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2,3,5-Triiodobenzoic acid, a contrast agent, induces caspase-dependent cell death through intrinsic pathway in NSCLC

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ABSTRACT

2,3,5-Triiodobenzoic acid (TIBA) is a well established inhibitor of polar transport in plant cells, a phenomenon that allows morphogenesis in plants, and due to this role in plant physiology it is considered as a pesticide. Another important feature of TIBA is that it can be also used as the basic structure for most of the clinically used contrast agents for computed tomography and other X-ray procedures, and more recently, it was used in nano-delivery systems. Even though there are several indications of the potential antitumoral activity of TIBA, its cell death-induced pathway has not been elucidated. The non-small cell lung cancer (NSCLC) cell line H460 was treated with the medium and TIBA at 100, 250 and 500 μM for 48 h. Also, cells were treated with zVAD and cyclosporine A (CSA), and cell cycle analysis and immunofluorescence were performed and loss of MMP was assessed. We show for the first time that TIBA itself is able to induce caspase-dependent apoptosis in lung cancer through an intrinsic pathway and that it is more active than carboplatin and betulinic acid against lung cancer. Thus, TIBA may constitute another therapeutic alternative to treat lung cancer and other tumors.

KEYWORDS: 2,3,5-Triiodobenzoic acid, TIBA, apoptosis, lung cancer, intrinsic pathway, H460.

INTRODUCTION

2,3,5-Triiodobenzoic acid (TIBA) is well established as an inhibitor of polar transport in plant cells [1], a phenomenon that allows morphogenesis in plants [2-3], and due to its role in plant development it is considered as a pesticide [4]. The presence of iodine causes TIBA and other iodine-containing compounds to absorb X-rays, thus making them potential sources of contrast agents [5]. Furthermore, TIBA derivatives such as omnipaque 350, and other contrast agents for computed tomography and other X-ray procedures are clinically available. Another diagnostic strategy involves the use of TIBA in single-photon emission computed tomography (SPECT)/Computed Tomography [6].

Additionally, TIBA was used as part of nanodelivery systems for doxorubicin (DOX) [7], was used for sorafenib delivery, using poly(lactic-co-glycolic acid) (PLGA) microspheres (MSs) for transarterial embolization (TAE), in liver cancer [8]. TIBA was also conjugated to hyaluronic acid (HA) for use in both cancer diagnosis and therapy [7]. The growing use of TIBA in nanodelivery systems is due, among other reasons, to its biocompatibility and radiopacity, allowing the possibility to track the delivery of the drug in real time [9]. Another feature of iodine-based contrast agents is their rapid renal clearance, and, due to high osmolality and viscosity of iodine aqueous solutions, the use of TIBA derivatives may lead to radiocontrast-induced nephropathy, the third most common cause of acute renal failure among patients [10].

The first reference to an antitumor activity of TIBA was made by Zilkah and co-workers in 1981 [11]. Sturzu *et al*. (2009) coupled TIBA to fluorescein isothiocyanate (FITC)-labelled peptides with the objective of evaluating nuclear uptake and if cellular accumulation of TIBA could result in cell death [5].

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They observed that, in fact, TIBA could be suitable for chemotherapy as well as for imaging applications.

Even though there are several indications of the potential antitumoral activity of TIBA, its cell death-induced pathway has not been elucidated. Herein, we show for the first time that TIBA itself is able to induce caspase-dependent apoptosis in lung cancer through an intrinsic pathway. Furthermore, we show that TIBA is more effective against lung cancer than carboplatin and betulinic acid.

MATERIALS AND METHODS

Cells and culture conditions

The human non-small cell lung cancer cell line H460 was maintained in RPMI 1640 Glutamax® (Gibco BRL, NY, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY, USA), 100 U penicillin and 10 μg/ml streptomycin in disposable plastic bottles at 37 °C with 5% CO_2.

Chemicals

2,3,5-Triiodobenzoic acid (TIBA) (sodium salt), propidium iodide, zVAD.fmk, Cyclosporin A and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich.

