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Original article

Selective cytotoxic and genotoxic activities of 5-(2-bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione against NCI-H292 human lung carcinoma cells

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ABSTRACT

Background: Thiazolidine-2,4-dione ring system is used as a pharmacophore to build various heterocyclic compounds aimed to interact with biological targets. In the present study, benzylidene-2,4-thiazolidinedione derivatives (compounds **2**–**5**) were synthesized and screened against cancer cell lines and the genotoxicity and cytotoxicity of the most active compound **(5)** was investigated on normal and lung cancer cell line.

Methods: For *in vitro* cytotoxic screening, the MTT assay was used for HL60 and K562 (leukemia), MCF-7 (breast adenocarcinoma), HT29 (colon adenocarcinoma), HEp-2 (cervix carcinoma) and NCI-H292 (lung carcinoma) tumor cell lines and Alamar-blue assay was used for non-tumor cells (PBMC, human peripheral blood mononuclear cells) were used. Cell morphology was visualized after Giemsa-May-Grunwald staining. DNA content, phosphatidylserine externalization and mitochondrial depolarization were measured by flow cytometry. Genotoxicity was assessed by Comet assay.

Results: 5-(2-Bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione **(5)** presented the most potent cytotoxicity, especially against NCI-H292 lung cancer cell line, with IC_{50} value of 1.26 μg/mL after 72 h incubation. None of the compounds were cytotoxic to PBMC. After 48 h incubation, externalization of phosphatidylserine, mitochondrial depolarization, internucleosomal DNA fragmentation and morphological alterations consistent with apoptosis were observed in NCI-H292 cells treated with compound **(5)**. In addition, compound **(5)** also induced genotoxicity in NCI-H292 cells (2.8-fold increase in damage index compared to the negative control), but not in PBMC.

Conclusion: Compound **5** presented selective cytotoxic and genotoxic activity against pulmonary carcinoma (NCI-H292 cells).

Introduction

Cancer is one of the leading causes of mortality worldwide characterized by sustained chronic proliferation of immortal cells, evasion of growth suppressors' programs, resistance to cell death, development of angiogenesis, tissue invasion and metastasis [1]. The most common sites of cancers diagnosed among men are lung, prostate, colorectal, stomach and liver. Meanwhile, most common sites of cancer in women are breast, colorectal, lung, cervix and stomach. The total number of cancer related deaths worldwide raised from 8.2 million in 2012 to 8.8 million in 2015 and lung cancers caused approximately 1.69 million deaths in 2015 [2–4].

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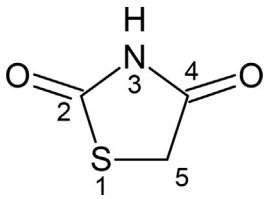

Fig. 1. Thiazolidine-2,4-dione ring.

Thiazolidine-2,4-dione ring (Fig. 1) system exhibits a broad spectrum of biological activities, such as hypoglycemic [5,6], anti-mycobacterial [7], anti-inflammatory [8], antileishmanial [9], antibiotics [7,10] and antitumor [11]. Much attention has been given to the construction of new derivatives of thiazolidinediones with anticancer activity [12–14].

Heterocyclic molecules belonging to the thiazolidinedione class represents a new alternative in the search for drugs with antineoplastic potential. Thiazolidinediones exhibit antitumor effects through both PPARγ-dependent and -independent mechanisms [15,16]. The role of PPARγ (peroxisome proliferator-activated receptors) in tumor cells has been extensively investigated and treatment with PPARγ agonists exert biological effects such as cell growth control, motility, differentiation and apoptosis [16,17] PPARγ-independent mechanism of tumor cell death induced by thiazolidinediones treatments includes proteasome-dependent degradation of cyclins (D1 and D3) with consequent cell cycle blockage at G_1-S transition, induction of cellular acidosis through inhibition of the Na^+/H^+ exchanger and release of apoptotic factors from the mitochondria due to the production of reactive oxygen species (ROS) [15].

Among many thiazolidinediones derivatives with distinct biological activities, 5-benzylidene-2,4-thiazolidinedione and its analogues presented cytotoxic activity against a variety of different cell lines: compound 2-[4-[(2,4-Dioxothiazolidin-5-ylidene) methyl] phenoxy]-*N*-[3-(trifluromethyl) phenyl] acetamide was active against K562 leukemia, MCF-7 (breast cancer), PC3 (Prostate cancer), KB (Nasopharyngeal cancer) and GURAV (Oral cancer) [18]; 5-(4-(2-(piperidin-1-yl)ethoxy)benzylidene)thiazolidine-2,4-dione was active against a panel of 60 human tumor cell [19]. In fact, a wide variety of substituents in the thiazolidine-2,4-dione (TZD) nucleus lead to a huge diversity of compounds targeting different signaling pathways related to cell proliferation or cell death [11].

One of the most important characteristic for a new molecule with action against cancer is its selectivity toward tumor cells, since many anticancer drugs cause diverse cytotoxic and genotoxic damages also in normal cells. Although there are many reports indicating a wide range of biological activities for compounds with a thiazolidine-2,4-dione nucleus, genotoxicity studies in normal cells are still insufficient. In this context, this study aimed to evaluate *in vitro* antitumor effects of 5-benzylidene-2,4-thiazolidinedione derivatives, as well as to investigate the possible mechanisms of action, and genotoxic outcomes of these compounds on human normal and tumor cells.

Materials and methods

Synthesis of thiazolidine-2,4-dione derivatives compounds

The synthesis methodology of compounds 1 to 5 was already described [9]. Briefly, the thiazolidine-2,4-dione **(1)** was obtained by the method already described [20,21]. This reaction occurs by condensation of monochloroacetic acid and thiourea in an aqueous medium under reflux for 24 h. The series of thiazolidine-2,4-dione derivatives were originated by changes in the 5 position of the heterocyclic ring (Fig. 1). The compounds5-(2-bromo-6-fluorobenzylidene)-thiazolidine-2,4-dione **(2)**, 5-(2-Hydroxy-3-bromo-5-chlorobenzylidene)-thiazolidine-2,4-dione **(3)**, 5-(2-Hydroxy-5-clorobenzylidene)-thiazolidine-2,4-dione **(4)** e 5-(2-bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione **(5)** were synthesized according to protocol described previously [9] (Fig. 2). A solution of thiazolidine-2,4-dione (0.2 g, 1.70×10^{-3} mol) in ethanol, (7.0 ml) containing piperidine (2 drops) and aromatic aldehyde (0.184 g, 2.25×10^{-3} mol) was heated (70 °C), under stirring, for 5–9 h. Afterward, the product was cooled in an ice bath, filtered and recrystallized with an appropriate solvent. The resulting precipitate was filtered off and recrystallized from acetic acid to give the compounds (2–5). All compounds were identified by IR spectroscopy method, NMR and HRMS (Supplementary material). The yields obtained ranged from 73 to 79%.

