinase (AMPK) pathway is a major sensor of the energetic status of the cell, which has been proposed as a promising therapeutic target in the cancer treatment.[2,8]

The metformin potential anticancer effects have already been demonstrated in various mammalian cancer cells lines including pancreas,[10,11] prostate,[12] lung,[13] ovary[2,14] and breast cancer (BC) cells.[9,15] In the human BC cells, metformin interacted synergistically with some molecularly targeted agents, such as the anti-Her2 monoclonal antibody trastuzumab, to suppress the proliferation of BC stem cells in the HER2-gene-amplified breast carcinoma cells.[16] In the B16 cell line of mouse, metformin showed an antimelanoma effect, causing a G2/M cell-cycle arrest associated with the apoptotic death of the melanoma cells.[17] The combination of classical chemotherapeutic agents with metformin has also been shown to be beneficial in the combinatorial cancer therapies. Preclinical studies in mouse xenographs showed that oral administration of metformin together with the widely used chemotherapeutic drugs doxorubicin, paclitaxel or carboplatin, is highly effective in blocking the tumour growth and in preventing relapse in a variety of cancer cell types.[18]

Taking into account that the metformin drug causes cell-cycle arrest at G1/S[2] and G2/M[17] and that it suppresses numerous mitosis-related gene families, including kinesins and tubulins,[19] and also assuming that events occurring during the cell cycle arrest may influence tumourigenesis,[20–22] this study evaluates the possible genotoxic effect of metformin in eukaryotic cells. The mutagenic potential of the drug was studied in human lymphocytes cultures using the chromosome aberrations and the micronuclei tests. Since the loss of heterozygosity (LOH) is a process associated with carcinogenesis, the metformin recombinogenic potential was evaluated by the homozigotization assay using a heterozygous diploid strain of *Aspergillus nidulans.*

Materials and methods

Chemicals and reagents

Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride CAS no. 1115-70-4), cisplatin (CAS no. 15663-27-1), colchicine (CAS no. 9754), cytochalasin B (CAS no. 14930-96-2), dimethyl sulfoxide (DMSO CAS no. 67-68-5) and mitomycin C (CAS no. 50-07-7) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gibco RPMI 1640 cell culture media supplemented with L-glutamine (2 mM), fetal calf serum (15%) and phytohemagglutinin (2%) were purchased from Invitrogen-Life Technologies (New York, USA). All other chemicals and solvents used in this study were of the highest purity. Metformin, cisplatin and mitomycin C were dissolved in sterile Milli-Q water. The selection of the metformin concentrations (12.5, 25.0 and 50.0 μM) in the genotoxic assays was based on the therapeutically relevant plasma concentration of metformin (20 μM).[23,24]

Lymphocyte isolation

This study has been approved by the ethics committee of the Universidade Estadual de Maringá, Maringá, PR, Brazil. Informed consent was obtained from the six healthy donors, aged 20–25 years (three males and three females), non-smoking, non-alcoholic, not under drug therapy and with no recent history of exposure to mutagens. The peripheral venous blood was collected in a heparin tube. After the centrifugation at 1000 rpm for 5 min, the lymphocyte layer was collected and added to 82% of RPMI 1640 medium supplemented with 15% fetal calf serum, 1% L-glutamine 200 mM and 2% phytohemagglutinin.

