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# Autophagy inhibited Ehrlich ascitic tumor cells apoptosis induced by the nitrostyrene derivative compounds: Relationship with cytosolic calcium mobilization

Andrana K. Calgarotto [a], Gustavo J. da Silva Pereira [b], Alexandre Bechara [b], Edgar J. Paredes-Gamero [c], Christiano M.V. Barbosa [c], Hanako Hirata [b], Mary L. de Souza Queiroz [a], Soraya S. Smaili [b], Claudia Bincoletto [b,*]

[a] Departamento de Farmacologia, FCM, Universidade Estadual de Campinas, UNICAMP, Campinas/SP, Brazil
[b] Departamento de Farmacologia, Escola Paulista de Medicina, Universidade Federal de São Paulo (UNIFESP), São Paulo/SP, Brazil
[c] Departamento de Biofísica, Escola Paulista de Medicina, Universidade Federal de São Paulo (UNIFESP) São Paulo/SP, Brazil

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ABSTRACT

Apoptosis induction is often associated with increased autophagy, indicating interplay between these two important cellular events in cell death and survival. In this study, the programmed cell death and autophagy induced by two nitrostyrene derivative compounds (NTS1 and NTS2) was studied using the tumorigenic Ehrlich ascitic tumor (EAT) cells. EAT cells were highly sensitive to NTS1 and NTS2 cytotoxicity in a dose-dependent manner. NTS1 and NTS2 $IC_{50}$ was less than 15.0 μM post 12 h incubation. Apoptosis was primarily induced by both compounds, as demonstrated by an increase in Annexin-V positive cells, concurrently with cytochrome *c* release from mitochondria to cytosol and caspase-3 activation. Although cytosolic $Ca^{2+}$ mobilization is involved in autophagy as well as apoptosis in response to cellular stress in many cancer cell types, from the two nitrostyrene derivative compounds studied, mainly NTS1 mobilized this ion and disparate autophagy in EAT cells. These results suggest that EAT induced cell death by NTS1 and NTS2 involved a $Ca^{2+}$-dependent and a $Ca^{2+}$-independent pathways, respectively. In accordance with these results, the treatment of EAT cells with 3 methyladenine (3-MA), an autophagy inhibitor; significantly increased the number of apoptotic cells after NTS1 treatment, suggesting that pharmacological modulation of autophagy augments the NTS1 efficacy. Thus, we denote the importance of studies involving autophagy and apoptosis during pre-clinical studies of new drugs with anticancer properties.



## 1. Introduction

One of the major clues today is the crosstalk between autophagy and apoptosis during tumor development since there is a complex relationship between these cellular events, determining whether a cell will live or die (Armstrong, 2010; Chen and Karantza, 2011; Danial and Korsmeyer, 2004; Todde et al., 2009). In addition, studies have demonstrated that resistance of tumor cells to apoptosis can also be overcome by autophagy inhibition (Armstrong et al., 2011).

A major form of autophagy is the macroautophagy, a process that occurs in all eukaryotic cells, in which parts of the cytoplasm and intracellular organelles and proteins are sequestered within a double autophagic membrane. Autophagosome, whose formation is controlled by the interaction and activity of proteins of autophagy-related genes (ATG), fuses with lysosomes to form autolysosomes, and the sequestered contents are degraded by lysosomal hydrolases (Klionsky, 2008; Levine and Klionsky, 2004; Yang and Klionsky, 2009). Autophagy is observed under physiological and pathological conditions including cancer (Galluzzi et al., 2008; Kondo et al., 2005), where its role is greatly tracting the interest of the scientific community.

The relationship between autophagy and tumorigenesis has been reported by many studies (Bialik and Kimchi, 2008; Eisenberg-Lerner and Kimchi, 2009). Liang et al., 1999 described that re-introduction the human orthologous gene of yeast ATG-6, into MCF7 breast cancer cell line induced autophagy and inhibited tumorigenicity. On the other hand, in tumor advanced stage, autophagy seems to promote survival of tumor cells, since those located in the tumor central area undergo autophagy to survive in low-oxygen and nutrient starvation conditions (Platini et al., 2010).

In breast cancer cells treated with tamoxifen, the autophagy inhibitor 3-methyladenine (3-MA) prevented cell death (Samaddar et al., 2008). On the other hand, mild heat shock (43 °C for 2 h) does not induce apoptosis in malignant glioma cell lines (U251-MG and U87-MG), but transient growth arrest with mild G2/M suggesting that autophagy is acting as a protective mechanism allowing cells to escape from apoptosis (Komata et al., 2004).

* Corresponding author. Tel./fax: +55 11 5576 4443.
*E-mail address:* claudia.bincoletto@unifesp.br (C. Bincoletto).

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Nitrostyrene derivative compounds are potent inhibitors of protein phosphatases, PTB1 (Park and Pei, 2004) and PP2A (Fathi et al., 2000) displaying an associated pro-apoptotic effect even in some multidrug-resistant tumor cells (Fathi et al., 2000; Kaap et al., 2003; Kim et al., 2003). Based on the nitrostyrene derivative compounds apoptosis inducer properties and in the fact that autophagy and apoptosis share common stimuli and signaling pathways, and exhibit some degree of mutual inhibition (Kang et al., 2011), in the present study, we investigated the effectiveness of two nitrostyrene derivative compounds, 1-((E)-2-nitrovinyl)benzene NTS1 and 1-nitro-3-((E)-2-nitrovinyl)benzene NTS2, to produce cytotoxic effects on EAT cells as well as their ability to induce apoptosis by caspase activation and cytochrome *c* release. The relationship of these events with autophagy and cellular $Ca^{2+}$ mobilization, which has been considered a modulator element among proliferation, apoptosis and autophagy in many tumor cells (Ferrari et al., 2002; Gerasimenko et al., 2002; Høyer-Hansen and Jäättelä, 2007) was also evaluated. Our results demonstrated that nitrostyrene derivative compounds induced EAT cells death, but only NTS1 was able to mobilize significantly cytosolic $Ca^{2+}$ and autophagy induction.

