



LNO3 AND L3 Are Associated With Antiproliferative And Pro-Apoptotic Action In Hepatoma Cells

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Abstract

The identification of antitumoral substances is the focus of intense biomedical research. Two structural analogues of thalidomide, LNO3 and L3, are two synthetic compounds that might possess such antitumor properties. We evaluated the toxicological effects of these substances, including cytotoxicity, genotoxicity and induction of apoptosis in HTC cells. Additionally, the production of free radicals (nitric oxide and superoxide) was investigated, and the expression of caspases genes 3, 8, and 9 were determined by RT-qPCR. The compounds exhibited cytotoxic effects that resulted in inhibited cell proliferation. LNO3 showed to be more effective and toxic than L3 in all assays. LNO3 stimulated the release of NO and superoxide, which was accompanied by the formation of peroxyxynitrite. Apoptosis was induced in a dose-dependent manner by both compounds; however, the expression of caspases 3, 8 and 9 was unchanged. These results suggested that L3 and LNO3 possess antiproliferative and pro-apoptotic effects in HTC cells. Additionally, although they exhibited cytotoxicity, L3 and LNO3 might be useful adjuvants in tumor treatment studies.

Keywords: thalidomide analogues, antiproliferative activity; cytotoxicity; free radicals; apoptosis.

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Introduction

Many *in vitro* methods are used to evaluate compounds of pharmacological interest, or to perform safety assessments. In many cases, these studies provide supplementary information on the specific mechanisms involved in a given toxic effect. This accumulation of scientific data supports future evaluations of *in vivo* studies both in animals and humans (Carere *et al.*, 2002). Most of these cellular models are either immortal cell lines or tumor-derived cells. Toxicity data concerning the effects on basic cellular functions and/or structures have shown a good correlation with *in vivo* data regarding human toxicity (Ekwall, 1999). At present, a large number of studies are directed at detecting DNA damage and expression changes in specific genes, cells and tissues, which are targets for chemotherapy.

The identification of chemotherapeutic substances for tumor inhibition represents a challenge to researchers. Potential antitumor compounds and newly synthesized molecules are being investigated for their ability to prevent or curtail disease progression (Mates *et al.*, 2012). Although cancers exhibit very heterogeneous characteristics, all malignant tumors possess the ability to grow beyond the normal limits of regular cells. The clonal expansion of a transformed cell depends on a high proliferative capacity and a resistance to apoptosis. Thus, despite the enormous variability of cancers, evidence shows that resistance to apoptosis is one of the most striking features of the major malignant tumors (Li *et al.*, 2012). Basic research and clinical trials have established that major anticancer agents induce apoptosis, and the inhibition of apoptotic programs may reduce the sensitivity of tumor cells to anticancer treatments (Mates *et al.*, 2012).

In recent years, thalidomide has shown potential for treating clinical conditions, including cancer. The clinical efficacy of thalidomide may be associated with its biochemical properties, which include the inhibition of TNF- α

synthesis, co-stimulation of T cells, and inhibition of angiogenesis (Ali *et al.*, 2012). However, treatment with thalidomide is associated with several toxic effects, such as peripheral neuropathy and thromboembolism, as well as well-known teratogenic effects. Due to these undesirable effects, the goal of current research is to identify agents with similar antitumor effects as thalidomide but with more tolerable toxic effects (Shortt *et al.*, 2013).

The design and synthesis of thalidomide analogues is aimed at obtaining compounds with similar activity profiles and enhanced cytotoxicity. Drugs with different structures and particular characteristics can induce morphological changes associated with apoptosis. This apoptotic process contributes to the cytotoxic action of most chemotherapeutic drugs (Fiandalo and Kyprianou, 2012). LNO3 [nitrate of 2-(1,3-dioxoisindolin-2-yl) ethyl] and L3 [2-(2-hydroxyethyl)isindolin-1,3-dione] are synthetic compounds that possess an isindolin-1,3-dione structure, characterizing them as derivatives of thalidomide (see Figure 1). LNO3 also possesses a radical group (NO_2) that produces nitric oxide (NO) when cleaved. Upon cleavage of the radical, L3 is formed as a metabolite of LNO3 (Aragon-Ching *et al.*, 2007).

In addition to the potential chemotherapeutic effects of thalidomide, nitric oxide (NO) is a radical known for its antitumor effects. There is a significant relationship between apoptosis and NO (Singh and Gupta, 2011). NO can interact with superoxide (O_2^-) to yield peroxynitrite (ONOO^-), and some studies suggest that the pro-apoptotic effects of NO are a result of the formation of peroxynitrite, which induces apoptotic DNA fragmentation and p53-dependent apoptosis (Pacher *et al.*, 2007).

The remarkable diffusibility and diversified chemical reactivity of NO in biological samples make this molecule unique amongst regulators of apoptosis. Understanding the factors that govern the effects of NO exposure on cell viability and gene expression and identifying the conditions under which the regulation of apoptosis by NO contributes to pathogenic processes are topics of great interest (Burke *et al.*, 2013).