In vitro *mammalian chromosome aberration test*

The *in vitro* chromosome aberration test was performed according to the International Programme on Chemical Safety (IPCS) guidelines.[25] The experiments were conducted in quadruplicate. The lymphocyte cultures were incubated at 37°C in humidified atmosphere with 5.0% CO_2 for 72 h. At 48 h incubation, metformin (12.5, 25.0 and 50.0 μM) and mitomycin C (0.3 μM), used as the positive control, were added to each culture individually. In all experiments, the untreated culture was used as the negative control. Colchicine (0.8 μM) was added to each flask culture during the last 2 h of the experiment. After 72 h incubation, the cells from the culture were treated with a hypotonic solution (75 mM KCl) for 20 min at 37°C and fixed with a cold solution of methanol: glacial acetic acid (3:1 v/v). The cells were fixed with two changes of fixative. Slides were prepared for the microscopic analysis by dripping three to four drops of the pre-fixed lymphocyte suspension from a distance of 30 cm, and then they were dried for five days at 22°C and stained with 5% Giemsa (pH 6.8 Sorensen's buffer). A total of 100 well-spread metaphases from each donor were analysed for structural (chromatid and chromosomal breaks, fragments and rearrangement) and numerical (polyploidy) chromosomal aberrations (a total of 400 metaphases per concentration). The cytotoxicity of the metformin concentrations (12.5, 25.0 and 50.0 μM) was determined by the mitotic index (MI) calculated by the number of dividing cells/total number of the cells × 100.[26] The data were expressed as mean ± standard error (SE) of the mean and they were statistically analysed by the non-parametric tests, Mann–Whitney *U*-test and Kruskal–Wallis test ($P < 0.05$).

In vitro *mammalian cell micronucleus test*

The OECD guideline no. 487[27] recommended the *in vitro* micronucleus (MNvit) assay. Experiments were done in duplicates. The lymphocyte cultures were incubated at 37°C in a humidified atmosphere with 5.0% CO_2, for 72 h. After 24 h, each lymphocyte culture was individually treated with metformin (12.5, 25.0 and 50.0 μM) and mitomycin C (0.3 μM) as the positive control, whereas an untreated culture was included as the negative control. The cytokinesis was blocked at 44 h of incubation with cytochalasin B (final concentration 12.5 μM), after the start of the culture. At the end of the incubation period (72 h), the lymphocyte cultures were treated with a cold hypotonic solution (75 mM KCl) and fixed three times in a cold solution of methanol: glacial acetic acid (3:1 v/v). In the second fixative solution, 1% of formaldehyde was added in order to preserve the cytoplasm. The slides were prepared for the microscopic analysis by dripping three to four drops of the pre-fixed lymphocyte suspension from a distance of 30 cm, dried in cold air and stained with 5% Giemsa (pH 6.8 – Sorensen's buffer). A total of 1000 binucleated cells were analysed for each treatment for the presence of micronuclei and buds (a total of 2000 cells per concentration). The cytokinesis block proliferation index (CBPI) was determined as recommended by Surralles *et al.*,[28] as follows: CBPI = N1 + 2N2 + 3 (N3 + N4)/500, where N1–N4 are the cells with one to four nuclei in 500 cells counted for each experiment. The data were expressed as mean ± standard deviation (SD) of the mean and they were statistically analysed by the Z-test and the non-parametric Mann–Whitney *U*-test ($P < 0.05$).