## 2. Materials and methods

### 2.1. Nitrostyrene derivative compounds

The nitrostyrenes 1-((E)-2-nitrovinyl) benzene (NTS1) and 1-nitro-3-((E)-2-nitrovinyl) benzene (NTS2) were synthesized by Villar et al., 2008 and kindly donated by these authors for the performance of these experiments. The compounds were dissolved in dimethylsulphoxide (DMSO), final concentration of 1% in RPMI.

### 2.2. Ehrlich ascitic tumor (EAT) cells preparation

Male BALB/c, 6–8 weeks old, were bred at the University Central Animal Facilities (UNICAMP, Campinas, SP), raised under specific pathogen-free conditions, and matched for body weight before use. Animal experiments were done in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Committee (Process: 1754-1). Mice were inoculated intraperitoneally (i.p.) on day 0 with 0.2 mL of a tumor cell suspension ($10^3$ cells/mL) prepared in balanced salt solution at pH 7.4. The cellular viability, assessed by the trypan0 blue dye exclusion method, was always found to be more than 95%. Tumor cell cultures were derived from ascitic tumor harvested by peritoneum from mice 8 to 10 days after tumor transplantation. Cells were plated in culture dishes with RPMI 1640 medium (Gibco BRL Co., Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 100 U/mL streptomycin, 100 U/mL penicillin G, and 2 mM L-glutamine and incubated in a humidified atmosphere of 5% $CO_2$ in air at 37 °C for 2 h. For all of the subsequent experiments, EAT cells were treated with NTS1 and NTS2 for 12 h. EAT cells autophagy and apoptosis were also evaluated in the presence of 3-methyladenina (3-MA) (20 mM) and rapamycin (RAP) (1 μM).

### 2.3. Cytotoxicity assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was done as described before (Mosmmann, 1983). Briefly $10^5$ viable EAT cells were seeded into 96-well flat microtit plates (Corning, USA) in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated with different concentrations of NTS1 or NTS2. After 12 h it was added 10 μg/well of MTT (5 mg/mL) and incubated for 4 h, when 100 μL of dimethyl sulfoxide DMSO (Merck, Germany) were added to each well to solubilize the formazan. Absorbance was measured at 560 nm.

### 2.4. Detection of apoptosis by flow-cytometry

EAT-nitrostyrene derivative compounds treated cells were stained with fluorescein isothiocyanate (FITC)-conjugated to annexin V/propidium iodide (PI) according to manufacturer's instructions (Annexin V/FITC Apoptosis Detection Kit, BD Pharmigen, CA, USA). The population of annexin $V^-PI^-$ viable cells and annexin $V^+$ apoptotic cells was evaluated by flow-cytometry. Data were collected in a FACS Calibur (Becton-Kickinson, Mountain View, Calif.) and analyzed by using Cell Quest software (Becton-Dickinson).

### 2.5. $Ca^{2+}$ measurements of EAT cells exposed to nitrostyrene derivative compounds

To evaluate the $Ca^{2+}$ handling, EAT cells were plated on coverslips after loaded with 3 μM of acetoxymethyl ester of fura-2 (Fura-2AM) (Molecular Probes, Eugene, OR, USA) in a buffer containing (mM): 130 NaCl, 5.36 KCl, 0.8 $MgSO_4$, 1 $Na_2HPO_4$, 25 glucose, 20 HEPES, pH 7.3 for 30 min. Cytoplasmic $Ca^{2+}$ measurements were evaluated by fluorescence microscopy (Nikon TE 300; Nikon, Osaka, Japan) coupled to a CCD camera (Quantix 512-Roper Scientific Inc., Princeton Instruments, Princeton, NJ). Images were acquired in BioIP software (Anderson Eng, Delaware, USA). Basal $Ca^{2+}$ levels were considered to be the first 15 images, and then exposed to NTS1 (13 μM) or NTS2 (6.5 μM) in the presence or absence of $Ca^{2+}$ external. Fura-2 fluorescence (emission = 510 nm) was monitored following alternate excitation at 340 and 380 nm. Percentages were expressed as ratio (340/380) values, normalized from the basal fluorescence and data were normalized by the $(F-F_0)/F_0 \times 100$ formula, in which $F_0$ represents the basal $Ca^{2+}$ level.

### 2.6. Caspase-3 activation

Active caspase-3 was evaluated in EAT cells treated with nitrostyrene derivative compounds by using flow cytometric analysis of endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 according to the manufacturer's instructions (Cell Signaling). Briefly, after treatment, EAT cells were washed with PBS and fixed in paraformaldehyde 2% in PBS (v/v) for 30 min. Cells were then permeabilized in PBS containing 0.01% saponin and 1% BSA. Afterwards, 10 μL of cleaved caspase-3 (Asp175) Alexa Fluor 488-conjugated antibody were added and cells were incubated in the dark at room temperature for 1 h. Cells were harvested and resuspended in 400 μL of PBS and analyzed ($10^5$ events were collected per sample) in a FACSCalibur Flow cytometer (Beckton Dickinson, CA, USA) using the CellQuest software.

### 2.7. Immunofluorescence staining of cytochrome c

NTS1 and NTS2-treated EAT cells were fixed with 2% of paraformaldehyde in ice-cold PBS for 30 min and permeabilized in PBS containing 0.1% saponin and 1% of BSA for 10 min. Cells were then incubated for 1 h with anti-cytochrome *c* polyclonal antibody (1:50 dilution), and then labeled with anti-rabbit IgG Alexa Fluor 488-conjugated antibody (1:300 dilution) for 30 min at room temperature in the dark. For imaging, cells were placed onto glass coverslips covered with Cell-Tak, washed in PBS and mounted in Fluoromount-G. Cells were then analyzed using an inverted laser scanning confocal microscope (LSM 510 META; Carl Zeiss, Germany). The sample was excited with argon laser 488 nm and emission detected using a bypass filter at 500–550 nm.