Viability inhibition

Cytotoxicity was assessed by the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide assay. Briefly, 180 μL of cell suspension (10^4/cells per well) was distributed in 96-well plates and pre-incubated for 24 h at 37 °C/5% CO_2 to allow stabilization of the culture. TIBA was dissolved in DMSO and diluted in the medium for future use. Cells were treated with the medium, 50, 100, 250 and 500 μM of TIBA, and DMSO (at the same final concentrations present in drug dilutions). After 48-h incubation MTT (5 mg/ml) was added and kept for 4 h at 37 °C. The formazan crystals produced by reduction of MTT by viable cell was dissolved in DMSO and the optical density was measured in an ELISA reader (Spectramax Multi-Mode Microplate Readers) at 570 nm (reference filter 630 nm). Results are expressed as mean ± SD of at least three different experiments performed in triplicate.

Cell cycle analysis

Apoptosis was assessed by cell cycle analysis using flow cytometry. After 24 h, plated cells (2 x 10^4/well) were treated with the medium or TIBA (100, 250 and 500 μM) for 48 h. Also, plated cells (2 x 10^4/well) were treated with the medium or TIBA (500 μM), with or without pretreatment with zVAD.fmk (20 μM) for 1 hour and incubated for 48 h, or treated with or without cyclosporin A (CSA) (40 μM) for 1 hour and incubated for 24 h. The cells were also incubated with cisplatin (33 μM), Carboplatin (539 μM) and Betulinic acid (548 μM), for 48 h. After this, cells were harvested, washed in phosphate-buffered saline (PBS) and suspended in a hypotonic fluorescent solution (50 μg/ml propidium iodide (PI) and 0.1% Triton X-100 in 0.1% Na Citrate buffer) for 1 h, at 4 °C in the dark and the sub-G1 population was measured by flow cytometry (FL2-A) using the Beckton Dikinson Accuri C6 flow cytometer. Results are presented as mean ± SD of subdiploid cells of at least three different experiments performed in triplicate.

Loss of MMP

Mitochondrial membrane potential (MMP) was assessed with the DIOC6(3) (40 nM). Cells were plated (2 x 10^4/well) and treated with or without TIBA (100, 250 and 500 μM) and incubated for 48 h. Stained cells were analyzed by flow cytometry (FL-1). The results represent the mean ± SD of three experiments performed in triplicate.

Immunofluorescence

H460 cells (5 x 10^5) were treated with TIBA (250 μM) and incubated for 24 h. After this, cells were washed and fixed in plates with paraformaldehyde 4% in PBS. Cells were then permeabilized with 0.1% Triton-X 100 (in PBS) for 10 min, blocked with 1% of bovine serum albumin (BSA)/PBS, and incubated with primary antibody (1:200) against Bax followed by incubation with secondary antibody conjugated with FITC. Four images of each well were captured in each experiment, and each experiment was performed three times. After the first staining, the cells were stained with a solution containing 25 μg/ml of propidium iodide (PI) and 0.05% Triton X-100 in 0.05% Na citrate buffer (hypotonic fluorescent solution 1:2 in PBS) for 15 min and washed twice with PBS. The images were captured using a Leica DMI 6000 Fluorescence Microscope and florescence intensity was evaluated with CellProfiler - Image Analysis Software [12] with a customized algorithm. The results for Bax are also presented as mean ± SD of three experiments performed in triplicate.

Statistical analysis

Data are presented as mean ± SD of at least three experiments made in triplicate. One-way analysis of variance (ANOVA) followed by Tukey's test, was performed using Graphpad Prisma 5.0 software. A value of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

TIBA reduced cell viability in a dose-dependent way

As can be seen in Figure 1, TIBA significantly reduced the viability of H460 cells in a dose-dependent way. The percentage of viability under TIBA was 102 ± 7, 95.9 ± 6, 84.5 ± 7 and 33.2 ± 4.9% at 50, 100, 250 and 500 μM of TIBA respectively, after 48 h of treatment.

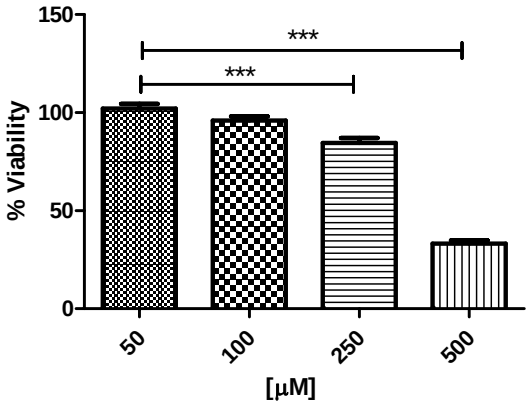

Figure 1. TIBA reduced cell viability in H460. Cells were treated with increasing concentrations of TIBA (50-500 μM) for 48 h and evaluated by MTT assay. ***: $p < 0.0001$.