Biological differences in the toxicity of thalidomide derivatives at specific endpoints may be attributable to differences in gene expression products. Such diversity could be the result of differential mRNA transcription, RNA splicing, RNA-protein turnover, post-translational modifi-

cations and proteolytic cleavages, alterations in protein-protein interactions, and sub-cellular localization of biologically active proteins (He and Chiu, 2003; Ge and He, 2009; Singh *et al.*, 2010).

Different kinds of assays (*in vivo* and *in vitro*) are utilized to determine the potential genotoxic effect of a particular substance. In *in vitro* experiments, several types of cell lines are utilized. However, there are differences in the results obtained with drug-metabolizing vs. non-drug-metabolizing cell lines. HTC cell lineage is a drug-metabolizing cell line that has been used for the direct and indirect identification of genotoxic agents (Maistro *et al.*, 2004; Matuo *et al.*, 2007; Niwa *et al.*, 2013). The aim of the present study was to determine the effects of LNO3 and L3 in drug-metabolizing cells for comparison of results with other cells lines. The tests were conducted in drug-metabolizing cells of hepatoma tissue of *Rattus norvegicus* (HTC), evaluated the cytotoxicity, free radicals production (superoxide and nitric oxide) and changes in *Casp3*, *Casp8*, and *Casp9* mRNA levels as potential pro-apoptotic and antitumor activity.

Materials and Methods

Cell culture

Rattus norvegicus-derived cells (HTC, a hepatoma cell line) were acquired from the Cell Bank of the State of Rio de Janeiro, Brazil. The cells were seeded into 25 cm² polystyrene flasks containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM; Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco®, Life Technologies, Carlsbad, CA, USA) and 1% sodium pyruvate (Gibco®, Life Technologies, Carlsbad, CA, USA) and were cultured in a controlled atmosphere (37 °C, 5% CO₂). Under such conditions, the cell cycle was approximately 24 h.

Chemical agents

The Study Group on Organic and Biological Chemistry at the Federal University of Minas Gerais (UFMG), Brazil, supplied the experimental drugs used in this study (LNO3 and L3). These drugs, chemically modified from thalidomide as shown in Figure 1, were diluted in dimethyl sulfoxide (DMSO 0.1%) (Mallinckrodt Chemicals, St. Louis, MO, USA). The damage-inducing agents doxorubi-

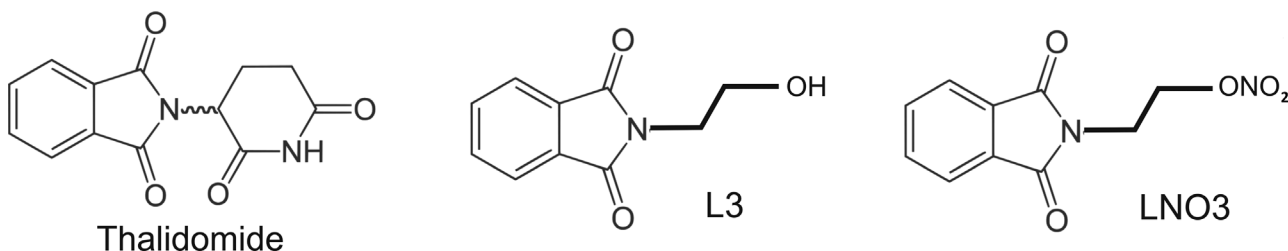


Figure 1 - Chemical structures of Thalidomide, L3 and LNO3.

cin (CAS 24316-40-9; Adriblastin®, Pharmacia, Italy), benzo[a]pyrene (CAS 50-32-8, Sigma-Aldrich, Saint Quentin Fallavier, France) and camptothecin (CAS 7689-03-4; Acros Organics, Fischer Scientific Latin America Headquarters, Suwanee, GA, USA) were used to assay for the induction of apoptosis.

Cytotoxicity assay

The MTT assay (Mosmann, 1983) was performed with some modifications. HTC cells were seeded into 24-well cell culture plates at a concentration of 2×10^4 cells/well in 500 μ L DMEM medium/well and incubated for 24 h. The medium was subsequently removed and replaced with the same volume of fresh medium along with the following treatments: 50, 100, 250 and 500 μ g/mL of LNO3 or L3/well. Doxorubicin (10 μ g/mL, CAS 25316-40-9, Adriblastina®- Pharmacia, Italy) served as positive control (DNA damaging agent). HTC cells exposed to 0.1% DMSO + DMEM served as negative control. The cells were then incubated for 24, 48 or 72 h, after which the medium was removed, and the cells were incubated with MTT

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, Invitrogen, Life Technologies, USA) solution (500 μ L of 0.1 mg/mL solution/well) at 37 °C for 4 h. After removal of the MTT solution, DMSO (500 μ L) was added to each well to dissolve the formazan crystals. The absorbance was determined at 540 nm in a spectrophotometer (Uniscience, São Paulo, SP, Brazil). Each experiment was performed in triplicate with three replicates per experiment.