### 2.8. Detection of acidic vesicular organelles

Acidic vesicular organelles which consist predominantly of autophagosomes and autolysosomes, were quantified by flow cytometry after cells staining with acridine orange (AO), a fluorescent weak

base that accumulates in acidic spaces and fluoresce bright red (Traganos and Darzynkiewicz, 1994). Nitrostyrene derivative compound-treated EAT cells were collected in FACS tubes (BD Biosciences Discovery Labware, MA, USA) and stained with AO (1 μg/mL) for 15 min at room temperature. Acidic vesicular organelles detection ($10^5$ events) was analyzed by using a FACSCalibur flow cytometer and the Cell Quest software.

### 2.9. GFP-LC3 overexpression and autophagy detection

EAT cells were transfected with GFP-LC3 construct by using Fugene® (Roche Applied Sciences) according to the manufacturers' instructions. After 24 h, EAT cells were exposed to NTS1 (13.0 μM) and NTS2 (6.5 μM) for 12 h. Images of NTS1 and NTS2-treated EAT cells were acquired by confocal unit (LSM 510 META; Carl Zeiss, Germany) and a microscope (Axiovert 200 M; Carl Zeiss, Inc.) equipped with a 10×0.3 NA Plan Neofluar objective, a 40× NA 1.3 Plan Neofluar oil immersion objective, and a 63×1.4 NA Plan Apochromat oil immersion objective. The excitation and emission wavelengths used were 488 and 505–550 nm for GFP constructs. The detection of punctuated staining of GFP-LC3 from the diffuse staining indicated the formation of autophagosomes.

### 2.10. Statistical analysis

Data for each assay mean ± SD (standard derivation) of three independent experiments run in triplicate were analyzed statistically by ANOVA. Multiple comparisons among group mean differences were checked with Tukey–Kramer post-test. Differences were considered significant when $p<0.05$.

## 3. Results

### 3.1. Nitrostyrene derivative compounds EAT cells cytotoxicity and apoptosis induction

Using the MTT end-pointy screening we studied the viability of EAT cells after 12 h in culture with NTS1 (Fig. 1A) and NTS2 (Fig. 1B). The $IC_{50}$ values for both compounds in these tumor cells were 13 and 6.5 μM, respectively (Fig. 1C). We next examined whether the cell death caused by NTS1 and NTS2 was due to apoptosis (Fig. 1D and E). The occurrence of apoptosis was determined in cells staining with annexin V-FITC/PI. NTS1 (13 μM) and NTS2 at (6.5 μM) significantly increased the percentage of apoptotic cells, comprising 39% ± 7.5 and 60% ± 8.83, respectively, after 12 h exposure (Fig. 1E).

### 3.2. Cytosolic $Ca^{2+}$ measurements

NTS1 exposure (13 μM) resulted in a significant increase of cytosolic $Ca^{2+}$ levels, which was represented by an elevation in fluorescence ratio (340/380) (Fig. 2A). NTS2 was not able to induce the same partner of NTS1 calcium mobilization (Fig. 2B). By using calcium free solution we also evaluate the participation of extracellular calcium influx in NTS1 and NTS2-induced $Ca^{2+}$ homeostasis alterations (Fig. 2C and D). Interestingly, in this circumstance, there was no $Ca^{2+}$ mobilization with both nitrostyrene derivative compounds, suggesting that both compounds studied are able to modify significantly cellular membrane calcium pumps. NTS1 causes statistical significant increase in cytosolic $Ca^{2+}$ levels when compared with $Ca^{2+}$ mobilization induced by NTS2 (Fig. 2E). These results suggest that $Ca^{2+}$ mobilization might be involved mainly in NTS1-induced EAT cell death as presented before.

### 3.3. NTS1 and NTS2-induced caspase-3 activation and cytochrome c release

Both nitrostyrene derivative compounds studied activated caspase-3 (Fig. 3A), denoting by the presence of a large endogenous fragment levels (17/19 kDa) of caspase-3 due to aspartic acid 175 adjacent cleavages. As expected, this event was preceded by NTS1 and NTS2 induced cytochrome *c* release from mitochondria to cytosol (Fig. 3B). While control non-treated EAT cells exhibited a punctuate distribution of green fluorescence due to mitochondrial cytochrome *c* co-localization, treatment of EAT cells for 12 h with NTS1 or NTS2 resulted in a diffuse green fluorescence distribution denoting cytochrome *c* release from mitochondria to cytosol.

### 3.4. Autophagy participation in nitrostyrene derivative compound-induced cell death

As a growing number of publications show that apoptosis induction is often associated with increased autophagy (Fimia and Piacentini, 2010), this event was evaluated in EAT cells treated with NTS1 and NTS2 for 12 h using acridine orange (AO) and GFP-LC3 transfection assays. NTS1, but not NTS2-EAT treated cells showed a high intracellular accumulation of AO, expressed by an increased red fluorescence in relation to control EAT-non treated cells (EAT basal acid compartment) and in relation to NTS1 EAT treated cells (Fig. 4A and B). As LC3 exists as two forms; an 18 kDa cytosolic protein (LC3-I) and a processed 16 kDa form (LC3-II) presented in cells engaged in autophagy when it is localize mainly in autophagosome membranes (Herman-Antosiewicz et al., 2006; Kabeya et al., 2000; Mizushima, 2007) fluorescence microscopy was used to evaluate the NTS1 and NTS2-induced autophagy in GFP-LC3 transfected EAT cells. A diffuse green fluorescence in EAT and NTS2 treated cells for 12 h revealed a localization of GFP-LC3 in the cytoplasm (Fig. 4C). On the other hand, EAT cells treated for 12 h with NTS1 (13 μM) produced a punctuate pattern for GFP-LC3 fluorescence, indicating recruitment of LC3-II to autophagosomes during NTS1-induced autophagy. NTS2 was not able to induced LC3-II recruitment, suggesting no autophagy activation (Fig. 4C).