Cell lines

The cell lines NCI-H292 (Mucoepidermoid pulmonary carcinoma), HL60 (Promyelocytic leukemia), HT-29 (Colon adenocarcinoma), K562 (Chronic myelogenous leukemia), HEp-2 (cervix carcinoma) and MCF-7 (Breast adenocarcinoma) were obtained from The Rio de Janeiro cell bank, Brazil. The cells were maintained in DMEM or RPMI 1640 medium. All cell lines were supplemented with 10% fetal bovine serum (GIBCO), 1% antibiotic solution (penicillin 5000 Units/mL + streptomycin 5000 μg/mL) and 1% L-glutamine 200 mM.

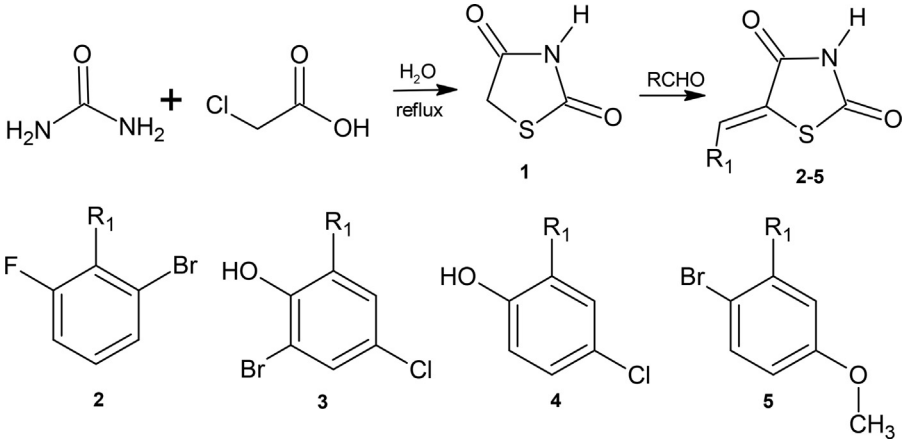

Fig. 2. Synthetic routes and structure of compounds 2–5.

Cytotoxic activity in tumor cells

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma Aldrich Co., St. Louis, MO, USA) reduction assay was used for cytotoxicity. Tumor cells were plated in 96-well plates (10^5 cells/mL for adherent cells or 3×10^5 cells/mL for leukemia). Tested compounds (0.39–25 μg/mL) dissolved in DMEM with DMSO 0.007% for the lowest concentration (0.39 μg/mL) to 0.5% for the highest concentration (25 μg/mL) of compounds were added to each well. Subsequently, cells were incubated for 72 h. The compound **5** was also evaluated after 24 and 48 h of incubation in NCI-H292 cells. Control groups received the same amount of DMSO. After 69 h of treatment 25 μL of MTT (5 mg/mL) was added, three hours later the supernatant was removed and the MTT-formazan product was dissolved in 100 μL of DMSO, and absorbance was measured at 570 nm in plate spectrophotometer. Doxorubicin (0.01–5 μg/mL) was used as positive control. Data are presented as IC_{50} (concentration that cause 50% of cell growth inhibition) values with their 95% confidence intervals (CI 95%) obtained by non linear regression after normalize the absorbance results against untreated control samples [22].

Peripheral blood mononuclear cells isolation and cytotoxic activity (PBMC)

Mononuclear cells (lymphocytes and monocytes) were obtained from the peripheral blood of healthy volunteers collected in sterile tubes with heparin solution (BD Vacutainer ™) as an anticoagulant. The protocol was approved by The Human Research Ethics Committee (N°CAAE 48809515700005208). The total blood (6 mL) was diluted in 3 mL PBS and layered onto 2 mL Ficoll®-Hystopaque (Sigma). For phase separation of the solution, the tube was centrifugated at 1500 rpm for 30 min. After centrifugation the mononuclear cells were concentrated in the middle layer located between the plasma (light phase) and erythrocytes (dark phase). Afterwards the PBMC were transferred to another tube with PBS to a final volume of 11 mL. The cells were pelleted by centrifugation and resuspended in RPMI 1640 medium supplemented with 20% fetal bovine serum and 1% antibiotics 1% antibiotic solution (penicillin 5000 Units/mL + streptomycin 5000 μg/mL). Phytohemagglutinin 2% (Sigma) was added to the medium to stimulate the proliferation of lymphocytes. Cells were seeded at 10^6 cells/mL in 96 well plates. After 24 h of incubation, compounds were dissolved in DMEM with DMSO and added to each well. Doxorubicin (0.078–10 μM) was used as a positive control. The negative control received the same amount of DMSO. Twenty-four hours before the end of the incubation period (total time of 72 h), 10 μL of Alamar Blue stock solution (0.312 mg/mL) was added to each well. The absorbances were measured at 570 nm (oxidized state) and 595 nm (reduced state) in a plate spectrophotometer. Cell proliferation that was calculated by the following formula: % proliferating = $ALW - (AHW \times R0) \times 100$, where ALW is the absorbance at the lowest wavelength (570 nm) e AHW is the absorbance at highest wavelength (595 nm), respectively. The R0 was calculated according to the formula: R0 = (absorbance of medium with Alamar Blue minus absorbance of medium without Alamar blue at 570 nm)/(absorbance of medium with Alamar Blue minus absorbance of medium without Alamar blue at 595 nm) [23].

