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ITH4012, a novel acetylcholinesterase inhibitor with "calcium promotor" and neuroprotective properties

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Abbreviations: AChE- Acetylcholinesterase; Aβ – amyloid β; AD – Alzheimer´s disease; BCA – bicinchoninic acid; BSA – Bovine serum albumin; CNS – Central nervous system; DMEM – Dulbecco´s Modified Eagle´s Medium; DMSO – Dimethylsulphoxide; ER – Endoplasmic reticulum; FCS – Fetal calf serum; HRP – Horseradish peroxidase; IgG – Immunoglobulin G; LDH – lactic dehydrogenase; nAChR - Nicotinic acetylcholine receptor; NEAAs – Non-essential amino-acids; PBS - Phosohate buffer solution; PI – propidium iodide; ; RIPA – Radioimmunoprecipitation ; SERCA – Sarcoplasmic endoplasmic reticulum ATPase; SDS – Sodium docecyl sulfate; PVDF – Polyvinylidene fluoride; VDCC – Voltage-dependent calcium channels.

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ABSTRACT

ITH4012 (ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-phenylbenzol[1,8]naphthyridine-3-carboxylate) is a novel tacrine derivative that can reduce cell death induced by various compounds with different mechanisms of action, such as thapsigargin (reticular stress), H_2O_2 (free radicals) and veratridine (calcium overload), in bovine chromaffin cell. Cell viability quantified as LDH (lactic dehydrogenase)-release, was significantly reduced by ITH4012 at concentrations ranging from 0.01 μM to 3 μM. In the human neuroblastoma cell-line SH-SY5Y, ITH4012 also reduced $A\beta_{25-35}$ (amyloid β_{25-35})-induced apoptosis, determined by flow cytometry. ITH4012 caused a slight elevation in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_c$) in fura 2-loaded bovine chromaffin cells, which could be related to the induction of protein synthesis relevant for cell survival. Blockade of protein synthesis by cycloheximide or blockade of Bcl-2´s active site with HA14-1 (ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate) reversed the cytoprotective action of ITH4012. Furthermore, exposure of bovine chromaffin cells for 24 h or 48 h to neuroprotective concentrations of this compound enhanced, nearly 3-fold, the expression of the antiapoptotic protein Bcl-2. In conclusion, ITH4012 is a tacrine derivative that maintains acetylcholinesterase-inhibiting activity (IC_{50}=0.8 μM), but has the additional property of acting as a calcium promotor, a property leading to neuroprotection through the induction of antiapoptotic proteins.

INTRODUCTION

Alzheimer´s disease (AD) is a degenerative disorder of the central nervous system (CNS) found primarily among the aged. It is the most common type of dementia in western societies and has been creating profound economic and social impacts as the elderly population continues to increase (Guttman et al., 1999). The hallmarks of the illness are extracellular deposition of neuritic plaques of amiloid-β peptide (Aβ), intracellular formation of neurofibrillary tangles and selective neuronal loss. Of all biochemical changes observed in AD patients, only the reduction in number of functional neuronal nicotinic receptors (nAChR) is related to neuropsychiatric symptoms (Schröder et al., 1995). For this reason the only pharmacotherapeutical strategy that, up to now, has proven to have some efficacy in slowing progression of the illness is that which improves cholinergic neurotransmission, counteracting the deficit of cerebral acetylcholine (Standaert et al., 1996). Therefore, acetylcholinesterase inhibition is currently the therapeutic strategy most commonly used to treat Alzheimer´s patients.

On the other hand, moderate chronic depolarization has demonstrated an ability to increase the survival of different neuronal types. Also, there is evidence that this effect is mediated by the mild and sustained elevation of $[Ca^{2+}]_c$, principally by Ca^{2+} influx through voltage-dependent Ca^{2+} channels of the L-subtype (Collins and Lile, 1989). Therefore, chemical synthesis of new compounds that combine both, a moderate “calcium-promotor” action and AChE inhibition could be useful in the treatment of AD.

A few years ago, we initiated the synthesis and pharmacological study of new tacrine derivatives. We were especially interested in derivatives which preserved their ability to inhibit AChE while possessing other properties, such as the modulation of

voltage-dependent Ca^{2+} channels (VDCCs) and/or nicotinic acetylcholine receptors (nAChR). As a result, we obtained a number of new compounds that were acceptable AChE inhibitors with the acquired additional properties (Marco et al., 2001; de los Ríos et al, 2002). One of these compounds, ITH4012 (ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-phenylbenzol[1,8]naphthyridine-3-carboxylate) (Fig.1a), was particularly promising because of its mild Ca^{2+} promotor action.