In vivo *homozygotization assay*

The diploid UT448//A757 strain of *A. nidulans*, carrying markers on chromosomes I, II and IV (Table 1), was prepared by the Roper's technique.[29] Since the diploid strain is heterozygous for the nutritional markers, it may grow in minimal medium (MM), consisting of Czapek-Dox medium, supplemented with 1% (w/v) glucose and solid medium containing 1.5% (w/v) agar. When growing on the complete medium (CM),[30] the diploid strain may originate auxotrophic mitotic segregants, recognized as normally growing yellow, green or white sectors on the UT448//A757 diploid green colonies. For the recombinogenic assay, metformin (12.5, 25.0 and 50.0 μM) was added to molten MM. For the toxicity measurements, the UT448//A757 diploid colonies diameters were determined during six days after incubation at 37°C. The growth rates in the presence (treatment) and in the absence (control) of the hypoglycemiant metformin were compared by the one-way variance analysis followed by the Bonferroni post-test ($P < 0.05$) (results not shown). The chemotherapeutic drug, cisplatin (6 μM), was used as the positive control for the recombinogenic test.[30] The colonies of the UT448//A757 diploid strain of *A. nidulans* were grown onto petri plates containing MM (negative control or untreated control) (Figure 1a), MM + cisplatin (positive control) and MM + metformin (treatment) (Figure 1b). These plates were incubated for six days at 37°C and, then, visually inspected for diploid sectors arising on the original diploid strains colonies. The obtained treated (metformin and cisplatin) and the untreated diploids were purified on the MM, individually transferred to CM plates and then processed by spontaneous haploidization. The haploidization process, or rather, the loss of one member of each chromosome pair through successive mitotic divisions, resulted in the haploid condition of the nuclei. After the haploidization, each diploid produced haploid mitotic segregants (Figure 1 (c) to (d)) which were individually transferred to different supplemented medium (SM) for phenotypic analyses. The SM consists of MM supplemented with all the nutritional requirements of the master strains, excepting one, in each medium type. The mitotic crossing-over causes homozygotization of the heterozygous-conditioned genes. If the metformin induces mitotic crossing-over in the original diploid strain, only heterozygotes (+/− or −/+) or homozygotes (+/+) diploids will develop in the MM and the nutritional markers will segregate among the haploids in the proportion of 4^+ to 2^-. However, if the drug fails to induce the crossing-over, the proportion will be 4^+ to 4^- (Figure 2). This is due to the fact that the initial selection process limits the growth of −/− diploids.[31] The ratio of prototrophic to auxotrophic segregants is described by the homozygotization index (HI), or rather, an HI equal to or higher than 2.0 indicates the recombinogenic effect of the metformin. The metformin recombinogenic potential was assessed by comparing the homozygotization indices of the nutritional markers by the Yates Corrected Chi-square test, Contingency Table ($P < 0.05$).

Table 1 Genotype and origin of *Aspergillus nidulans* strains

Strain	Genotype	Origin
A757	*yA2*(I), *methA17*(II), *pyro A4*(IV)	FGSC
UT448	*wA2*(II), *ribo A1* (I), *paba A124* (I), *bi A1* (I), *Acr A1* (II)	Utrecht[a]

Note: FGSC: Fungal Genetic Stock Center; *meth*: methionine; *pyro*: pyridoxine; *ribo*: riboflavin; *paba*: paraminobenzoic acid; *bi*: biotine; *Acr*: acriflavin resistance; *y*: yellow conidia; *w*: white conidia.
[a]Holland.

Results

In vitro *mammalian chromosome aberration test*

Table 2 shows the results of chromosome aberration analysis. As expected, the frequency of chromosome aberrations in the positive control (mitomycin C) was higher when compared with the negative control. On the other hand, metformin at all tested concentrations (12.5 μM, 25.0 μM or 50.0 μM) did not show any significant difference in the frequency of structural and numerical aberrations when compared to the negative control (Table 2, Figure 3). With regard to the mitotic index, metformin did not significantly affect the mitotic activity when compared to the negative control at all tested concentrations (Table 2).