Nitric oxide (NO) dosage with cadmium (Cd²⁺)

Nitrite samples were measured to estimate NO levels. The measurements were performed using the method of Panis *et al.* (2011) adapted to cell culture. LNO3 and L3 were tested at a concentration of 500 μ g/mL. The treatment included a control without cells to determine whether NO was released directly by the drug or as a result of cellular metabolism. As such, two 24-well plates were used: The first plate was seeded with 10^4 HTC cells in 300 μ L DMEM medium supplemented with FBS. The second plate received only DMEM medium with FBS (cell free). Both plates were incubated for 24 h following the addition of 300 μ L of DMEM medium containing the following treatment: 500 μ g/mL of L3 or 500 μ g/mL of LNO3. The treatments were added in triplicate to the wells with and without cells. After one hour of treatment, 60- μ L plasma aliquots were deproteinized by adding 50 μ L of 75 mM ZnSO₄, followed by a centrifugation (9,500 g for 2 min at 25 °C). Next, we added 70 μ L of 55 mM NaOH and centrifuged the samples at 9,500 g for 5 min at 25 °C. The supernatant (250 μ L) was recovered and diluted in glycine buffer solution (45 g/L, pH 9.7) at a proportion of 5:1. Cadmium granules (stored in 100 mM H₂SO₄) were added to a 5 mM CuSO₄ solution in

glycine-NaOH buffer (15 g/L, pH 9.7) for 5 min. The granules were then added to the sample and suspended for 10 min while the nitrate from the sample was converted to nitrite. Then, sample aliquots were recovered, and the same volume of Griess reagent was added (Reagent I, 50 mg of *N*-naphthyl ethylenediamine in 250 mL of distilled water; Reagent II, 5 g of sulfanilamide in 500 mL of 5% phosphoric acid), and 100 μ L of the sample was used to determine the nitrite concentration. A calibration curve was prepared by the dilution of 7.8 μ M NaNO₂. The absorbance was determined at 550 nm.

Determination of O₂⁻ production by chemiluminescence:

HTC cells (5×10^4) seeded in tubes containing 1 mL of DMEM with fetal bovine serum (10%) were used to monitor the production of superoxide anions in real time by chemiluminescence. After 24 h of stabilization, 10 μ L of one of the following treatments was added to each tube: phorbol myristate acetate (PMA) at 2 μ g/mL (positive control) or LNO3 or L3 at 500 μ g/mL. To amplify the photon emissions, 5 μ M of lucigenin was added to the reaction. The readings were monitored on a TD 20/20 Glomax luminometer (Promega, Madison, USA) employing a protocol that allowed 1 reading per second for 15 min. The results were expressed as the integrals of the emission area of each sample. The experiment was performed in triplicate.

Kinetics of cell proliferation and cell viability

For proliferation kinetics assays, we used 10 cm² seeded tubes (10^5 cells/tube). After stabilization, the cells underwent four incubation rounds of 24, 48, 72 and 96 h and were subsequently counted. Subsequently, the cells were trypsinized (0.1% trypsin-ethylenediaminetetraacetic acid at 37 °C). The cell suspension was centrifuged at 1080g for 5 min, resuspended in 500 μ L of DMEM medium and counted in a Neubauer chamber. For this assay, the cells were exposed to 125, 250 and 500 μ g/mL of LNO3 or L3. The kinetics of cell proliferation were investigated in two independent experiments. Additionally, based on cell viability measurements performed using trypan blue (5%)/ultrapure water (v/v), 80% or more living cells were considered viable.

Cytokinesis-block micronucleus assay and nuclear division index

For this experiment, HTC cells (10^6) were grown in 25 cm² culture flasks and stabilized for 24 h before treatment. The HTC cells were then treated as follows: (a) control (DMSO, 0.1%); (b) DNA damage-inducing agent (Benzo-[a]-pyrene, 20 μ g/mL); or (c) LNO3 or L3 (125 μ g/mL, 250 μ g/mL and 500 μ g/mL). In all cases, the cells were exposed to the compounds for 24 h. The treated cells were washed with PBS buffer, and cytochalasin-B (3

$\mu\text{g/mL}$ - Sigma-Aldrich) was added for 26 h to obtain binucleated cells. The cells were then washed with PBS buffer, lysed with 0.1% trypsin-EDTA (Gibco, BRL) at 37 °C, inactivated with culture medium and centrifuged at 360g for 5 min. Next, the cells underwent gentle homogenization with 1% sodium citrate hypotonic solution, followed by the addition of formaldehyde (15 μL) to the cell suspension, which was immediately homogenized. The cells were again centrifuged, the supernatant was discarded, and the cells were fixed with methanol:acetic acid (3:1 v/v) for 10 min. Next, the solution was centrifuged again for 5 min at 360g, yielding a cell pellet that was used for the histological staining. For the detection of micronuclei, the cells were treated with Giemsa stain (5%). A total of 3,000 binucleated cells per treatment were analyzed per repetition to determine the micronuclei frequency (MN), and 1,000 binucleated cells were analyzed to determine the nuclear division index (NDI). The criteria for the selecting binucleated cells, identifying micronuclei and calculating the NDI followed the protocol provided by Fenech (2000). Three repetitions of each experiment were performed.