We describe here the cytoprotective effect of ITH4012 on primary cultures of bovine chromaffin cells. Like neurones, these are excitable cells that synthesize, store and release catecholamines and express different ionic voltage dependent channels (García et al., 2000). We also used the human neuroblastoma cell-line SH-SY5Y and a variety of toxic stimuli that are relevant to the pathophysiology of Alzheimer´s disease.

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MATERIAL AND METHODS

Material

Thapsigargin, veratridine, H_2O_2 (hydrogen peroxide) and Bay K8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-pyridine-3-carboxylic acid methyl ester) were purchased from Sigma (Madrid, Spain) and beta-amyloid$_{25-35}$ from Calbiochem (Germany). Dulbecco´s Modified Eagle´s Medium (DMEM) and fetal calf serum were obtained from GIBCO (Madrid, Spain). Fura-2 AM was purchased from Molecular Probes (Holland).

Concentrated solutions of drugs were prepared in dimethylsulfoxide (DMSO). Appropriate dilutions were then made in Krebs-Hepes solution containing (in mM): NaCl, 144; KCl, 5.9; $MgCl_2$, 1.2; $CaCl_2$, 2; Hepes, 10; glucose, 11; pH 7.3, titrated with NaOH. DMSO, at the final concentration used (less than 0.1%), had no effect on any of the parameters tested. Aβ_{25-35} was dissolved in ultrapure water (Mili-Q standard) and kept frozen until use.

Preparation and culture of bovine chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated using collagenase digestion and Percoll gradients, as previously described (Moro et al., 1990). Cells were suspended in DMEM supplemented with 5% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin (reagents from GIBCO, Madrid, Spain). The cells were pre-plated for 30 min and proliferation inhibitors (10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 10 μM leucine methyl esther) were added to the medium to prevent excessive growth of fibroblasts, which would interfere with chromaffin cell-death

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measurements; $5x10^5$ cells/well were plated in 24-well dishes. Cultures were maintained in an incubator for 2-4 days at 37°C in a water-saturated atmosphere with 5% CO_2. Cell treatments were performed in DMEM, free of serum and inhibitors of cell proliferation.

Culture of SH-SY5Y cells

The neuroblastoma cell-line SH-SY5Y, at between 3 and 16 passages after thawing, were maintained in DMEM containing 15 non-essential amino-acids (NEAAs) and supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Cultures were seeded into flasks containing supplemented medium and maintained at 37ºC in 5% CO_2/humidified air. Each stock culture was passaged 1:4 weekly and fed twice weekly. For assays, SH-SY5Y cells were sub-cultured in 6-well plates at a seeding density of $5x10^5$ cells/well. Cells were treated with drugs before confluence in serum-free DMEM.

Incubation of drugs

To study neuroprotective action, the drugs were all incubated 24 h before adding cytotoxic stimuli (veratridine, thapsigargin, Aβ, or H_2O_2); then cells were co-incubated for another 24 h with the drug in the presence of the stimuli. All toxic stimuli were incubated in serum-free DMEM.

Measurement of lactic dehydrogenase (LDH) activity

Extracellular and intracellular LDH activity was spectrophotometrically measured using a Cytotoxicity Cell Death kit (Roche-Boehringer Mannheim), according to

manufacturer's instructions. Total LDH activity was defined as the sum of intracellular and extracellular LDH activity; released LDH was defined as the percentage of extracellular activity compared to total LDH activity.