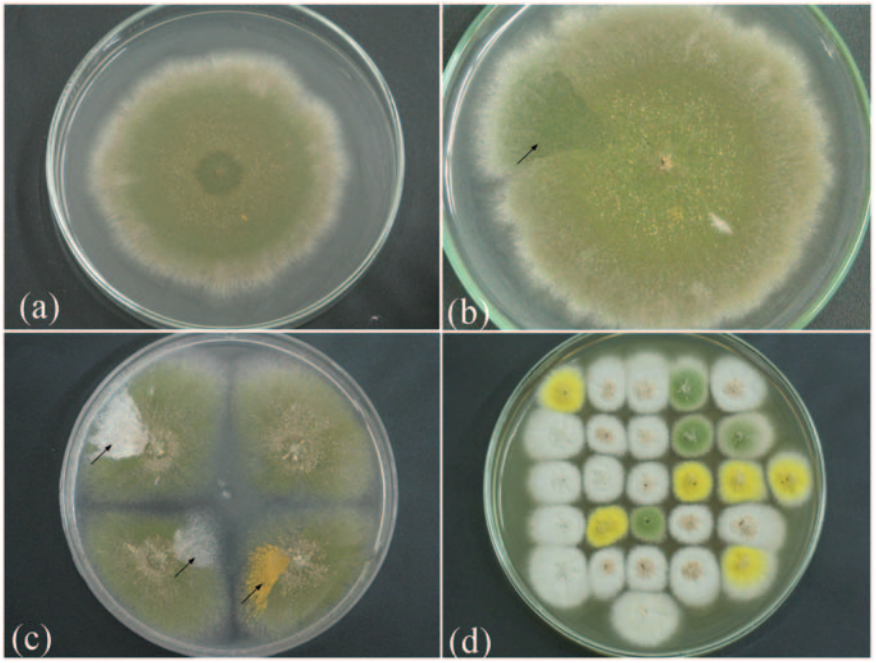

Figure 1 Diploid and haploid segregants obtained after the treatment of the UT448//A757 strain with metformin. (a) Growth of the UT448//A757 diploid strain in the absence of metformin. (b) Origin of a metformin-treated diploid (arrow) in plates containing MM + 25 μM metformin. (c) Haploid mitotic segregants (arrows) derived from the metformin-treated diploid shown in (b). (d) yellow, white and green haploid segregants derived from the diploid obtained with 50 μM of metformin. (A color version of this figure is available in the online journal)

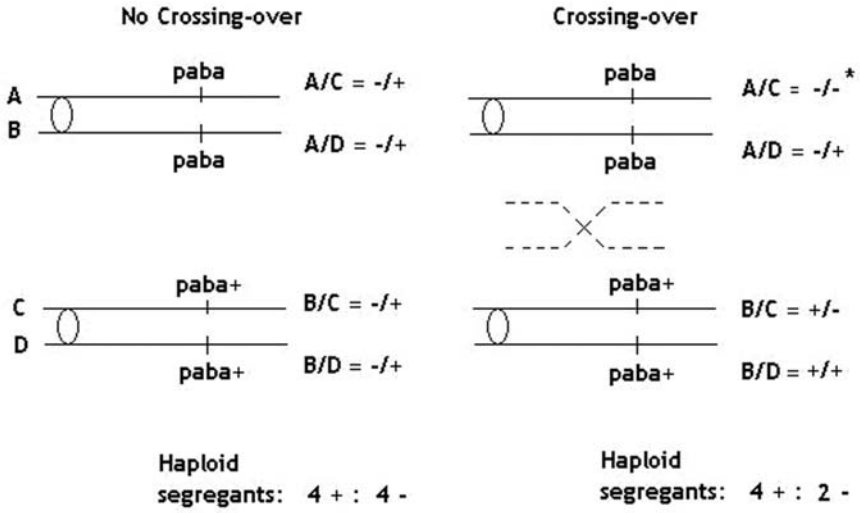

Figure 2 Origin of heterozygous (+/− and −/+) and homozygous (+/+) diploids caused by mitotic crossing-over between *paba* gene and centromere. (*) Not grow in MM (Pires and Zucchi[31])

Table 2 Total chromosomal aberrations (CA) and Mitotic Index (MI) in human lymphocytes treated with metformin

Treatment (48 h)	Concentrations (μM)	Chromosome aberrations							Total CA	Abnormal cells (mean ± SE)	CA/cell (mean ± SE)	Mitotic index (mean ± SE)
		Cog	Cag	Cob	Cab	Frag	Rear	Pol				
Negative Control	0	0	0	0	0	0	0	0	0	0	0	2.7 ± 0.38
Positive Control	0.1	8	8	31	37	4	3	0	75	14.50 ± 3.75	0.187 ± 0.061*	2.0 ± 0.32
Metformin	12.5	0	1	1	2	1	0	1	5	0.75 ± 0.75	0.012 ± 0.012	2.9 ± 0.18
	25	0	1	0	4	0	0	0	4	1.00 ± 0.75	0.010 ± 0.007	3.18 ± 0.49
	50	0	2	3	1	0	0	2	6	1.50 ± 1.50	0.015 ± 0.015	3.05 ± 0.53