Apoptosis induction assay

For the morphological detection of apoptosis, HTC cells (5×10^4 cells/well) were grown in a 6-well plate containing 5 mL of DMEM medium and a coverslip (20 x 20 mm) in each well. Apoptotic cells were identified by analyzing the chromatin condensation pattern after staining with Acridine Orange. After 24 h of stabilization, the medium was replaced, and LNO3 or L3 (125, 250 and 500 $\mu\text{g/mL}$) was added to the wells. HTC cells were treated with camptothecin 10 $\mu\text{g/mL}$ (CAS 7689-03-4; Acros Organics, Fischer Scientific Latin America Headquarters, Suwanee, GA, USA) as a positive control, and with 0.1% DMSO as a negative control. After 24 h of treatment, the HTC cells were harvested according to the protocol of Tsuboy *et al.* (2010) to remove the coverslip and fixed with Carnoy's solution (methanol:glacial acetic acid, 3:1 v/v) for 5 min. Afterwards, the coverslip was hydrated using a descending series of ethanol washes (from 95% to 25%).

Next, the coverslips were washed for 5 min with McIlvaine buffer (0.1 M citric acid and 0.2 M disodium phosphate, pH 7). The HTC cells were then stained with acridine Orange (0.01%) for 5 min and washed a second time with McIlvaine buffer. Chromatin condensation was analyzed to identify the apoptotic cells. Three independent experiments were performed, and 1,000 cells were analyzed per treatment. The analyses were conducted via fluorescence microscopy (excitation filter 420-490 nm, barrier 520 nm; Nikon 027012, Melville, NY, USA).

Isolation of total RNA and RT-qPCR

HTC cell cultures (10^6 /treatment) were pre-incubated for 24 h and treated for 12 h as follows: (a) control (b) LNO3 (250 $\mu\text{g/mL}$), or (c) L3 (250 $\mu\text{g/mL}$). Total RNA was extracted using TRIzol LS reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was conducted with reverse transcriptase (RT M-MLV, Invitrogen, Life Technologies, USA) using 2 μg of total RNA as the template, according to the manufacturer's protocol. The qPCR amplifications were performed in a PTC 200 DNA Engine Cycler using a Chromo4 Detection System (MJ Research, Bio-Rad, Waltham, MA, USA). Table 1 provides a list of the oligonucleotides utilized in these experiments. Platinum® SYBR Green qPCR Supermix-UDG (Invitrogen Life Technologies, USA) was used in the reaction mixture, to which 2 μL of each primer (1 μL forward and 1 μL reverse primers) and 2 μL of template cDNA were added to produce a final volume of 20 μL . The PCR thermal cycling conditions included an initial step at 50 °C for 1 min, 95 °C for 3 min and 35 cycles of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 20 s, followed by 95 °C for 10 s and 40 °C for 1 min. A melt-curve analysis was consistently performed at the end of each reaction, with temperatures increasing from 50 to 95 °C at a rate of 0.5 degrees per 5 s. The data were normalized to β -actin and glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*) cDNA amplified in each set of PCR experiments. The choice of the normalizer gene was

Table 1 - List of primers used in relative gene expression.

Gene	Sequence 5' - 3'	Access n°. /NCBI
<i>Gapdh</i>	F- ACA AGA TGG TGA AGG TCG GTG TCA R- AGC TTC CCA TTC TCA GCC TTG ACT	M17701 / Tso <i>et al.</i> , 1985
β -actin	F- TTG CTG ACA GGA TGC AGA AGG AGA R- ACT CCT GCT TGC TGA TCC ACA TCT	BC_063166 / Present study
<i>Casp3</i>	F- TCA TGT CCA CCA CTG AAG GAT GGT R- AGA GTT GGA GCA CTG TAG CAC ACA	NM_012922 / Whidden <i>et al.</i> , 2010
<i>Casp8</i>	F- AAA GCA AGG ACC ACA AGG GCA AAG R- AGG GCA CTT TGA GCC AGT GAA GTA	NM_022277 / Bai and Meng, 2010
<i>Casp9</i>	F- ATG TCT GGT TGG CAG AAC TCA GGA R- ACC ACG GAC ACA TGA GGT TGT GTA	NM_031632 / Bai and Meng, 2010

made based on the results of the expression obtained in all treatments. Both endogenous genes used showed no significant change. All cDNA samples were analyzed in three technical replicates for each primer pair.