### 3.5. Relationship between apoptosis and autophagy induction in EAT-NTS1 treated cells

Next, we raised the question whether induction of autophagy affects NTS1 induced cell death. We addressed this question using 3-MA, a specific autophagy inhibitor (Seglen and Bohley, 1992). Fig. 5 shows that NTS1-induced apoptosis was increased from 39.0% (only NTS1) to 99.8% in the presence of 3-MA, whereas 3-MA treatment alone did not induce apoptosis. The 3-MA did not affect NTS2-induced apoptosis. From these results, we suggest that autophagy is a mechanism of NTS1-EAT cells resistance to apoptosis induction.

## 4. Discussion

Although the roles of autophagy in protein and organelle catabolism are well-accepted, the involvement of this process in cell death is controversial (Tsuchihara et al., 2008). The presence of dying cancer cells with morphological evidence of autophagosomes accumulation in response to chemotherapy has been observed suggesting that autophagy might be a non-apoptotic form of programmed cell death (PCD), called autophagic cell death (ACD) or type II PCD (Puissant et al., 2010). According to this context, it is possible to observe that apoptosis is not the only way the cells regulate the process by which it undergoes self-elimination, since death can occur by several mechanisms and the phenotypic changes that accompany cell death can vary depending on the cell setting and cytotoxic stimulus (Hail et al., 2006).

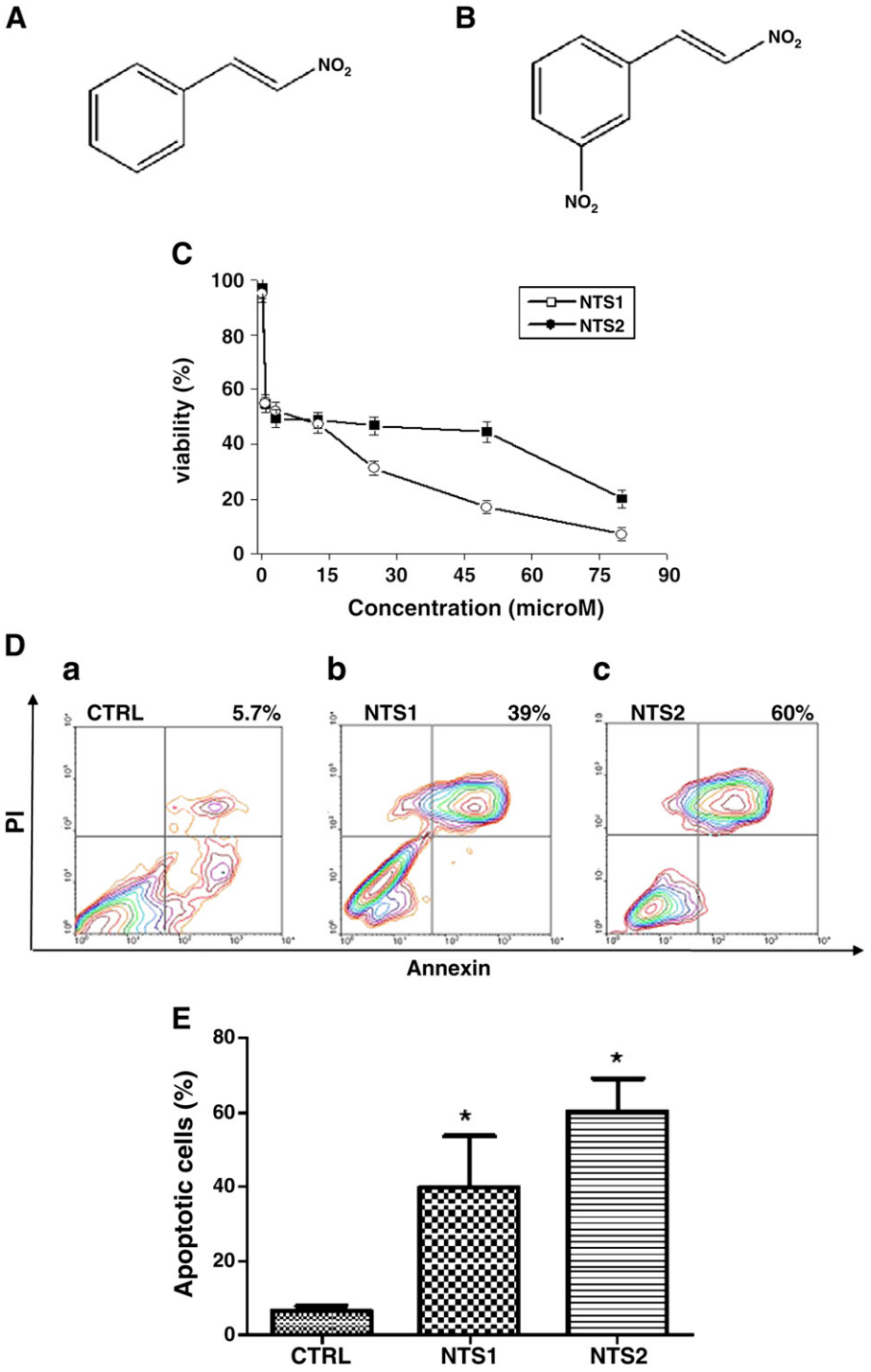
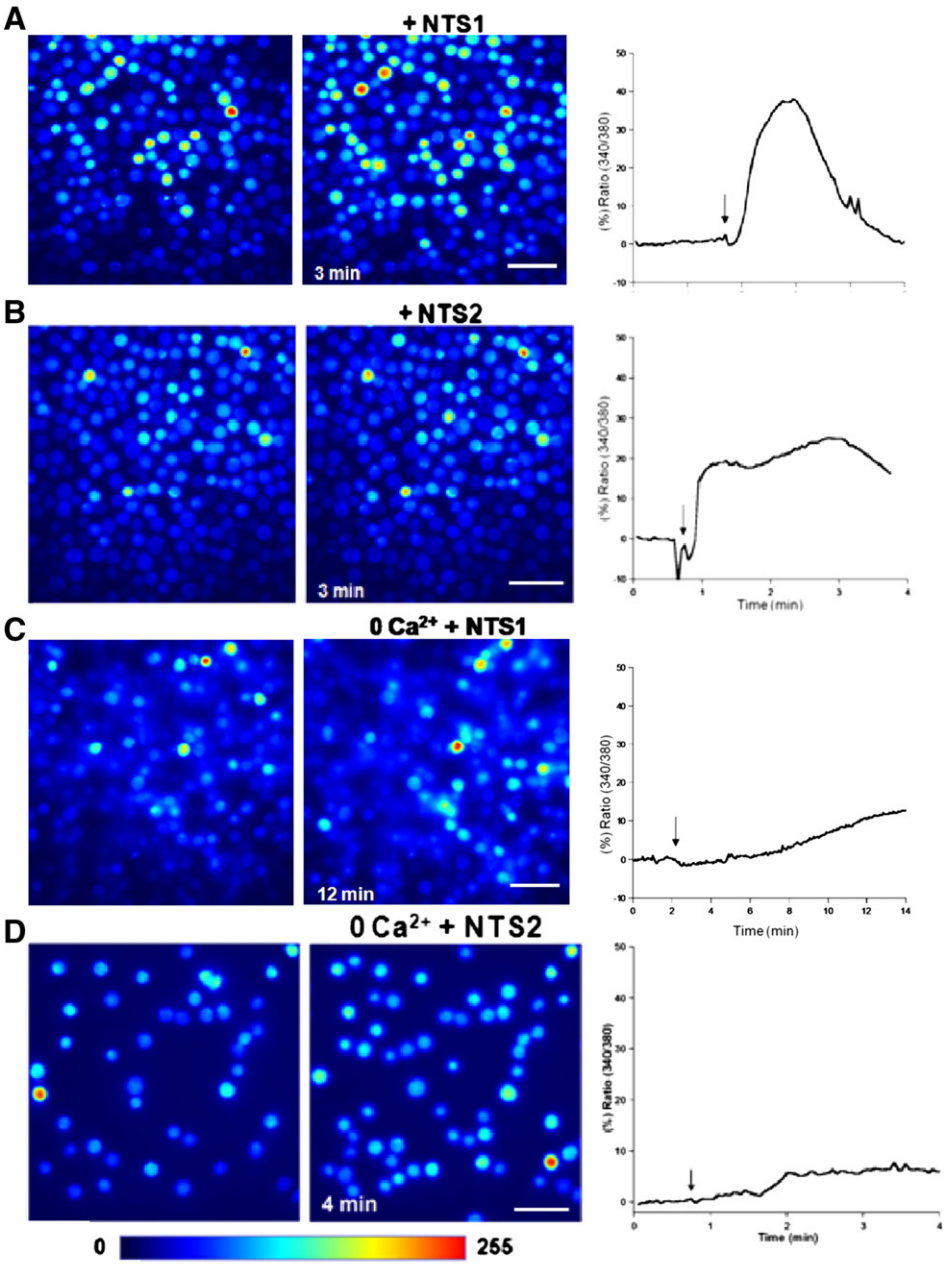
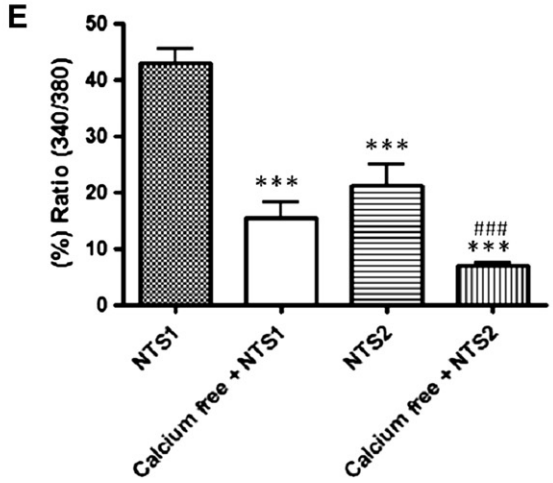