Evaluation of hemolytic potential in human erythrocytes

Blood was collected from healthy volunteers. Erythrocytes were pelleted by centrifugation (3000 rpm/5 min) and resuspended in saline (0.85% NaCl + 10 mM $CaCl_2$) to obtain 2% erythrocytes solution (SE) 2%. The 96-well plates were prepared according to the following: 100 μL of saline (negative control); 50 μL of saline solution and 50 μL of vehicle with 1% DMSO (Blank); 80 μL saline + 20 μL Triton X – 100 1% (positive control); 50 μL of saline solution and 50 μL of compounds (1.95–250 μg/mL) diluted in 1% DMSO. Then 100 μL of the solution of erythrocytes was plated in each well. After incubation for 1 h under constant agitation at room temperature, the supernatant was analyzed in automatic plate reader (450 nm). The 50% effective concentration (EC_{50}) and their 95% confidence intervals were determined from nonlinear regression.

*Morphological changes induced compound **5** (5-(2-bromo-5-methoxybenzylidene)-thiazolidine-2,4-dione) on lung adenocarcinoma (NCI-H292)*

Compound **5,** the most active compound, was selected for further investigation of cytotoxicity on lung carcinoma NCI-H292. Cells cells (10^5 cells/mL) were treated for 48 h with Compound 5 at 8.0 and 16.0 μg/mL, the IC_{50} and $2\times$ IC_{50} for 48 h, respectively. Afterward, slides were prepared by cytospin centrifugation. To evaluate the morphology, the cells were fixed with methanol for 1 min and stained with May-Grunwald-Giemsa. Doxorubicin (0.5 μg/mL) was used as a positive control.

Analysis by flow cytometry

Externalization of phosphatidylserine in NCI-H292 after treatment with compound 5

The externalization of phosphatidylserine was evaluated by annexin/propidium iodite staining. NCI-H292 cells were treated with compound 5 at 8 and 16 μg/mL for 48 h, the IC_{50} and $2\times$ IC_{50} for 48 h, respectively. Doxorubicin (0.5 μg/mL) was used as positive control. Cells were harvested and incubated with AnnexinFitV-FITC kit according to the manufacturer (Sigma). Cell fluorescence was then determined by flow cytometry on a BD cytometry – FACS Calibur – CBA. Twenty thousand events per experiment were acquired and cell debris were omitted from the analysis. The percentages of viable cells, early apoptotic, late apoptotic and necrotic cells were graphed using Prism 5.0 (GraphPad Software Inc.).

*Measurement of mitochondria transmembrane potential in NCI-H292 after treatment with compound **5***

NCI-H292 cells treated with compound 5 at 8 and 16 μg/mL for 48 h, the IC_{50} and $2\times$ IC_{50} for 48 h, respectively and doxorubicin (0.5 μg/mL) were centrifuged at 2000 rpm for 5 min, washed with PBS and stained with 200 μL of rhodamine 123 (1 μg/mL) for 15 min. Subsequently, cells were pelleted again and incubated with PBS for 30 min. Cell fluorescence was then determined by flow cytometry. Twenty thousand events per experiment were acquired and cell debris were omitted from the analysis [24].

*DNA content of NCI-H292 cells (DNA fragmentation and cell cycle) after treatment with compound **5***

Cells were collected, pelleted and fixed with cold ethanol (70%) for 1 h. After washing with phosphate buffer saline to remove ethanol, treated and untreated cells were incubated with 100 μL of lysis solution (0.1% sodium citrate, 0.1% triton X – 100 and 2 μg/mL of propidium iodide). After 30 min cell fluorescence of the samples were analyzed on a BD cytometry – FACS Calibur – CBA. FlowJo was used to calculate the cell cycle parameters, based on the cells PI emission (analyzed in the FACSCalibur FL2 channel that captures 564–606 nm emissions). Twenty thousand events per experiment were acquired and cell debris were omitted from the analysis [25].

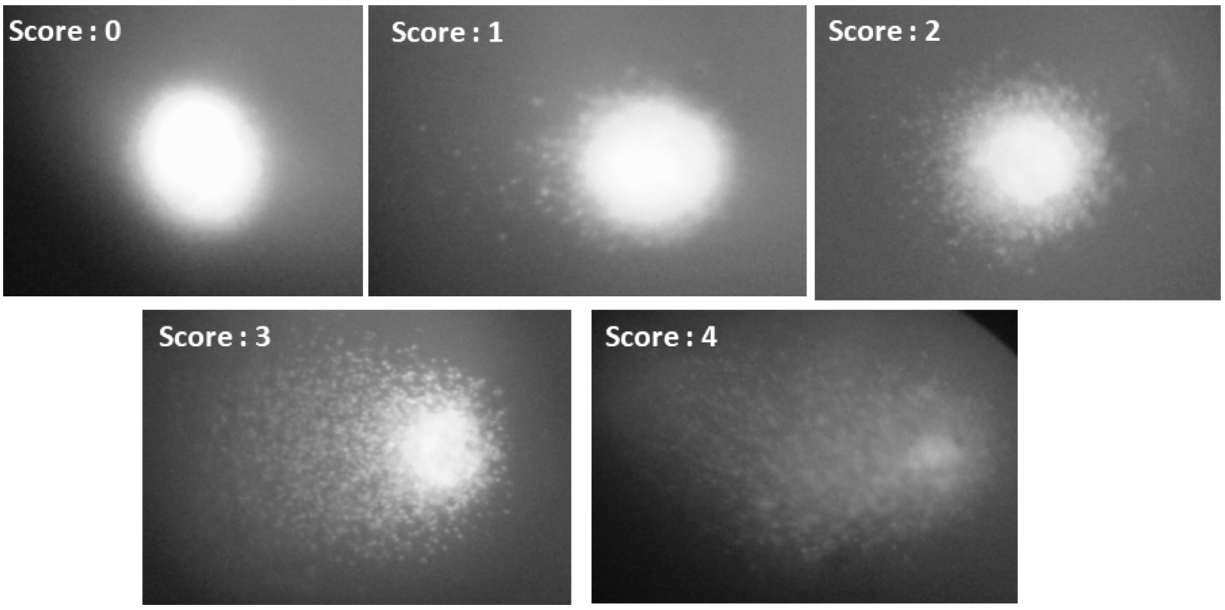

Fig. 3. Photomicrograph of comets classified by visual inspection into five categories: score zero representing undamaged cells and score 1–4 representing increasing damage. 1000× magnification.