Measurement of apoptosis by flow cytometry

Apoptosis was determined by flow cytometry analysis of the cell cycle after DNA staining with propidium iodide (PI, Molecular Probes) (Robinson et al., 1997). Cells were grown in 6-well plates until they reached 50% confluence (typically, after 24-48 h in culture). After treatment, cells that remained attached to plates were harvested in 5 mM EDTA in PBS and collected together with floating (detached) dead cells. Cells were then centrifuged for 5 min at 200xg, the supernatant was discarded, and the cell pellet suspended in 0.5 ml PBS by repeated pipetting to avoid cell clumping. The cell suspension was transferred to 4.5 ml 70% cold ethanol and kept in this fixative for a minimum of 2 h. Ethanol–fixed cells were centrifuged for 5 min at 200xg and washed once with 10 ml PBS. Finally, the cell pellet was suspended in 1 ml of PI/Triton X-100 staining solution (0.1% Triton X-100, 20 μg/ml RNase in PBS) and incubated for 15 min at 37ºC. Samples were analyzed by flow cytometry (FACS Calibur, Beckton Dickinson). Analysis of samples included a first selection (gate 1) in which events (cells) with appropriate size (forward scatter) and complexity (side scatter) were selected. Then selected events, were analyzed to discard doublets, using a PI intensity-width versus PI intensity-area dot plot (gate 2). Finally, events that were contained in gates 1 and 2 were plotted on a histogram representing the number of events containing a specific PI intensity-area (e.g., specific

amount of DNA). Apoptosis was measured as the percentage of cells with a sub G0/G1 DNA content on the PI intensity-area histogram plot.

Measurement of single-cell cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_c$

For these experiments, cells were plated on 1cm-diameter glass coverslips at a density of $5x10^4$ cells/coverslip. Cells were loaded with fura-2/AM (4 μM) for 45 min at 37 °C in Krebs-Hepes solution. Loading with fluorescent dye was terminated by washing the coverslip containing the attached cells twice with Krebs-Hepes; then cells were kept at room temperature for 15 min before placing them in the headstage of a Nikon Diaphot microscope to measure their fluorescence.

The fluorescence of fura-2 in single cells was measured with a photomultiplier-based system (Neher et al, 1989), which produces a spatially averaged measurement of $[Ca^{2+}]_c$. Fura-2 was excited with light, alternating between 360 and 390 nm, using a Nikon 40x fluorite objective. Emitted light was transmitted through a 425 nm dichroic mirror and a 500-545 nm barrier filter before being detected by the photomultiplier. $[Ca^{2+}]_c$ was calculated from the ratios of the light emitted when the dye was excited by the two alternating excitation wavelengths (Grynkiewicz et al., 1985).

Immunostaining of Bcl-2

Bovine chromaffin cells were plated on 2cm glass coverslips ($3x10^5$/coverslip) in 6-well plates and fixed with 3.7% formaldehyde at room temperature for 20 min. Formaldehyde was removed by washing the cells twice with PBS; cells were then permeabilized with 0.1% Triton X-100/PBS for 2 min and rinsed twice with PBS. Blocking solution, 1% bovine serum albumin (BSA) in PBS, was applied to cells for 20 min and

removed with PBS. The antibody against Bcl-2 (1:500) (Santa Cruz Biotechnology, Calif., USA) in the blocking solution was incubated for 1 h at room temperature; then cells were rinsed three times with PBS. A secondary antibody BODIPY-conjugate against Bcl-2 (goat anti-mouse IgG; dilution 1:100) (Molecular Probes) was applied for 1 h at room temperature. Then cells were washed with PBS and mounted in glycerol-PBS (1:1 vol/vol). Fluorescent antibody staining was visualized using a Leica multispectral laser confocal microscopy imaging system and a 40x/1.4 oil-immersion objective. A blue excitation filter (excitation 503 nm; emission 512 nm) and a green excitation filter (excitation 596 nm; emission 615 nm) were used to visualize stained samples.

Western blot analysis

Immunoblot analysis was performed on protein extracts from chromaffin cells that were previously incubated with DMEM, either alone or in the presence of the different treatments used (see Results). Cells were seeded at a density of 5 x 10^6/10cm plate and lysed in radioimmunoprecipitation (RIPA) buffer (PBS with 1% Nonidet P40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate, SDS) at a dilution of 1:100 for 30 min. The lysed cells were centrifuged for 2 min at 12,000xg and the supernatant was discarded. Protein concentration in the pellet was determined using a bicinchoninic acid (BCA) protein quantification kit (Pierce). 50 μg of protein was resolved on 12% SDS-polyacrylamide gels and electronically transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with blocking solution, 5% non-fat milk in Tris buffer solution Tween, TBS-T (10 mM Tris, pH 7.4; 150 mM NaCl; 0.2% Tween-20) at room temperature for 1 h. Then the membrane was washed with TBS-T and incubated with

the antibody against Bcl-2 (1:500) which was dissolved in blocking solution for 24 h at 4 ºC. The membrane was washed and incubated in mouse anti-IgG-HRP antibody for Bcl-2 for 1 h at room temperature (the secondary IgG antibody peroxidase-conjugate was purchased from ICN Biomedicals Inc., Ohio, USA). The blot was washed and samples were subjected to chemilluminescence (ECL, Amersham). Bcl-2 expression was quantified by densitometric scanning using the NIH image program (version 1.61). In each gel, the amount of β-actin was also determined; therefore, levels of expression of Bcl-2 were corrected for the amount of β-actin. The results are expressed as percentage of density with respect to control basal levels.