**Fig. 1.** Chemical structures of NTS1 (A) and NTS2 (B). EAT cells cytotoxicity of nitrostyrene derivative compounds after 12 h incubation (C), evaluated by the MTT assay. NTS1 and NTS2 $IC_{50\%}$ of 13 and 6.5 μM, respectively was obtained. Annexin V/PI-positive populations analyzed by flow cytometry in NTS1 and NTS2 EAT exposed cells (D and E). All data are representative of three independent experiments in triplicate and express as mean ± standard deviation.*$p<0.05$ in relation to control group.

Synthetic nitrostyrene derivative compounds have relevant biological activities *in vitro*, including cytotoxicity against human cancer cell lines exhibiting a pro-apoptotic effect and a selective human telomerase inhibition property (Fathi et al., 2000; Kaap et al., 2003; Kim et al., 2003). In this study, using the MTT assay, we demonstrated that two nitrostyrene derivative compounds (NTS1 and NTS2) produce a pronounced cytotoxic effect in a dose-dependent manner to EAT cells. In nitrostyrene derivative compounds EAT exposed cells, a typical sign of apoptosis was observed as reflected by an increase of Annexin V-FITC/PI double-positive cells after 12 h exposure.

In addition, both nitrostyrene derivative compounds stimulated the EAT intrinsic pathway of apoptosis, by cytochrome *c* release and caspase-3 activation. It is well known that the pro-apoptotic protein cytochrome *c* binds to and activates APAF-1, which binds to ATP/dATP forming the apoptosome (Cain, 2003; Cao et al., 2004; Lora et al., 2004), which mediates the caspase-9 triggering a cascade of


A
+ NTS1
3 min
(%) Ratio (340/380)
B
+ NTS2
3 min
Time (min)
C
0 Ca2+ + NTS1
12 min
D
0 Ca2+ + NTS2
4 min
0
255