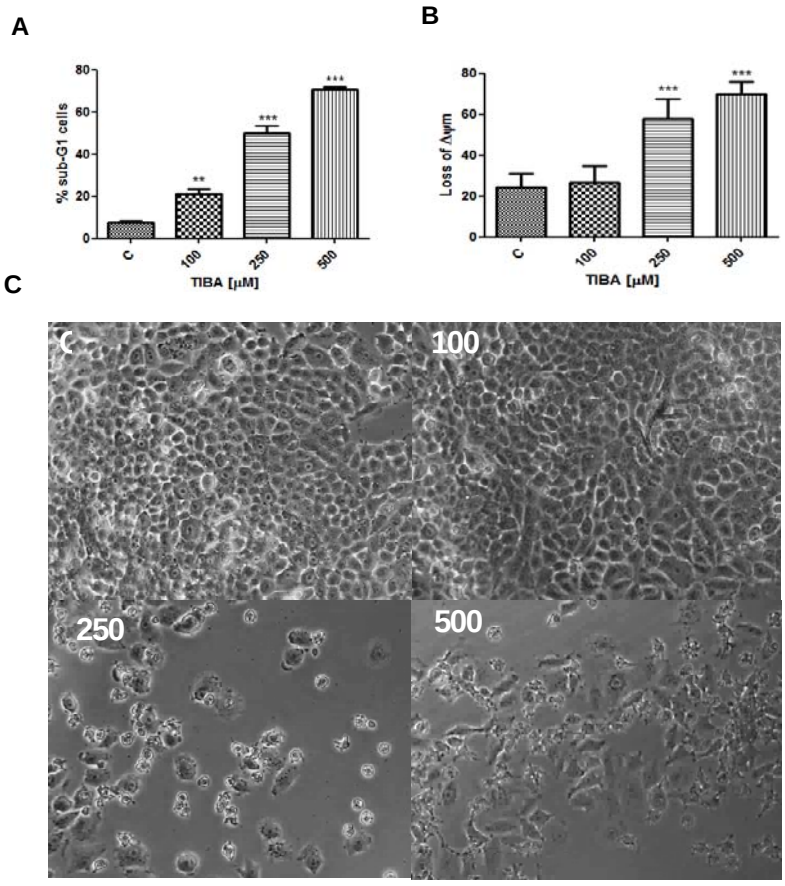

Figure 2. TIBA induced apoptosis in H460. Cells were treated with increasing concentrations of TIBA (100-500 μM) for 48 h. **A**. Sub-G1 population measured by flow cytometry; **B**. Cells treated with TIBA (100-500 μM) were harvested and incubated with DioC6(3) and the loss of MMP was measured by flow cytometry. **C**. Bright field microscopy showing the dose-dependent morphological alterations in H460. The columns represent mean ± SD of three experiments performed in triplicate. **: $p < 0.01$, ***: $p < 0.001$.

TIBA induces apoptosis dependent on caspase activation and mitochondrial membrane potential

The widespread use of TIBA as contrast agent and its recent use in nano-delivery systems have raised the question about its intrinsic toxicity against tumor. Due to its impact on public health, non-small cell lung cancer cell line H460 was chosen for this study. As can be seen in Figure 2A, cells were treated with 100, 250 and 500 μM of TIBA for 48 h and cell cycle analysis was performed with flow cytometry. TIBA induced the appearance of the sub-G1 populations in a dose-dependent manner.

As the intrinsic pathway is the major route activated by available chemotherapeutic drugs [13], we evaluated if the death induced by TIBA involved loss of mitochondrial membrane potential. And, as can be seen in Figure 2B, TIBA induced dose dependent loss of MMP after 24 h of treatment. Furthermore, the photomicrographs of H460 treated with 100, 250 and 500 μM of TIBA for 48 h show the loss of cell adhesion and shrinkage, indicative of apoptotic cell death (Figure 2C). Zilkah *et al.* (1981) have already observed that TIBA was able to reduce viability of tumor cells, and more recently, along with the evaluation of nanoassemblies containing TIBA [11], Lee and co-workers showed that TIBA alone reduced cell viability in NIH3T3 (embryonic fibroblast) and SCC7 (squamous cell carcinoma), but the nature of this inhibition was not investigated [7]. Herein, we showed that TIBA induced the appearance of sub-G1 population in a dose-dependent way, indicative of apoptosis [14].