Statistical analysis

In the cytotoxicity assay, the measured parameters of NO levels and cell proliferation kinetic values were compared by analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. The latter test was used to identify group differences using GraphPad Prism® version 5 software (GraphPad Software, San Diego, California, USA). The threshold of significance was set at $p \leq 0.05$ to compare LO and LNO3 concentrations with control HTC cells. The production of superoxide anions by chemiluminescence was verified by qualitative and quantitative production expressed as integrals of the broadcast area of each sample in GraphPad Prism® 5 software. The data obtained from the apoptosis and micronucleus assays were compared in GraphPad Prism® 5 using the chi-square (X^2) test, with $p \leq 0.05$. The relative gene expression levels were determined according to Pfaffl (2001). The Pair-Wise Fixed Reallocation Randomization Test was used in the Software REST© 384 (Relative Expression Software Tool-384) for comparison and statistical analysis of relative expression results in real-time PCR (Pfaffl *et al.*, 2002). Statistical significance was set at $p < 0.05$.

Results

MTT cytotoxicity assay

L3 showed a cytotoxic effect only at a concentration of 500 $\mu\text{g/mL}$ after 72 h of exposure. In contrast, LNO3 was

able to reduce cell viability within 48 h at a concentration of 500 $\mu\text{g/mL}$ and within 72 h at a concentration of either 250 $\mu\text{g/mL}$ or 500 $\mu\text{g/mL}$ (Table 2).

Determination of nitric oxide (NO) by cadmium (Cd^{2+})

L3 did not release NO nor did it stimulate the cellular production of NO, as observed when comparing the treated and control cells (Figure 2). However, LNO3 was capable of producing approximately 20 times more NO than L3, and this increase was due to the release of NO by LNO3 upon contact with the culture medium. This effect was not dependent on the cellular machinery, as evidenced by the concentrations of NO in both treatments with LNO3 (with and without cells), which were very similar.

Superoxide anion production (O_2^-)

L3 was unable to stimulate a significant level of O_2^- production over a period of 15 min compared with the control. Conversely, LNO3 exhibited high levels of O_2^- production (Figure 3).

Kinetics of cell proliferation

L3 was found to have little effect on cell proliferation. At a concentration of 500 $\mu\text{g/mL}$, L3 decreased cell proliferation within 48 and 72 h compared with the control, whereas the other concentrations tested did not alter the cell cycle (Figure 4).

Conversely, LNO3 altered the kinetics of cell division at all concentrations tested in 48 h of treatment. The effect of LNO3 was even more evident than that of L3 at 72 h at all concentrations examined. The viability of the cells in all treatments was similar, indicating that the cytotoxic ef-

Table 2 - The average cell viability (%) of HTC cells exposed to L3 and LNO3 for 24, 48 and 72 hours obtained from cytotoxicity assay (MTT). The cell viability percentage was calculated from absorbance values obtained from cytotoxicity assay. The positive control was doxorubicin (10 $\mu\text{g/mL}$).

Treatments	[$\mu\text{g/mL}$]	Times of Exposure		
		24 hours	48 hours	72 hours
CTRL		100 \pm 3.158	100 \pm 2.580	100 \pm 0.7976
DXR	10	59.48 \pm 4.214 ^{a*}	26.00 \pm 1.620 ^{a*}	10.34 \pm 0.9262 ^{a*}
L3	50	111.3 \pm 3.698	102.5 \pm 6.041	98.00 \pm 2.527
	100	106.7 \pm 1.764	94.06 \pm 5.536	97.13 \pm 1.815
	250	106.6 \pm 1.809	93.64 \pm 3.592	94.83 \pm 1.719
	500	99.82 \pm 5.079	89.76 \pm 4.653	92.07 \pm 1.780 ^{a*}
	LNO3	50	97.73 \pm 2.795	98.39 \pm 2.674
	100	94.41 \pm 4.886	92.61 \pm 0.6698	99.39 \pm 2.865
	250	86.05 \pm 5.918	84.25 \pm 0.4903	87.38 \pm 2.212 ^{a*}
	500	80.06 \pm 6.226	67.52 \pm 2.168 ^{a*}	78.23 \pm 0.4306 ^{a* b*}

CTRL: Control; DXR: Doxorubicin; a: Statistically significant compared to control; b: Statistically significant compared to L3 at the same concentration. * $p \leq 0.05$.