E
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NTS1
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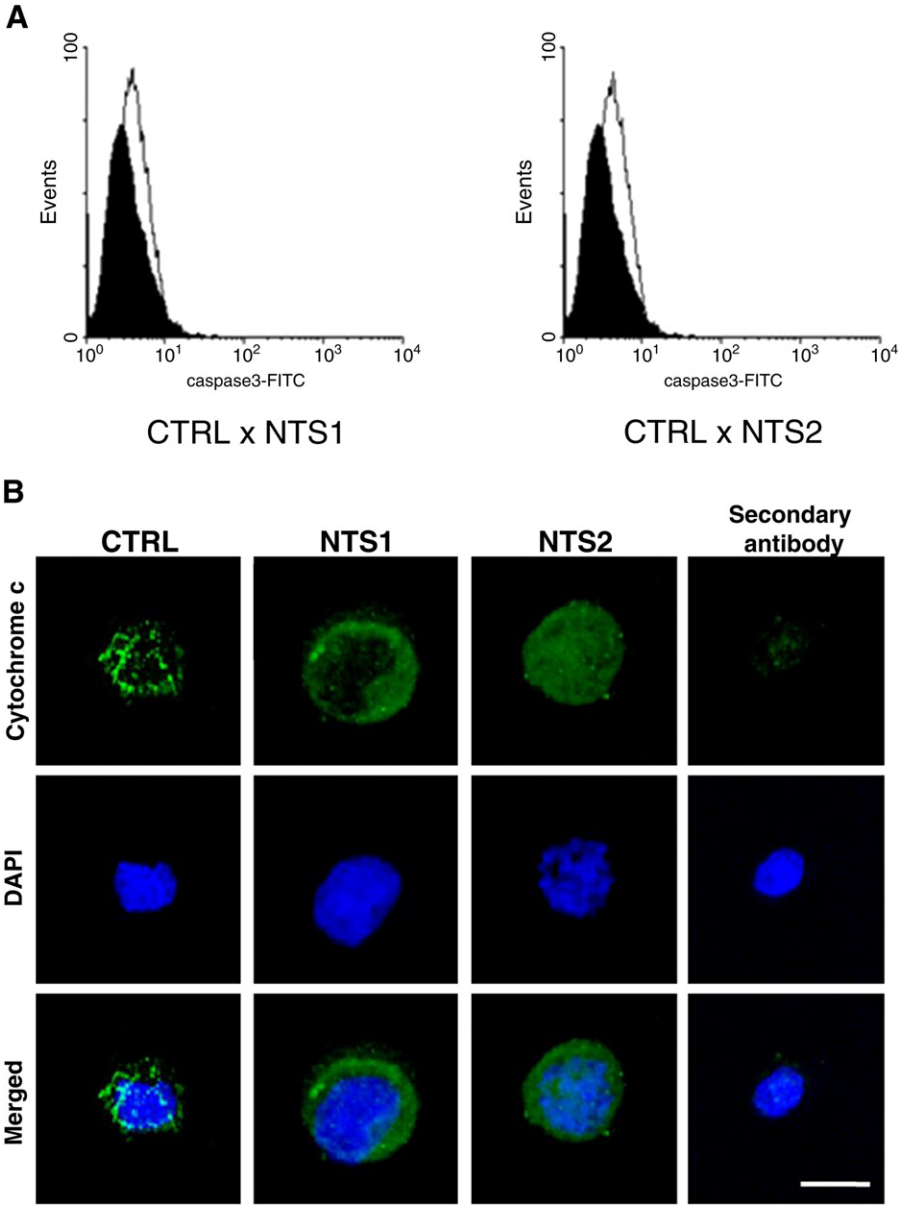


**Fig. 3.** Caspase-3 activation expressed by endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 (A) and cytochrome *c* releases from mitochondria to cytosol (B) of EAT cells exposed to 13 and 6.5 μM of NTS1 and NTS2, respectively after 12 h incubation. A. Black histogram represents control cells treated with medium only. B. Control non-treated EAT cells exhibited a punctuate distribution of green fluorescence due to mitochondrial cytochrome *c* co-localization. Treatment of EAT cells with both nitrostyrene derivative compounds resulted in a diffuse green fluorescence distribution denoting cytochrome *c* release from mitochondria to cytosol. Representative results of three independent experiments are presented.

caspase activation (Bromberg et al., 2010; Cain, 2003; Cao et al., 2004; Matés et al., 2008).

As several lines of evidence suggest that an increase in cytosolic $Ca^{2+}$, might be associated with apoptotic signaling (Pan and Gollahon, 2011; Saris and Carafoli, 2005; Shi et al., 2010; Smaili et al., 2000, 2003; Waring, 2005), changes on the homeostasis of this ion was evaluated in EAT cells exposed to two nitrostyrene derivative compounds. Interestingly, although NTS1and NT2 induced caspase-3 activation and cytochrome *c* release, from the two nitrostyrene derivative compounds studied, mainly NTS1 significantly increased the extracellular $Ca^{2+}$ influx in EAT cells. As mentioned before, NTS2 was not able to induce the same partner of NTS1 calcium mobilization. These findings demonstrated that NTS1 and NTS2 apoptosis-induced may involve $Ca^{2+}$-dependent and $Ca^{2+}$-independent pathways, respectively. In accordance with our results, studies have demonstrated $Ca^{2+}$-independent apoptosis induced in thymic lymphoma cells (Matuszyk et al., 1998) and neutrophils (Chakraborti et al., 1999).

Several signals denoting that pathways involved in autophagy are in common with apoptosis (Giansanti et al., 2011). Mitochondria, an organelle of great interest on the regulation of programmed cell death, is also especially sensitive to autophagy (Klionsky, 2008;

**Fig. 2.** Cytosolic calcium mobilization of EAT cells loaded with Fura-2 and exposed to NTS1 (13 μM) and NTS2 (6.5 μM) in the presence of Ca2+ external (A and B), but not in free Ca2+ medium (C and D). Histogram shows averaged of cytosolic Ca2+ responses from cells stimulated with 13 μM of NTS1 and 6.5 μM of NTS2 in the presence or absence of external Ca2+ (E). Note that mainly NTS1 mobilizes significantly this ion. Averaged were obtained from cytosolic Ca2+ responses (amplitude) from individual cells in relation to baseline. All data are expressed as mean fluorescence ratio ± s.e.m of a number of cells (in brackets) from at least 3 different experiments ***$p<0.0001$ in relation to NTS1 group; ### $p<0.0001$ in relation to NTS2.