The loss of MMP is a hallmark of apoptotic cell death; however, this event may also be observed in the caspase-independent cell death, such ferroptosis [15]. In canonical apoptotic pathway, loss of MMP leads to the release of cytochrome c and formation of apoptosome. To evaluate if TIBA treatment leads to the canonical pathway, the cells were pre-treated with 20 μM of the pan caspase inhibitor zVAD, for 1 h, prior to the addition of 500 μM of TIBA and cell cycle analysis was performed. The results showed that zVAD inhibited sub-G1 population appearance (Figure 3A).

Using the same conditions, cells were pretreated with 40 μM of cyclosporin A for 1 h and then, were also treated with the medium and 500 μM of TIBA for 24 h.

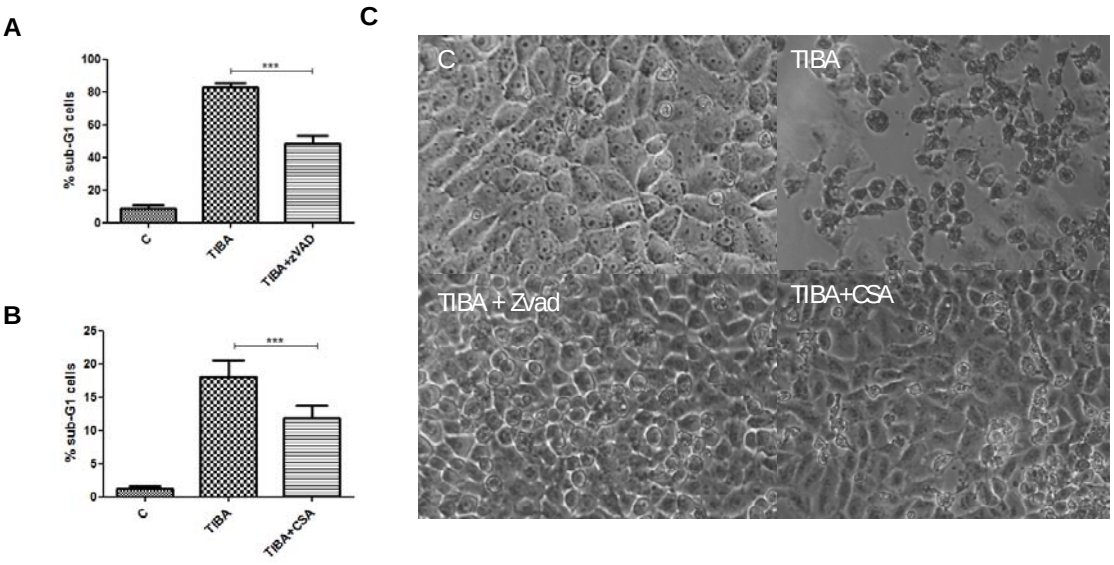

Figure 3. Apoptosis induced by TIBA is inhibited by zVad and cyclosporin A. **A**. H460 cells were pre-treated with zVAD.fmk (20 μM) for 1 h and incubated for 48 h with the medium and TIBA (500 μM). Cells were harvested and the sub-G1 population was measured by flow cytometry. **B**. H460 cells were pre-treated with cyclosporin A (40 μM) for 1 h and incubated for 24 h with the medium and TIBA (500 μM). Cells were harvested and the sub-G1 population was measured by flow cytometry. **C**. Bright field microscopy showing morphology alterations induced by TIBA in the presence of zVAD and cyclosporin A. The columns represent mean ± SD of three experiments performed in triplicate. ***: $p < 0.001$.

Flow cytometry analysis showed that the presence of cyclosporine also reduced cell death (Figure 3B). The morphological analysis (Figure 3C) of H460 cells under the same treatment as in Figure 2A indicates that the cell death is not deviated to necrosis, since the cells remained attached to the substrate and with morphology similar to the control, evidencing the reduction of cell death mediated by CSA. These results indicated that TIBA induces apoptosis with the involvement of the loss of MMP.