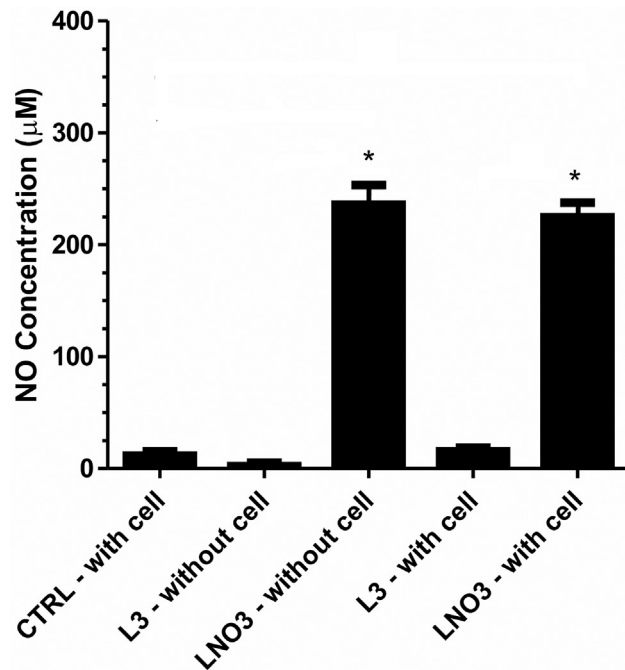


Figure 2 - Nitric oxide concentrations in DMEM. Average concentration of nitric oxide (NO) in the culture medium of HTC cells exposed to 500 µg/mL of L3 or LNO3 for 60 min. NO concentration (µM) measured by chemiluminescence. The bars represent the mean ± SD of three independent experiments. Significant differences: * $p \leq 0.05$ compared with control cells.

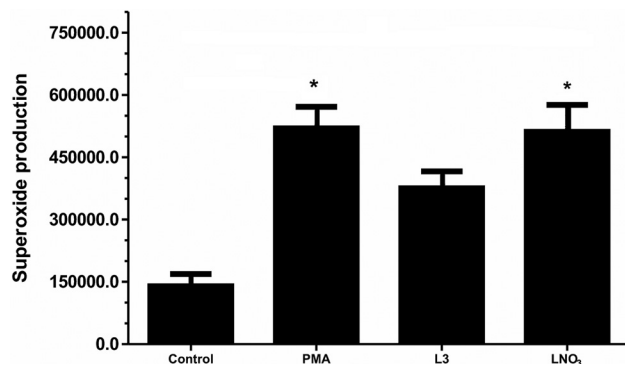


Figure 3 - Superoxide production. Quantification of average concentration of superoxide anions (O_2^-) in the culture medium of HTC cells exposed to 500 µg/mL of L3 or LNO3 for 15 min (1 reading per second). The O_2^- concentration (µM) was measured by chemiluminescence. PMA (phorbol myristate acetate; 2 µg/mL) was used as a positive control. The results were expressed as the integral area. The bars represent the mean ± SD of three independent experiments. Significant differences: * $p \leq 0.05$ compared with control cells.

fect on the cells is not likely to occur primarily through the induction of cell death.

The data in Table 3 show the values for R2 and the Doubling Time for each treatment. The doubling time values achieved indicated that control cells required less time to duplicate than L3-treated cells by up to 4 h. In addition, LNO3 delayed the cell cycle at all concentrations tested, re-

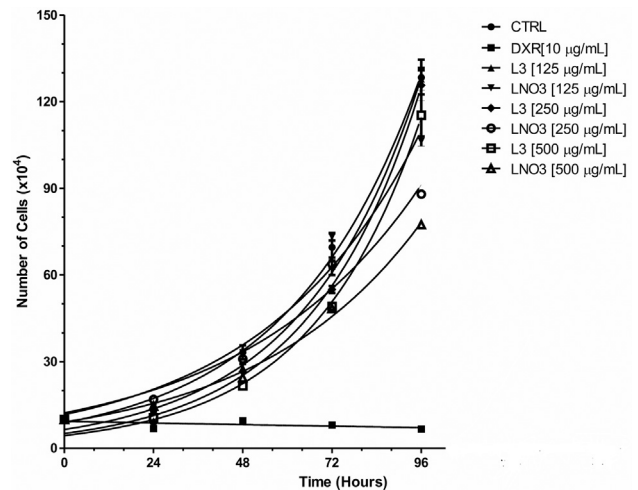


Figure 4 - Cell growth curve determined by counting HTC cells after 24, 48, 72 and 96 h of exposure to L3 or LNO3. Doxorubicin (10 µg/mL) was used as a control. The bars represent the mean ± SD of two independent experiments. CTRL: HTC cell control, DXR: doxorubicin.

Table 3 - Doubling Time (DT) in kinetics of cell proliferation.

Treatments	[µg/mL]	R2	DT
CTRL		0.9627	24.78
DXR		0.2397	-248.6
L3	125	0.9911	22.28
250	0.9901	20.79	
500	0.9816	20.39	
LNO3	125	0.9734	29.65
250	0.9745	33.11	
500	0.994	30.89	

CTRL: Control; DXR: Doxorubicin.

quiring an average of six more hours for cell duplication than the control group.

Induction of apoptosis

As shown in Figure 5, apoptosis was induced in all treatments tested (L3 and LNO3 at 125, 250 and 500 µg/mL). LNO3 activated the apoptosis pathway more effectively than its metabolite L3 only at the highest concentration (500 µg/mL). On the other hand, the other tested concentrations produced similar levels of apoptotic cells

Micronucleus assay

L3 produced more cells with chromosomal damage compared with controls at the highest concentration tested (500 µg/mL), whereas LNO3 produced mutagenic effects at 250 and 500 µg/mL. Although the induction of micronuclei was higher for LNO3, their rates did not show statistically significant differences compared with L3 (Figure 6).