*Genotoxic effect of compound **5** in tumor and normal cell lines – comet assay*

We evaluated the genotoxic effect of compound **5** using the comet assay [26]. Approximately 2×10^5 cells/mL of the NCIH-292 were treated with compound 5 for 48 h. To investigate the initial effects on DNA damage induced by compound 5 we used the 72 h IC_{50} and 2× IC_{50}, 1.26 and 2.5 μg/mL, respectively. For normal cells, we choose 2× IC_{50} and 4× IC_{50} found in cancer cells. PBMC were treated with compound **5** with 2.5 and 5.0 μg/mL for 48 h. Doxorubicin (0.5 μg/mL) was used as a positive control. The negative control received the same amount of DMSO as treated samples (0.5%). After treatment, cells were harvested and 15 μL of the cell suspension was embedded in low melting point agarose at 37 °C. The homogenate was then casted on agarose-coated glass slide. Slides were stored in the dark at 4 °C for 20 min before adding electrophoresis buffer. Gel electrophoresis was performed at 40 V for 20 min and 300 mA. The slides were neutralized in 0.2 M Tris buffer (pH 7.5) for 15 min and fixed with 98% ethanol for 5 min. After drying, the slides were stored in a refrigerator until staining. Staining was performed through 1:1000 Gel Red (BiotargetGelRed®) addition onto each slide. We analyzed the slides using a fluorescence microscope (Olympus – BX series). Approximately 100 cells per treatment were analyzed and scored from 0 to 4 points depending on the degree of damage in the nucleoid as the following criteria: (a) class 0: undamaged, with no tail; (b) class 1: with tail shorter than the diameter of the head (nucleus); (c) class 2: with tail length between one and two times the diameter of the head; (d) class 3: with tail longer than two times the diameter of the head; and (e) class 4: comets with no heads. The damage index (DI) can vary from 0 (0 × 100) to 400 (4 × 100) [27]. Examples of comets from our experiment are provided for better understanding of the classification used (Fig. 3).

Statistical analysis

For cytotoxicity assays, IC_{50} values and their 95% confidence intervals were obtained by non-linear regression. In order to determine the differences, the data were compared by analysis of variance (ANOVA) followed by Dunnett's test ($p < 0.05$). The GraphPad Prism version 5.00 was used for statistical analysis.

Results

Cytotoxic activity of thiazolidine-2,4-dione derivatives in tumor cells

Four compounds were tested in six human tumor cell lines HEp-2, HT-29, HL-60, MCF-7, K562, and NCI-H 292 and cytotoxicity was determined by MTT assay after 72 h of incubation. Compounds **2**, **3** and **4** showed low cytotoxic activity with IC_{50} values ranging from 9.0 μg/mL to 25.0 μg/mL (Table 1). Compound **5** was the most active compound with lowest IC_{50} value (1.2 μg/mL) for lung cancer (NCI-H292). All cell lines were inhibited by the positive control doxorubicin, being HEp-2 and HT-29 the most resistant cell lines. In order to investigate the time

Table 1
Cytotoxicity of thiazolidine-2,4-dione derivatives after 72 h of incubation. The compound concentration that causes 50% inhibition of cell growth and their 95% confidence interval (CI95%) are expressed in μg/mL. Doxorubicin was used as positive control (DOX).

	Tumor cell line IC_{50} (CI95%) μg/mL					
Compound	HEp-2	HL-60	HT-29	K-562	MCF-7	NCI-H292
2	>25	9.0 (6.8–11.9)	>25	>25	20.2 (16.4–24.7)	15.4 (12.9–18.4)
3	>25	16.1 (13.9–18.9)	>25	11.6 (8.7–15.4)	17.9 (15.6–20.6)	10.9 (8.8–13.5)
4	>25	10.2 (7.7–13.3)	>25	17.0 (8.2–35.1)	>25	16.9 (12.8–22.4)
5	>25	2.0 (1.5–2.7)	>25	6.8 (0.2–14.5)	3.5 (1.8–6.3)	1.26 (0.9–1.7)
DOX	1.2 (0.5–2.4)	0.03 (0.02–0.03)	0.7 (0.3–1.0)	0.24 (0.16–0.39)	0.5 (0.36–0.74)	0.3 (0.17–0.9)

IC_{50}: Concentration that causes 50% inhibition of cell growth, and (CI95%) confidence interval. Dox: doxorubicin.

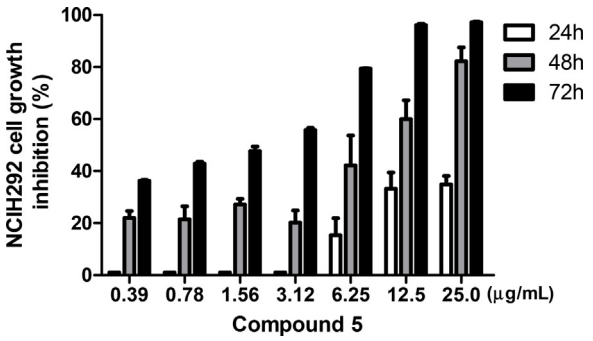

Fig. 4. Time-dependent growth inhibition effect of compound 5 in NCI-H292 cell line. Tumor cells were treated with 7 different concentrations at 24, 48 and 72 h.

dependency of compound 5 cytotoxic activity, NCI-H292 cells were treated for 24 h and 48 h. Compound **5** presented IC_{50} > 25 μg/mL after 24 h of treatment and IC_{50} of 7.9 μg/mL after 48 h of treatment (Fig. 4).

Cytotoxic activity of thiazolidine-2,4-dione derivatives on peripheral blood mononuclear cells (PBMC) and hemolytic activity

Compounds that cause non-specific cell death targeting the cell plasmatic membrane are very toxic. A positive hemolytic assay excludes the compound from being used as therapeutic drug. None of the compounds tested were toxic to human erythrocytes (EC_{50} > 125 μg/mL). In addition, the compounds were tested in a model of human normal cells: peripheral blood mononuclear cells. After isolation from human blood, PBMC cultivated under mitogenic stimulus works as a model of normal cells. Compounds **2** to **5** were not cytotoxic to PBMC after 72 h of treatment (IC_{50} > 25 μg/mL). The results indicated that the compound **5** showed selectivity for tumor cell lines since it causes tumor cell growth inhibition but it does not cause hemolysis of human erythrocytes neither cytotoxicity toward PBMC.