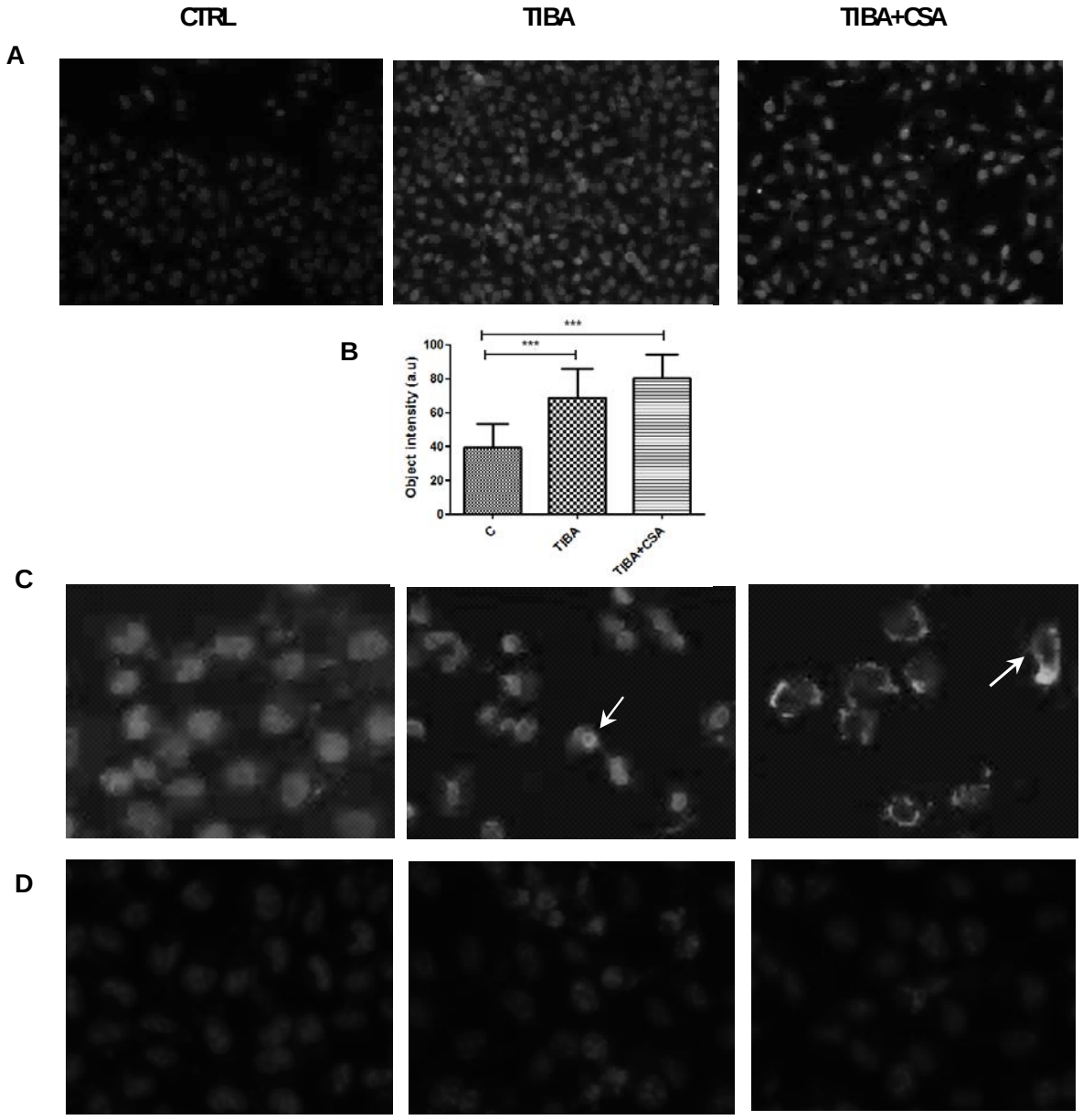

Figure 4. Apoptosis induced by TIBA involves Bax translocation. Cells were pre-trated with cyclosporin A (40 μM) for 1 h, followed by the medium or TIBA (500 μM) for 24 h and immunofluorescence was performed. **A**. TIBA enhanced Bax expression which was not reduced by CSA. **B**. Measurement of the fluorescence intensity of the images presented in A, using cell profiler software; **C**. TIBA induced Bax translocation to the perinuclear site, which was not affected by CSA, and **D**. CSA reduced TIBA-induced chromatin condensation. The columns represent mean ± SD of three experiments performed in triplicate. ***: $p < 0.0001$.