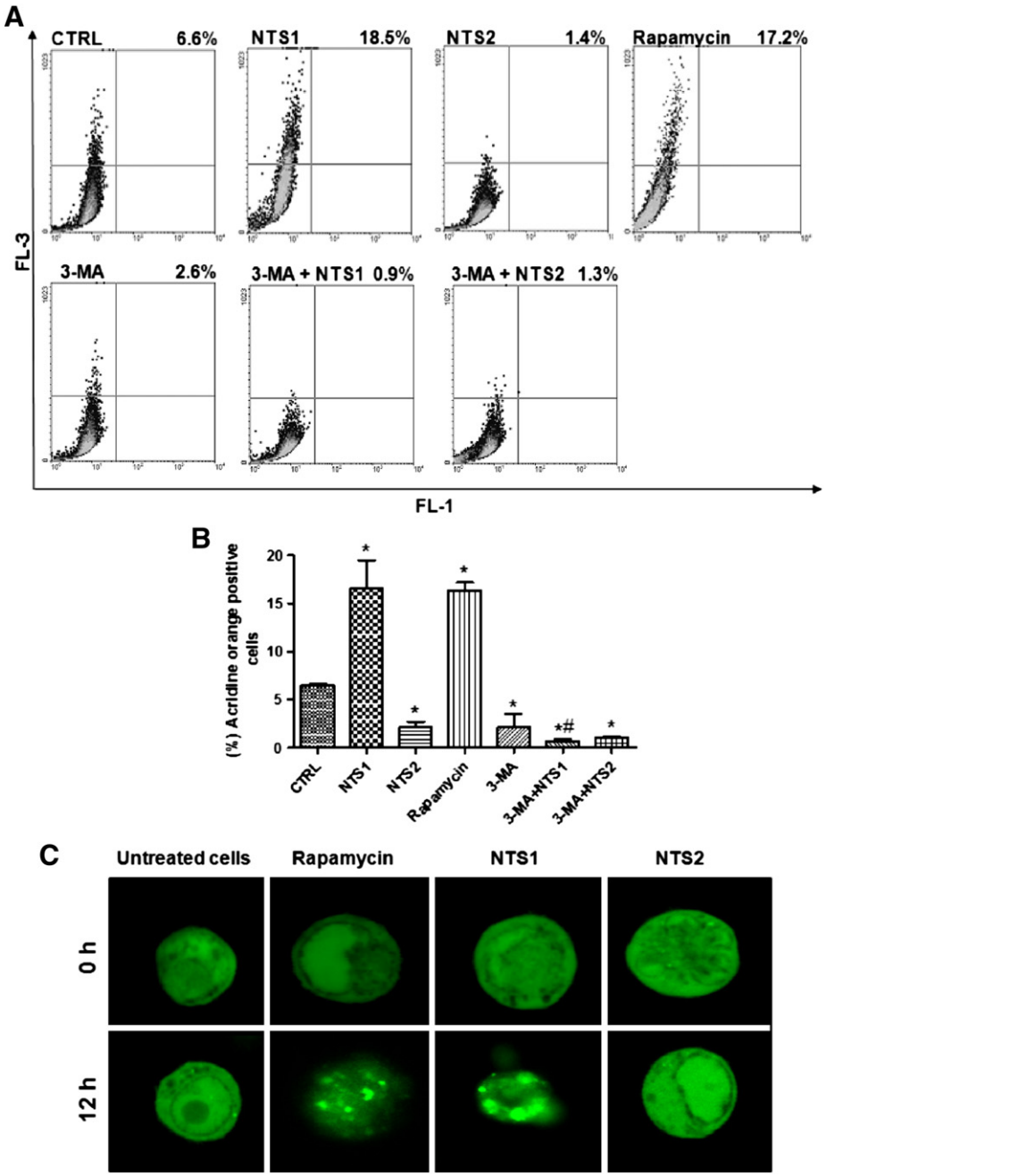


**Fig. 4.** Development of acidic vesicular organelles (A and B) in EAT cells firstly stained with AO and then treated with 13 and 6.5 μM of NTS1 and NTS2 respectively, for 12 h following acidic vesicular organelles detection using flow cytometry. The values mean media ± SD. $^{*}p<0.05$ in relation to control group; $\#p<0.05$ in relation to NTS1 treated cells. C — Involvement of LC3B in NTS1 and NTS2 induced autophagy in EAT cells transfected with GFP-LC3 and exposed to NTS1 (13 μM) and NTS2 (6.5 μM) for 12 h. The detection of punctuated staining of GFP-LC3 from the diffuse staining indicated the formation of autophagosomes. Rapamycin (RAP) was used as a positive control of autophagy induction. Images of EAT-NTS1 and EAT-NTS2 treated cells were acquired by confocal unit, which is representative of at least two independent experiments.

Smaili et al., 2000), a catabolic dynamic process for degradation and turnover of cytoplasmic organelles (Lemasters et al., 1998) described before. Based on these findings and in our results showing that nitrostyrene derivative compounds induced-apoptosis is dependent on the intrinsic pathway, we hypothesized that NTS1 and NTS2 may also induce autophagy. This hypothesis was examining by acidic vesicular organelles formation evaluation, which is a feature of autophagy engaged cells following different stimulus (Daido et al., 2004; Paglin et al., 2001; Tan et al., 2009). It was observed that NTS1, but not NTS2 increased significantly the EAT cells acidic vesicular organelles formation. The induction of autophagic process by NTS1 treatment produced a punctuate pattern for GFP-LC3 fluorescence in EAT cells, indicating recruitment of LC3-II to autophagosomes during NTS1 induced autophagy. Collectively, these results provided further evidence that NTS1 treatment causes apoptosis induction and autophagy in EAT cells.