Analysis of cell morphology – May – Grünwald – Giemsa staining

The cell morphology was analyzed by optical microscopy after 48 h of incubation. NCI-H292 untreated cells (negative control – CN) showed typical cellular morphology of adherent cells with intact nuclear and plasmatic membrane and the presence of mitotic cells (Fig. 5). Cells treated with compound **5** at 8.0 μg/mL and 16.0 μg/mL exhibit morphological changes consistent with apoptosis including the cell volume reduction and nuclear fragmentation (Fig. 5). Doxorubicin (0.5 μg/mL) reduced the number of cells, induced reduction of cell volume, chromatin condensation and nuclear fragmentation in NCI- H292 cells.

Phosphatidylserine externalization analysis and mitochondrial transmembrane potential by flow citometry

The percentage of NCI-H292 apoptotic cells was significantly higher ($p < 0.05$) after 48 h treatment with compound **5** at 16 μg/mL (64%) compared to the negative control (15%). Doxorubicin 0.5 μg/mL also induced apoptosis (51%) compared to the negative control ($p < 0.05$). None of the treatments induced increase on necrotic cells (Figs. 6 and 7). The mitochondrial membrane potential evaluation was performed by flow cytometry. Rhodamine 123, a green-fluorescent dye, is sequestered by active mitochondria. When mitochondria depolarizes due to membrane damage, the dye is lost and the average fluorescence decrease. After exposure of NCI-H292 to compound **5** (16.0 μg/mL) for 48 h a

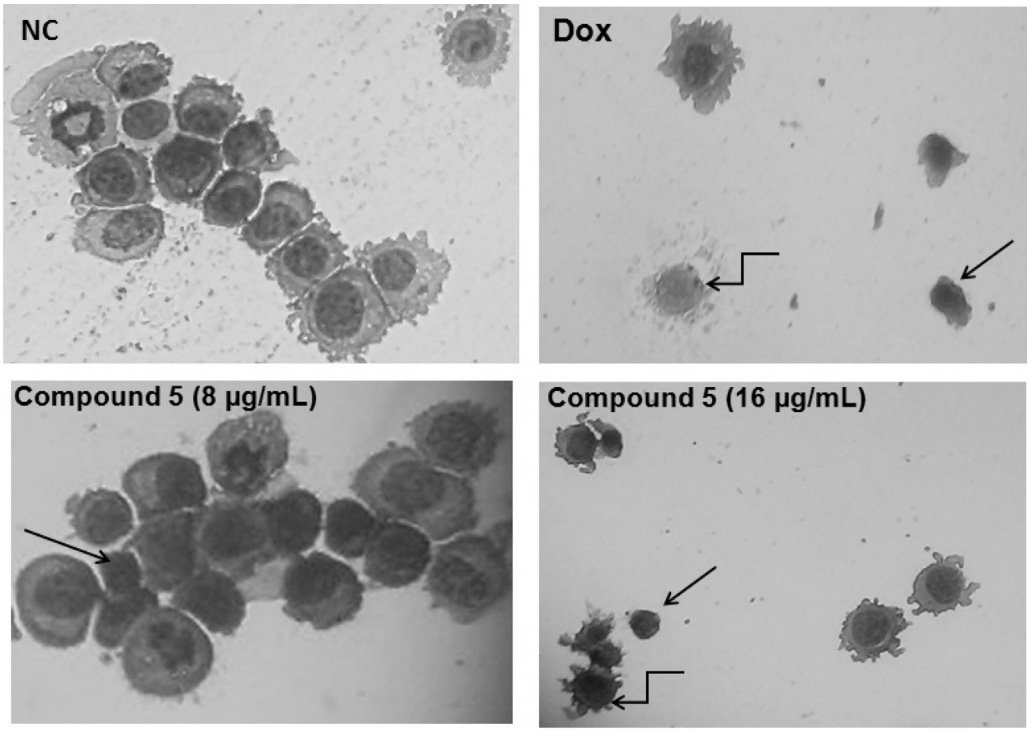

Fig. 5. Cell Morphology of NCI-H292 cells after 48 h of treatment. (NC) negative control treated with the vehicle alone DMEM 0.5% DMSO. Doxorubicin 0.5 μg/mL was used as positive control (Dox). Cells were treated with compound **5** at the concentrations 8.0 μg/mL and 16.0 μg/mL. Arrows indicate the aspects observed: volume reduction (short arrows) and cellular debris (curved arrows). Cells were stained with May – Grunwald – Giemsa and visualized by light microscopy with 400× magnification.

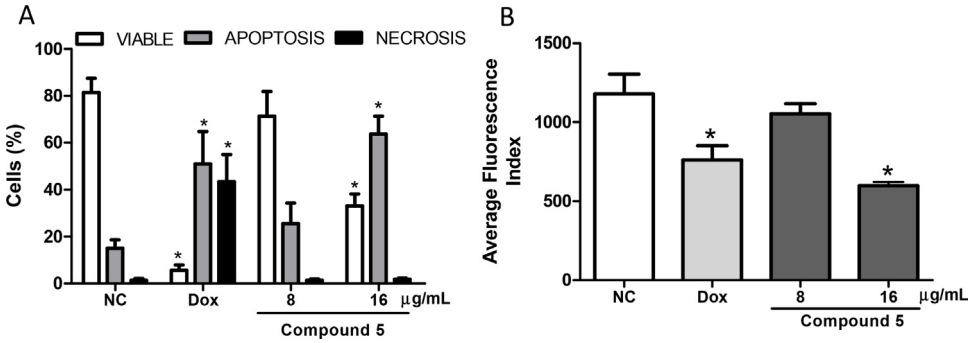

Fig. 6. A – Externalization of phosphatidylserine on lung cancer cell line NCI-H292 analyzed by flow cytometry after 48 h incubation with compound **5**. **B** – Effect of compound **5** in NCI-H 292 transmembrane mitochondrial potential determined by flow cytometry after 48 h of treatment. The negative control (NC) was treated only with DMEM 0.5% DMSO. Doxorubicin (0.5 μg/mL) was used as positive control (Dox). Values are mean ± standard deviation of 3 independent experiments (n = 6). $^{*}p < 0.05$ compared to negative control by ANOVA followed by Dunnett's test.

significant increase ($p < 0.05$) in mitochondrial depolarization was observed (Figs. 6 and 7). Doxorubicin (0.5 μg/mL) was also able to promote significant mitochondrial depolarization at the concentration tested ($p < 0.05$) (Fig. 6).