Note: Positive Control: mitomycin C; Cog: chromosome gap; Cag: chromatid gap; Cob: chromosome break; Cab: chromatid break; Frag: fragment; Rear: rearrangement; Pol: polyploidy. Four hundred metaphases were scored for each treatment; to mitotic index 4000 lymphocytes were scored for each treatment.
*$P < 0.05$ versus negative control (Mann–Whitney *U*-test).

In vitro *mammalian cell micronucleus test (MNvit)*

The number of micronuclei for the metformin treatment, the positive (mitomycin C) and the negative (untreated culture) controls are shown in Table 3. The treatment with metformin in concentrations ranging from 12.5 μM to 50.0 μM did not lead to a significant increase in the number of micronuclei when compared to the negative control (Table 3, Figure 4). On the other hand, the positive control (mitomycin C) increased the number of micronuclei in comparison to that of the negative control. The CBPI values, obtained with the metformin treatment, were not decreased significantly when compared to those with the negative control (Table 3).

In vivo *homozygotization assay*

The metformin recombinogenic potential was evaluated by the homozygotization indices (HI) for the *riboA1*, *pabaA124*, *biA1* and *pyroA4* nutritional markers (Table 4). HI rates for untreated diploids (negative control) were lower than 2.0 for all analyzed markers. The values of HI for treatment with 6 μM of cisplatin (positive control) were higher than 2.0 for the *paba*, *bi* and *pyro* markers and significantly ($P < 0.05$) different from the HI negative control rates. The treatment in MM did not allow the development of auxotrophic diploids, specifically those which were homozygous for the nutritional markers *ribo*, *paba* and *bi*. The treatment of the original UT448//A757 diploid strain with the metformin (12.5 μM, 25.0 μM or 50.0 μM) drug produced nine prototrophic diploids, all of them with green conidia ($y+//y$). The HI rates for the treated diploids were less than 2.0 for all the analyzed markers.

Discussion

This study evaluated the mutagenic and recombinogenic potentials of metformin in eukaryotic cells by using diploid cells of *A. nidulans* and human lymphocytes. The data collected in this study demonstrated that the treatment of human lymphocytes with increasing concentrations of drug tested did not increase the frequencies of micronuclei or chromosomal aberrations. This fact indicated that the hypoglicemiant agent is not a genotoxic compound. Our results are in agreement with the reports of Attia *et al.*[4] and Aleisa *et al.*,[32] which show the lack of genotoxic activity of metformin in rats and mice bone marrow cells, respectively. On the other hand, the drug had a significant antimutagenic effect on mouse embryonic fibroblast cells by

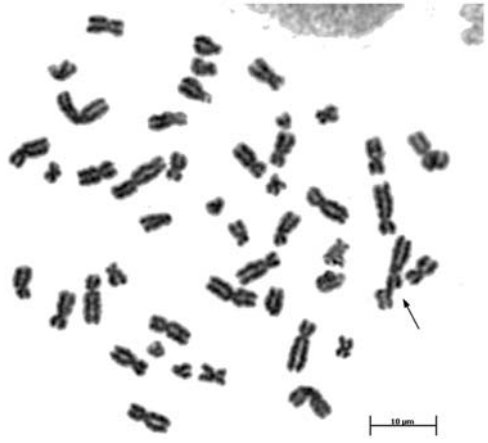

Figure 3 Chromatid break (arrow) observed in a lymphocyte cell after the treatment with 25 μM of metformin