Regarding cancer treatment, autophagy can promote cells adaptation and survival against antitumor therapy (Kong and Yamori, 2008). Indeed, the stimulation of autophagy in cancer cells was often observed in response to anticancer treatments (Bauvy et al., 2001), which could be attributed to the recycle of proteins and organelles damaged during the anticancer therapy. Thus, in this specific situation autophagy inhibition can improve the anticancer cytotoxic effects (Kanzawa et al., 2004). As we found that in NTS1 EAT treated cells, the pharmacological autophagy inhibitor 3-MA increased the Annexin V/PI positive cells, it is possible that autophagy inhibitors

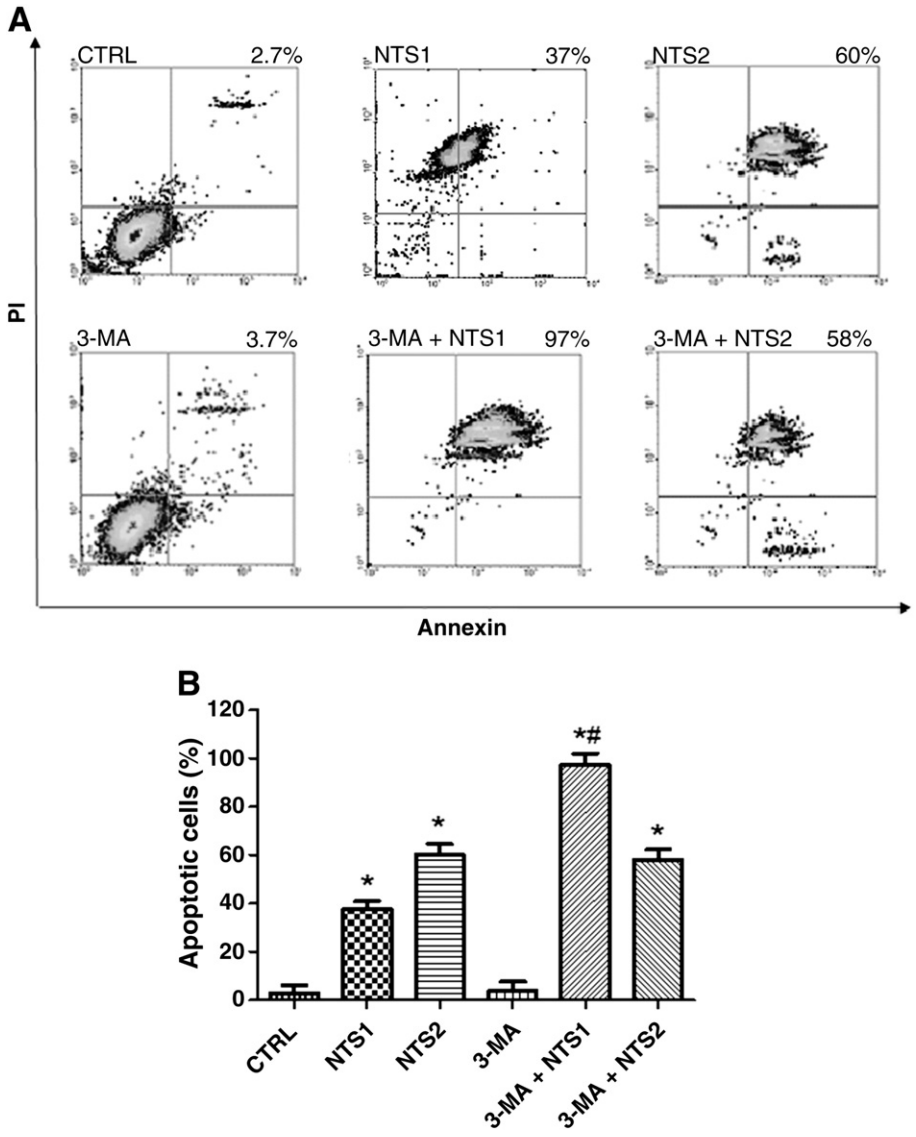


**Fig. 5.** Relationship between apoptosis and autophagy in NTS1 and NTS2 EAT treated cells (A and B). Apoptosis in EAT cells exposed to 13 and 6.5 μM of NTS1 and NTS2 respectively for 12 h in the absence or presence of 3-methyladenine 3-MA (20 mM) were analyzed by flow cytometry using Annexin V/PI-positive cells. The value means media ± SD. $^{*}p<0.05$ in relation to control group; $\#p<0.05$ in relation to NTS1 group.

might sensitize EAT cells to NTS1 treatment by enhancing the rate of apoptotic cell death or by converting the autophagy to an apoptotic process. Similar results were obtained by Bauvy et al., 2001 in a study showing that autophagy delayed sulindac sulfide-induced apoptosis in colon cancer cells by sequestering mitochondrial death-promoting factors, such as cytochrome *c*. An increase in the percentage of apoptotic cells induced by chemotherapy or radiotherapy was also observed when various cancer cells were previously exposed to Bafilomycin A1, another autophagy inhibitor that prevents the fusion of autophagosomes with lysosomes (Kanzawa et al., 2004). Potentiation of 5-fluorouracil (5-FU) anticancer effects on colon cancer cells by chloroquine, a well-known lysosomotropic agent, was also demonstrated in *in vitro* experiments (Sasaki et al., 2010). Chen et al., 2011 have also reported that autophagy inhibition significantly augments the cytotoxic effect of BO-1051 an N-mustard derivative compound, suggesting that autophagic inhibitors offers a potentially new therapeutic modality for the treatment of cancer.

Exploration of a new chemotherapy strategy is very important to overcome cancer cells resistance. From our results, we found that the autophagy inhibitor 3-MA enhances NTS1-induced EAT cells death. Interestingly, although both nitrostyrene derivative compounds (NTS1 and NTS2) induced the intrinsic pathway of cell death, mainly the NTS1 was able to induce autophagy and mobilize extracellular calcium influx in EAT cells. From these results, it is possible to suggest that the ion calcium is a key step of autophagy mobilization in our model. Further studies are necessary to better understand the relationship between calcium, apoptosis and autophagy and tumor cell resistance. Moreover, autophagy inhibition might be a promising strategy to potentiate NTS1 anticancer effects.

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