Analysis of cell cycle and DNA fragmentation

NCI-H292 cells were treated with compound **5** for 48 h (Fig. 8) and 72 h (Fig. 9) at IC_{50} and 2× IC_{50} and DNA content was quantified by flow cytometry. After 48 h of treatment, an increase on subdiploid DNA was observed when cells were treated with compound **5** at 16 μg/mL (16.12% after 48 h) compared to negative control (2.4% after 48 h) ($p < 0.05$). After 48 h there were no significant differences at cells cycle phases (G0/G1, S and G2/M) between treated and control cells (Fig. 8). After 72 h of treatment compound **5** at 1.26 μg/mL caused an increase of cells on G0/G1 (59.8% after 72 h) vs. negative control (41.3% after 72 h) ($p < 0.05$). When cells were treated with 2.52 μg/mL of compound **5** during 72 h, an increase on subdiploid DNA (27.4% after 72 h) was observed (negative control 3.79% after 72 h) ($p < 0.05$).

Genotoxicity assessment in PBMC and NCI-H292

The *in vitro* genotoxic activity on both PBMC and lung cancer cell line NCI-H292 was assessed after 48 h by the Comet assay. To analyze the extent of damage to DNA molecules, data were expressed as damage index (ID) ranging from 0 (no damage) to 400 (maximum damage) (Fig. 10). No DNA damage was detected on PBMC after 48 h of treatment at any tested concentration of Compound **5** (2.52 μg/mL e 5.0 μg/mL) compared to the negative control; Conversely, Doxorubicin (0.5 μg/mL) caused significant DNA damage. For lung cancer cell line NCI-H292, compound **5** (1.26 μg/mL e 2.52 μg/mL) caused an increase on damage index of DNA at both tested concentrations (1.26 μg/mL e 2.52 μg/mL). Doxorubicin also increased DNA damage on NCI-H292.

Discussion

Recently, compounds 2, 3, 4 and 5 were synthesized in satisfactory yields and tested against pteridine reductase 1 (PTR1), a promising enzyme as target for the development of antileishmanial drugs. Compound 3, the first non-competitive inhibitor of PTR1, presented a two-digit micromolar potency (less than 50 uM) in comparison with compounds 2, 4 and 5 [9]. Nonetheless, cytotoxic and genotoxic potentialities on neoplastic or normal cells have not been described yet.

Compounds 2 to 5 were screened against six tumor cell lines (HEP-2, HL-60, HT-29, K562, MCF-7 and NCI-H292) and 5 presented lowest IC_{50} values. The most sensitive cell line treated with compound 5 was NCI-H292 (IC_{50} value of 1.26 μg/mL). The presence of an electron releasing substituent methoxy group increased the activity of compound 5, while in compound 2 the presence of two strong electron withdrawing atoms (bromine and fluorine) reduced the cytotoxic activity. Previously, some studies have reported *in vitro* cytotoxic activities of thiazolidine-2,4-dione derivatives upon tumor lines of different histological types [18,28,29]. Amongst ten compounds synthesized, (2-[4-[(2,4-Dioxothiazolidin-5-ylidene) methyl] phenoxy]-*N*-[3-(trifluromethyl) phenyl] acetamide) showed potent cytotoxicity against five of the seven cell lines tested (breast cancer MCF-7, prostate cancer PC3, nasopharyngeal KB, cancer oral cancer GURAV and leukemia K562) [18]. Another group of derivatives where the substitution reaction occurs at the third position of the 2,4- thiazolidinedione ring presented two active compounds against MCF-7 cell line [28]. Recently, two series of thiazolidinediones were synthesized and their cytotoxicity evaluated in prostate adenocarcinoma PC-3, breast adenocarcinoma MDA-MB-231, and fibrosarcoma HT-1080 cancer cell lines. Most of the compounds synthesized were active against at least one cell line, being 5-{4-[(3-(4-Chlorophenyl)-3,4-dihydro-4-oxoquinazolin-2-yl)methoxy]benzylidene}thiazolidine-2,4-dione the most active compound. Moreover, mechanistic investigation revealed pro-apoptotic activity of the most cytotoxic compound [29].

In order to verify whether the cytotoxicity is related to direct cell membrane damage, the compounds were incubated with human erythrocytes. None of the compounds induced lysis of human erythrocytes ($EC_{50} > 125$ μg/mL). Probably, the cytotoxicity of compound 5 toward tumor cells occurs by a more specific mechanism other than an immediate and direct disruption of cell membrane. In fact, treatment of NCI-H292 cells for a brief period such as 24 h with compound 5 did not reduce cell growth. Only after 48 h of treatment, a significant cytotoxicity on NCI-H292 was observed with maximum effect achieved after 72 h. Lung cancer is currently the leading cause of cancer death worldwide and for most patients with this disease current treatments do not promote cure [3,30]. Additionally, cytotoxic action on normal cells (peripheral blood mononuclear cells) was not observed with compounds 2 to 5 ($IC_{50} > 25$ μg/mL). Compound 5 demonstrated antiproliferative selectivity to lung cancer cells, since the tumor cells were affected by the treatment but not normal cells.

Morphological and biochemical patterns in NCI-H292 human lung cancer cells incubated with compound 5 were investigated.