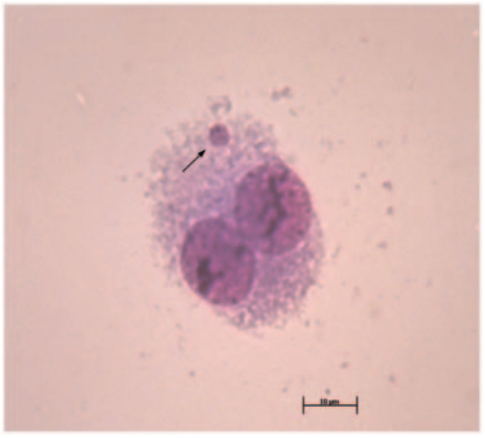

Figure 4 Micronucleus (arrow) observed in a lymphocyte binucleated cell after the treatment with 50 μM of metformin. (A color version of this figure is available in the online journal)

Table 3 Effect of metformin on micronucleus induction in human lymphocytes *in vitro*

Test substance	Treatment (48 h) Concentration (μM)	BNC scored	No. of MN			No. of buds			CBPI[a]		
			Experiment			Experiment			Experiment		
			I	II	Mean ± SD	I	II	Mean ± SD	I	II	Mean ± SD
Negative control	–	2000	3	1	2.00 ± 1.41	0	0	0	2.10	2.27	2.19 ± 0.12
Positive control	0.1	2000	83	92	87.0 ± 6.36*	8	5	6.5 ± 2.12*	1.92	1.91	1.92 ± 0.01
Metformin	12.5	2000	1	3	2.00 ± 1.41	0	1	0.5 ± 0.70	2.16	2.29	2.23 ± 0.09
	25	2000	2	2	2.00 ± 0.00	0	0	0	2.22	2.06	2.14 ± 0.11
	50	2000	2	4	3.00 ± 1.41	1	0	0.5 ± 0.70	2.17	2.17	2.17 ± 0.00

Note: BNC: binucleated cells.
[a]CBPI: Cytokinesis blocked proliferation index mean from 1000 cell per treatment for the study; Positive control: mitomycin C.
*Significantly different from negative control (Z-test).

Table 4 Homozygotization index (HI) values for markers from UT448//A757 diploid strain after treatment with 50, 25 and 12.5 μM of metformin

					Metformin					
	Negative control		Positive control[a]		50 μM		25 μM		12.5 μM	
Markers	NS[b]	HI	NS[b]	HI	NS[b]	HI	NS[b]	HI	NS[b]	HI
ribo+	291	1.7	265	1.4	108	1.7	253	1.9	294	1.9
ribo	168		189		65		134		152	
paba+	288	1.7	349	3.3*	109	1.7	253	1.9	293	1.9
paba	171		105		64		134		153	
bi+	288	1.7	321	2.4*	109	1.7	253	1.9	285	1.8
bi	171		133		64		134		161	
pyro+	249	1.2	354	3.5*	95	1.2	236	1.6	286	1.8
pyro	210		100		78		151		160	

Note: *ribo* = riboflavin; *paba* = *p*-aminobenzoic acid; *bi* = biotin and *pyro* = pyridoxine.
[a]Positive control, diploids treated with cisplatin (6 μM).
[b]Number of mitotic segregants.*Significantly different from control (Contingency Table, Yates Corrected Chi Square, $P < 0.05$).

its ability to prevent the reactive oxygen species accumulation.[33]

The chromosomal aberrations and the micronuclei are biomarkers of the chromosomal damage and of the genomic instability events measured in human lymphocytes.[34–36] As cytogenetic aberrations are directly involved in cancer etiology,[37] high chromosomal aberrations frequencies have been showed to be predictive of an increased risk of many types of cancer, regardless of the cause of the initial chromosomal aberrations.[38–40] In our analysis, structural and numerical chromosomal aberrations were in fact induced by metformin, but this datum was not statistically significant. In addition, statistical differences were not observed in the MI values, which indicate that metformin in concentrations ranging from 12.5 μM to 50 μM is not cytotoxic.