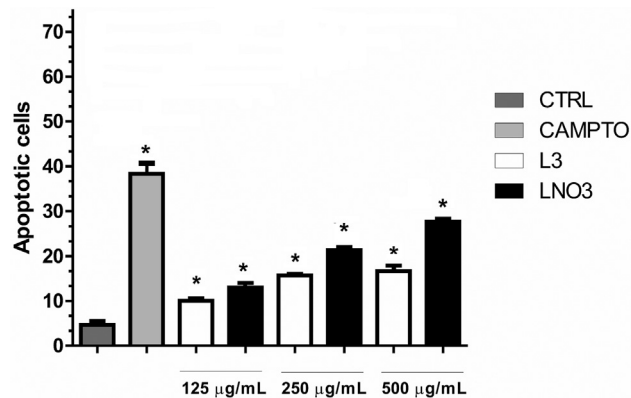


Figure 5 - Assay of induction of apoptosis in situ in HTC cells after 24 h of exposure to L3 or LNO3. Camptothecin (10 µg/mL) was used as a positive control. The bars represent the mean \pm SD of three independent experiments. Significant differences: * $p \leq 0.05$ compared with control cells.

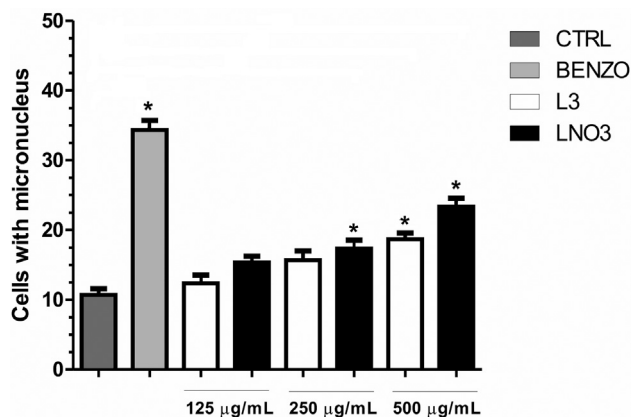


Figure 6 - Average of number of micronuclei (MN) in HTC cells exposed to L3 or LNO3 for 26 h. The treatments were as follows: control HTC cells, HTC cells with Benzo[a]pyrene (20 µg/mL) (DNA-damage inducer), HTC cells with L3 or LNO3. The bars represent the mean \pm SD of three independent experiments. Significant differences: * $p \leq 0.05$ compared with control HTC cells.

RT-qPCR analysis

After analyzing and normalizing the relative gene expression values using the reference gene, the gene expression of *Casp3*, *Casp8* and *Casp9* was found to increase by a factor of 0.091, 0.13 and 0.099, respectively, following LNO3 treatment. Nevertheless, these differences were not statistically significant. Similarly, gene expression increased by a factor of 0.184 for *Casp3*, 0.405 for *Casp8* and 0.117 for *Casp9* upon L3 treatment. However, these differences were also not statistically significant when compared with control cells (Figure 7).

Discussion

The identification of chemotherapeutic substances to induce tumor regression represents one of the major chal-

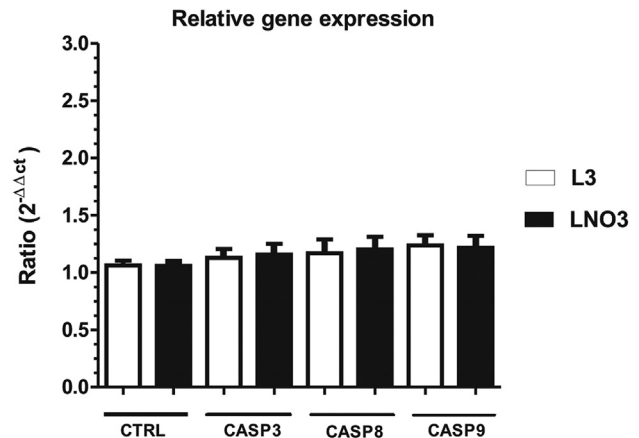


Figure 7 - Relative expression of the *Casp3* (caspase), *Casp8* and *Casp9* genes using RT-qPCR after 12 h of exposure to 250 µg/mL L3O or LNO3. The bars represent the mean \pm SD of three independent experiments.

lenges for modern medicine. Currently, new agents and potential antitumor compounds for the prevention or containment of disease progression are under investigation, and new molecules are being synthesized (Havrylyuk *et al.*, 2010).

The combined antiangiogenic and anti-TNF- α properties of thalidomide may represent a promising strategy for cancer treatment. However, treatment with thalidomide carries a series of side effects that occur with cumulative doses (Shortt *et al.*, 2013). Therefore, the search for agents with similar or improved action but better tolerability is underway. In this regard, structural analogues of thalidomide are being synthesized and tested for their antiangiogenic, anti-TNF- α and chemotherapeutic properties. Aragon-Ching *et al.* (2007) showed that a class of thalidomide analogues (SelCID) was consistently effective in reducing the viability of tumor cells in a variety of solid tumors.