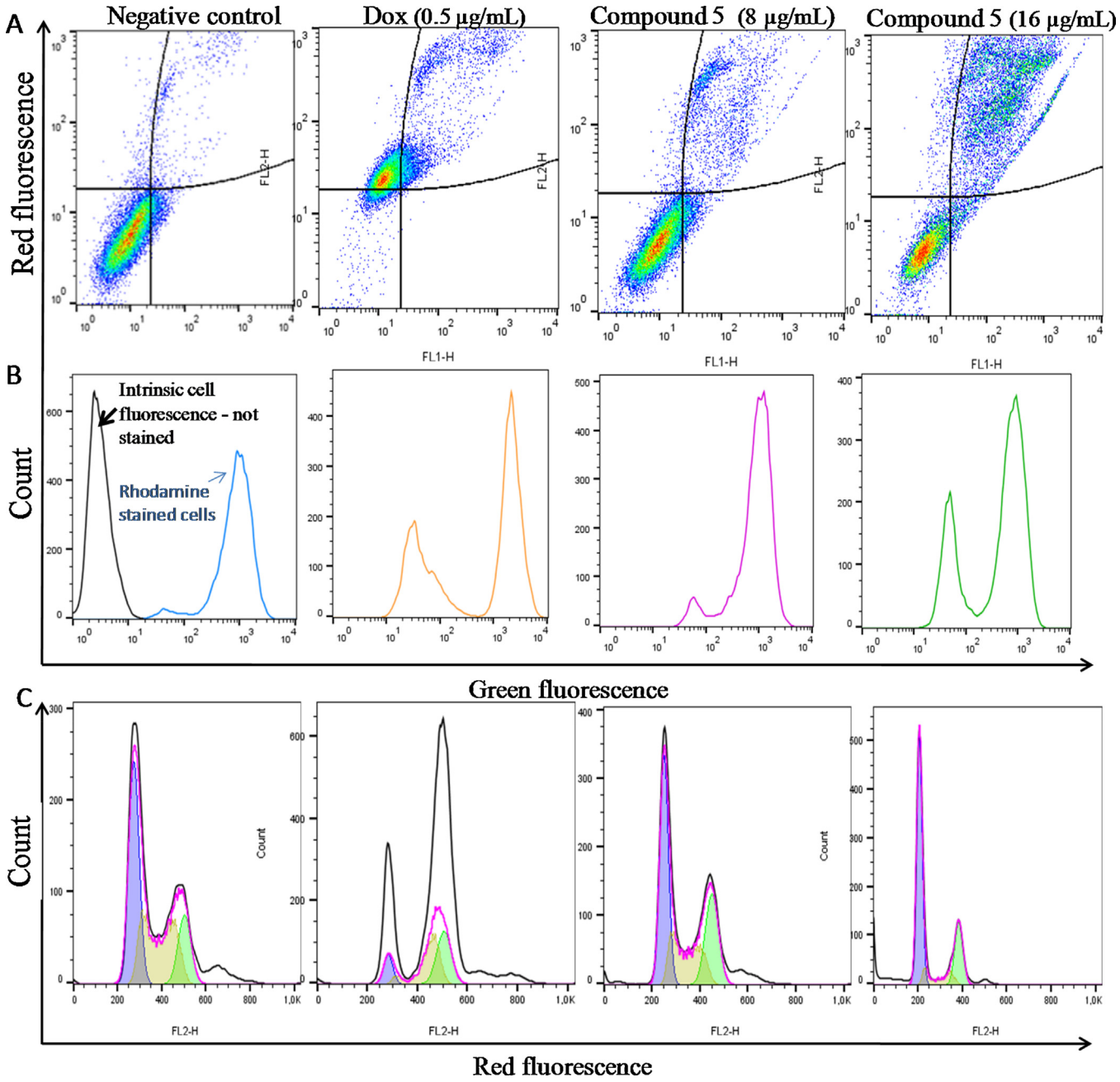

Fig. 7. Effect of Compound 5 in NCI-H292 cell line apoptosis parameters and DNA content after 48 h treatment. **A)** Representative flow cytometric dot plots showing the percentage of cells in viable, apoptotic and necrotic stages. **B)** Representative flow cytometric histograms of cells with mithochondrial despolarization. **C)** Cell cycle histograms. Dox: doxorubicin.

NCI-H292 treated cells exhibited cell volume reduction and DNA fragmentation (8 and 16 µg/mL), suggesting activation of apoptotic events. The process of cell death by apoptosis has some characteristics such as cell volume reduction, chromatin condensation, and nuclear fragmentation without extravasation of the cellular content [31]. So, unlike what happens in necrosis, no inflammatory process occurs [32]. Mitochondrial membrane depolarization and loss of plasma membrane phospholipid asymmetry also occur during the apoptosis process [33].

To confirm if morphological alterations in NCI-H292 treated cells were consistent with apoptosis, phosphatidylserine (PS) externalization was evaluated. An increase on NCI-H292 apoptosis was observed after 48 h of treatment with compound 5 and such findings were accompanied by mitochondrial depolarization and DNA fragmentation (16 µg/mL). After 72 h of incubation, compound 5 caused DNA fragmentation in NCI-H292 cells at 6-fold lower concentration (2.5 µg/mL) than that used for 48 h analyses and cell cycle arrest at G0/G1 at 1.26 µg/mL. A report has shown that hybrid molecules containing 5-benzilidene thiazolidine-2, 4-dione induced apoptosis on leukemia cells by activation of the extrinsic and the intrinsic pathways of cell death [14]. Pioglitazone (PGZ), a thiazolidinedione compound, induced growth inhibition and apoptosis of human B lymphocytic leukemia (SD1 cells). Treatment of these cells with the PPARγ ligand pioglitazone resulted in growth inhibition in a dose-dependent manner which was associated with a G1 to S cell cycle arrest after 3 days of treatment. After 4 days of treatment PGZ caused significant apoptosis in lymphocytic leukemic cell lines [34]. TZD18, Another

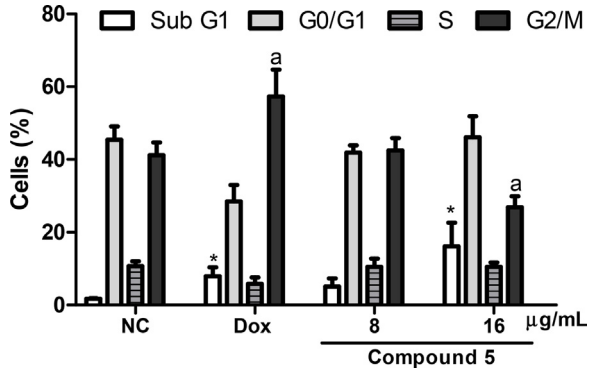

Fig. 8. The effect of compound 5 at IC_{50} and 2× IC_{50} concentrations on the cell cycle of NCI–H292 was determined by flow cytometry after 48 h of incubation. The negative control (NC) was treated only with DMEM 0.5% DMSO. Doxorubicin (0.5 μg/mL) was used as positive control (Dox). Values are mean ± standard deviation of 3 independent experiments (n = 6). $^{*}p < 0.05$ when Sub G1 was compared to negative and $^{a}p < 0.05$ when G2/M was compared to negative control by ANOVA followed by Dunnett's test.