The human cytokinesis-block micronuclei analysis, which allows micronuclei to be scored specifically in binucleated cells prior to the cytokinesis, provides a simple and fast method to detect and measure chromosomal DNA damage and chromosomal instability phenotype in mammalian cells. The micronuclei arise mainly from chromosome or chromatid acentric fragments or from whole chromosomes that fail to be included in the daughter nuclei during the mitosis anaphase. The micronucleus is an ideal biomarker in human cells both for *in vitro* and *in vivo* genetic toxicology studies and has been prospectively associated with an increased cancer risk.[40–43] The current data demonstrate that the metformin drug does not induce any chromosomal DNA damage or instability. Furthermore, the metformin did not affect significantly the CBPI rates, revealing a lack of cytostatic effects of the antidiabetic drug.

The homozygotization assay using *A. nidulans* diploid cells is a bioassay extensively used to detect genotoxic effects of several physical and chemical agents such as X-rays, environmental volatile pollutants, herbicides and cancer chemotherapeutic compounds, thus providing relevant information about their recombinogenic potentials.[21,30,44–46] *A. nidulans* is a filamentous fungus with a well-characterized genetic system whose cells spend most of their cell cycle in the G2 phase, a phase in which the chromosomes are duplicated, favouring the mitotic recombination event.[47] This study evaluated, for the first time, the recombinogenic potential of metformin by using the homozygotization assay. The HI rates for the genetic markers from metformin-treated diploids were not statistically different from the negative control (non-treated diploids) and demonstrated the lack of recombinogenic activity of this antidiabetic drug in non-cytotoxic concentrations. Metformin did not induce the production of homozygous diploids for nutritional or conidia colour markers and consequently it did not increase the frequencies of prototrophic haploid segregants derived from such metformin-treated diploids. This explains the origin of the HI rates which were lower than 2.0 as shown in Table 4.

The homologous recombination (HR) in mammalian cells has been shown to be important in the repair of several types of DNA lesions such as double-strand breaks (DSB), damage generated during DNA replication and DNA interstrand cross links. The HR, which occurs in the S and G2 phases of the cell cycle, leads to a precise DNA damage repair since the sister chromatid may be used as a repair template.[48–50] Alternatively, although the HR may use the homologous chromosome for the DSB repair when the cells are in the G1 phase of the cell cycle, this would indeed lead to the LOH or to other gene rearrangements, such as translocations. Since DNA damages may induce recombination between homologous or non-homologous chromosomes, the recombination process is strongly suppressed in mitosis.[21,49–51]

The LOH, which is the loss of the functional allele at a heterozygous locus, is a process commonly associated with several genetic disorders such as cancer initiation and progression, neurofibromatosis type 1-associated glomus tumours, dermal neurofibromas and ichthyosis with confetti.[52–55] Although the LOH events may be caused by several mechanisms including deletion and nondisjunctional chromosome loss, the mitotic recombination process has

been shown to be the major pathway leading to the LOH *in vivo*.[56,57] The mitotic recombination is known to be an important mechanism for the LOH in the neurofibromatosis type 2 and in the schwannoma tumours, which are benign peripheral nerve sheath tumours that occur within the context of the schwannomatosis.[58] In the familial adenomatous polyposis, the mitotic recombination has been described as an important process of the LOH at the *APC* tumour suppressor gene, leading to the loss of critical APC functions.[59]

The current results demonstrate that when different analysis systems, both *in vivo* and *in vitro*, are used, the metformin drug have no genotoxic activity. Since the metformin alone[60] or in combination to chemotherapeutic drugs has been proposed as a novel treatment for different cancer types,[14] the data in the current study suggest that metformin is not a secondary cancer inducer, since it did not show mutagenic or recombinogenic activities when used in pharmacological concentrations.

Author contributions: All authors participated in the design, interpretation of the study, analysis of the data and review of the manuscript. SJR, YJPRS, RLJ, FCCS, PJA, MPCF, CPMAA conducted the experiments, CPMAA and SJR wrote the manuscript.

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