The MTT cytotoxicity assay is one of the most sensitive methods for the detection of *in vitro* cytotoxicity (Fotakis and Timbrell, 2006). However, this assay not only evaluates cell death, but can also indicate the inhibition of cell growth, i.e., the cytostatic effect. We showed that L3 was cytotoxic only at the highest concentration (500 µg/mL) after 72 h of exposure, whereas LNO3 showed cytotoxicity at 500 µg/mL after 48 h of exposure. LNO3 was also able to induce cytotoxic effects at 250 µg/mL within 72 h, indicating that a lower concentration of this compound can disrupt cellular behavior compared to its metabolite (L3).

Cell proliferation assays yielded further data supporting the results obtained from the MTT assay. Specifically, these assays revealed that the cell proliferation changed when the cells were treated with 500 µg/mL of L3 between 48 and 72 h. Similarly, an even greater antiproliferative effect with LNO3 was observed at all concentrations tested (125, 250 and 500 µg/mL) for 48 h of treatment. The inhibi-

tion of cell proliferation was proportional to the dose tested and the length of exposure. The effects induced by LNO3 may be attributable to the blockage of cell proliferation in situations where the genomic integrity is compromised. However, these effects need further investigation to compare these compounds with different chemotherapeutic agents. In addition, their action on tumor cells must be compared with their action on healthy cells.

Nitric oxide (NO) can display antitumor activity (Carpenter and Schoenfisch, 2012). However, the anti-tumor activity of NO depends on the amount of NO generated, the individual sensitivity of the cells and the duration of the phenomenon (Singh and Gupta, 2011). In this study, LNO3 caused a significant release of NO in a short period, which is indicative of potential anti-tumor activity.

Recently, the reaction between nitric oxide and superoxide to form peroxynitrite has received considerable attention, especially as a potentially deleterious reaction. Here, we detected a high level of superoxide anion production in HTC cells treated with LNO3. According to Pacher *et al.* (2007), several studies have shown that the reaction of NO with oxygen or superoxide can form mutagenic molecules (NO-derived species). The production of superoxide combined with the NO released by LNO3 results in the formation of peroxynitrite (ONOO⁻), which is the agent that is likely to be responsible for the mutagenicity observed in the micronucleus test.

The apoptosis assay provided additional evidence supporting the results of the micronucleus test. This assessment revealed that the three concentrations of LNO3 and L3 tested (125, 250 and 500 µg/mL) can induce apoptosis in HTC cells.

In addition to the cytotoxic and mutagenic effects caused by NO and its derivatives, the isoindoline structure present in thalidomide and its analogues also merits discussion for its effect in combating cancer. Li *et al.* (2009) showed that STA-35, another analogue of thalidomide, as well as thalidomide itself, can exert cytotoxic effects in acute myeloid leukemia culture cells (HL-60). In their study, these authors found that the inhibitory effect on cell growth is due to the induction of apoptosis by the cleavage of PARP-1 (Poly (ADP-ribose) polymerase 1), as evidenced by the increased number of cells in Sub-G1.

The cytotoxic effects, apoptosis induction and micronucleus formation induced by L3 indicate that the effects of this compound are due to its structure similarity to thalidomide rather than to the effects of nitric oxide and its derivatives. The strongest effects produced by LNO3 suggest that this compound promotes apoptosis through the substantial release of NO, peroxynitrite formation and the generation of L3, which also has pro-apoptotic activity.

We conducted gene expression analyses of the major initiator caspases of both the extrinsic (*Casp8*) and intrinsic (*Casp9*) apoptotic pathways to determine which pathway could be triggered by L3 and LNO3. We also assessed the

main effector caspase (*Casp3*). Relative expression levels of all three caspases were similar to those of the control cells, indicating that basal levels of expression were sufficient for the occurrence of apoptosis in treatments of the HTC cells with these analogues.

These data suggest that free radicals, such as those produced by L3 and LNO3, can activate programmed cell death mechanisms. Similarly, these results show that, in addition to its mutagenic action, LNO3 can arrest the cell cycle. The deleterious effects on the genomic material revealed by the micronucleus assay may explain the presence of a larger number of apoptotic cells after treatment. These results show that LNO3 has cytotoxic and mutagenic activities, and induces apoptosis at a higher level than does its metabolite L3. In addition, the free radicals NO and O₂⁻ are present only after LNO3 treatment, potentially causing the increased antitumor effects.

In conclusion, there is a high interest in the development of new compounds based on the structure of thalidomide to improve its pharmacokinetic properties and reduce its teratogenic effects. In this study, we showed that LNO3 and L3 presented antiproliferative and pro-apoptotic effects in HTC cells and, thus, may be target substances for *in vivo* antitumor studies. Understanding their mechanisms of action will help to improve their development, optimize clinical applications, and finally, translate their effects into beneficial activity in specific cancers.

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