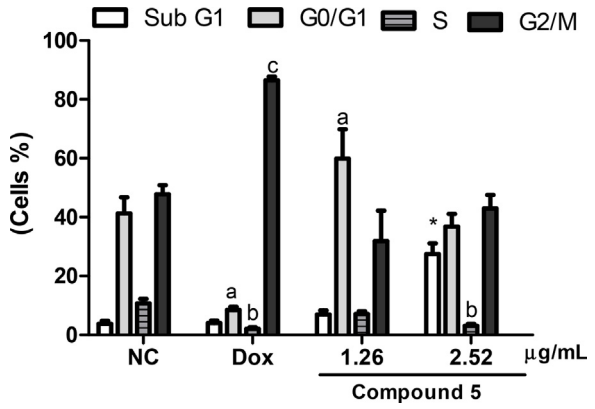

Fig. 9. The effect of compound 5 at IC_{50} and 2× IC_{50} concentrations on the cell cycle of NCI-H292 determined by flow cytometry after 72 h of incubation. The negative control (NC) was treated only with DMEM 0.5% DMSO. Doxorubicin (0.5 μg/mL) was used as positive control (Dox). Values are mean ± standard deviation of 3 independent experiments (n = 6). $^{a}p < 0.05$ when G0/G1 was compared to negative control, $^{b}p < 0.05$ when S phase was compared to negative control, $^{c}p < 0.05$ when G2/M was compared to negative control, $^{*}p < 0.05$ when Sub G1 was compared to negative control. All of them were compared by ANOVA followed by Dunnett's test.

PPARα and PPARγ agonist structurally related to the thiazolidinedione, induced G1 cell cycle arrest in Ph+ lymphocytic leukemia cell line at 10 uM and at higher concentration (20 μM) induced apoptosis in a time-dependent fashion [35]. Thus, G1 arrest may represent one of the underlying mechanisms for subsequent cell apoptosis.

Thiazolidine-2,4-dione (TZDs) were evaluated regarding the expression of proteins that control the transition from the G1 to S cell cycle's phase in human prostate cancer cell lines [36]. Inhibitory concentrations of TZDs rosiglitazone and ciglitazone induced expression of p21 (Inhibitor of cyclin-dependent kinase) and decrease of cyclin D1 levels in PC-3 cells. Cell cycle arrest occurred due to the decrease in the level of cyclin proteins that regulate cell cycle progression [36].

The genotoxicity of compound 5 was accessed by alkaline comet assay. This technique allows quantitative evaluation of recent damage to DNA by single and double breaks [37] and has advantages over others DNA damage methods such as the micronucleus test because of its high sensitivity and early detection of DNA injury. Compound 5 did not cause genotoxicity in PBMC even for a prolonged period of treatment with 5 μg/mL (48 h), but NCI-H292 cells presented significant DNA damage at 4-fold lower concentrations than that used for normal cells (1.25 μg/mL). Doxorubicin (0.5 μg mL) also produced DNA damage in normal cells. DNA damage induced by compound 5 in NCI-H292 initiated with low concentration as detected by the comet assay and became more extensive with higher concentrations of compound 5, as detailed by flow cytometry assay. Although 5-benzylidene-thiazolidine-2,4-dione derivatives have been reported in a large number of biological activities, there are still a few studies that investigate possible genotoxic effects in normal lines. Recently, some thiazacridine derivatives (ATZD), a new class of cytotoxic agents combining one acridine nucleus with thiazolidine group, were described as non-genotoxic upon human lymphocytes [38]. Progress in the treatment of locally stage III Non–Small Cell Lung Cancer has been achieved with two chemotherapy doublets in combination with thoracic radiotherapy: 1) full-dose cisplatin plus etoposide (PE) for two cycles; and 2) weekly low-dose paclitaxel plus carboplatin (PC). However, advanced lung cancer has been resistant to traditional chemotherapy [39]. This data reinforce the need for encouragement of smoking cessation as a preventive approach and the development of new chemotherapeutic agents to treat lung cancer. Hence, we have identified compound 5- (2-bromo-5-methoxybenzylidene) thiazolidine-2,4-dione as a selective cytotoxic and pro-apoptotic

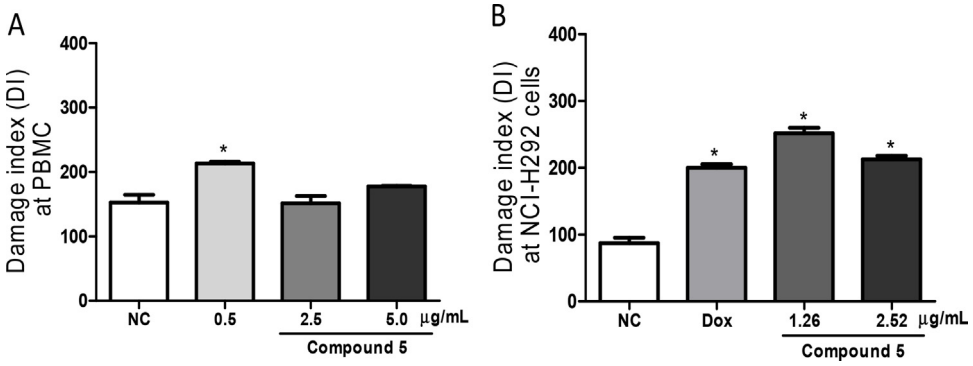

Fig. 10. Damage Index (ID) obtained by Comet assay. A) Peripheral blood mononuclear cells (PBMC) isolated from healthy human volunteers. B) NCI-H292 lung cancer cells. Both normal (PBMC) and cancer cells (NCI-H292) were treated with DMEM 0.5% DMSO (NC); Doxorubicin 0.5 μg/mL as positive control (Dox) and compound **5** for 48 h. Values (damage index of 100 cells at 2 slides; n = 2) corresponds to the mean ± SD of two independent experiments.* $p < 0.05$ compared to the negative control (NC) and analyzed by ANOVA followed by Dunnett's *post* test.

agent against lung carcinoma cells. Furthermore, compound 5 did not show genotoxicity to normal cells.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.pharep.2017.11.008.

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