Loss of MMP involves, among several features, the translocation of Bax, a pro-apoptotic member of the Bcl-2 family of proteins. In order to evaluate the Bax involvement in TIBA-induced cell death, we pre-treated H460 cells with 40 µM of cyclosporin A for 1 h, followed by treatment with the medium and 500 µM of TIBA for 24 h and immunofluorescence was performed. As can be seen in Figure 4A-B, TIBA enhanced the expression of Bax. Additionally, TIBA induced translocation of Bax from the cytosol to the perinuclear site, which is related to its interaction with mitochondria [16] (Figure 4 C). This feature was already observed in several studies involving other drugs such as cisplatin [17], simvastatin [18] and ceramide [19]. The perinuclear localization of Bax is thought to occur in order to facilitate the concentration of the apoptogenic molecules that would lead to nuclear alterations characteristic of apoptosis [16]. Incubation with CSA did not reduce the Bax translocation. Also, the presence of CSA reduced chromatin condensation, in accordance to the cell cycle analysis, as evidenced by PI staining (Figure 4D). These results are in agreement with previous studies, where the use of CSA reduced cell death, but not induced Bax translocation [16]. These studies also found that Bax translocation is related to pH acidification and heat stress [20].

Since we found that TIBA induces apoptosis in lung cancer, we inquired how potent TIBA is compared to other drugs under study or already in the clinic, and we found that TIBA is significantly more effective against lung cancer than carboplatin and betulinic acid (Figure 5). The latter, is a pentacyclic triterpene that showed activity against several tumor types including lung cancer. Betulinic have been recently employed in nanodelivery systems to improve their distribution and therefore their efficiency [21]. Carboplatin is less toxic than cisplatin, but also faces lung cancer resistance [22], and cisplatin, in use for the last 30 years, showed higher activity.

The frequent use of TIBA as the base for the synthesis of new contrast agents and its application in nanodelivery systems have raised concerns about its intrinsic ability to induce cell death. Ko and co-workers showed that iohexol (ominipaque), one of the most used TIBA derivatives, induced cell death dependent on caspases, and that this

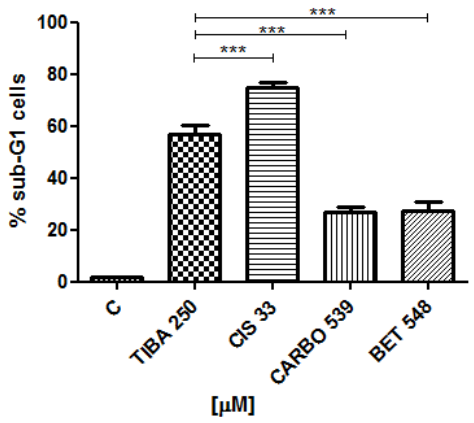

Figure 5. TIBA is more effective against lung cancer than clinically available carboplatin. Cells treated with TIBA (250 µM), Cisplatin (33 µM), Carboplatin (539 µM) and betulinic acid (548 µM). After 48 h, cells were harvested and the sub-G1 population was measured by flow cytometry. The columns represent mean ± SD of three experiments performed in triplicate. ***: $p < 0.001$.

effect was involved in radiocontrast-induced nephropathy in mice [10]. In another study, the efficiency of sorafenib release and the capacity of the MSs, and tumor reduction *in vivo* were evaluated using sorafenib and TIBA-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres (MSs); however, TIBA was not tested for its intrinsic toxicity [8]. Herein, we showed that TIBA alone is able to induce cell death in tumor cells, and that this cell death is a consequence of the apoptotic route. If this toxicity is shared by its derivative iohexol, other derivatives may also exhibit similar effects.

CONCLUSION

The studies performed lead us to conclude that TIBA induces intrinsic apoptotic cell death in a dose-dependent way. This death is dependent on the mitochondrial loss of MMP, inhibited by zVAD and cyclosporine A, with participation of Bax translocation. Whether this potent antitumoral activity is shared in some extent by its derivatives remains to be elucidated.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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