Statistical analysis of the results

Data are expressed as means ± SEM. Differences between non-paired groups were compared by means of ANOVA with the statistical program StatView. A p value equal to or smaller than 0.05 ($p \leq 0.05$) was taken as the limit of statistical significance.

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RESULTS

Effects of ITH4012, tacrine and Bay K8644 on thapsigargin-induced cytotoxicity

Thapsigargin is an irreversible SERCA inhibitor that releases Ca^{2+} from the endoplasmic reticulum (ER) and causes apoptotic cell death (Wei et al., 1998; Arias et al., 2004). While ER stress (i.e., Ca^{2+} depletion) seems to be an initial and crucial step to induce apoptosis and neuronal cell death (Nath et al., 1997), it has also been involved in neuronal degeneration in the brain of AD patients (Jellinger and Stadelmann, 2001). Therefore, thapsigargin-induced toxicity seemed a good *in vitro* cytotoxicity model for studying the possible protective properties of ITH4012.

Twenty-four hours after applying 5 μM thapsigargin to bovine chromaffin cells, LDH released to the extracellular medium increased from 5.8±0.3%, under basal conditions, to 24.5+0.9%. Under these experimental conditions, concentrations of the drugs ranging from 0.001 μM to 10 μM were evaluated; significant cytoprotection against thapsigargin-induced toxicity was achieved at concentrations of ITH4012 from 0.1 μM to 10 μM (Fig. 2a). In the case of the L-type calcium-channel agonist Bay K8644 (a well-known "calcium promotor"), significant protection was achieved only at 0.01 μM; this cytoprotective effect was lost at higher concentrations (Fig.2b). The AChE-inhibitor tacrine showed no significant protection at any of the concentrations used (0.01 μM -10 μM) (Fig. 2c).

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Effect of ITH4012 on free radical-induced cytotoxicity

We used hydrogen peroxide (H_2O_2) to generate free radicals and cell death. In a previous study, we showed that 0.1-1 mM H_2O_2 caused the death of bovine chromaffin cells (Abad et al., 1995). Thus, we selected 500 μM H_2O_2 to perform the present experiments. In figure 3a, control cells appear forming typical acini, with a strong birrefringency and smooth cytosol. Figure 3b shows the drastic effects of H_2O_2; cells have stopped forming acini, and they present a dark rough cytoplasm. Figure 3c shows partial recovery of the shape of control cells after exposure to H_2O_2, but in the presence of ITH4012 (1 μM from 24h before, and during, H_2O_2 exposure). Figure 3d shows quantitative data from pooled experiments performed with the protocols shown in figure 3 a,b,c. When bovine chromaffin cells were exposed for 24 h to 500 μM H_2O_2, cell death, measured as LDH released to the extracellular medium, increased from 6.4±0.7% (control release) to 48.9±5% (H_2O_2-exposed cells). ITH4012 showed a tendency to protect at 0.1 μM and provided significant protection at 1 μM; above that concentration, no cytoprotective effect was seen (Fig. 3).

Effect of ITH4012 on beta-amyloid-induced cytotoxicity in a human neuroblastoma cell line

For these studies, we used the human neuroblastoma cell-line SH-SY5Y. The cells were maintained in serum-free medium; under these conditions, apoptosis increased from 10±0.5% (basal) to 24±2% in cells treated for 24 h with $A\beta_{25-35}$. ITH4012 at 0.01 μM and 0.1 μM was preincubated for 24 h before, and during, apoptotic stimuli with $A\beta_{25-35}$. At

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0.01 μM, ITH4012 showed a tendency to reduce apoptosis, and at 0.1 μM the reduction was statistically significant (Fig. 4); higher concentration did not show further protecetion. If basal apoptosis is subtracted, we come to a value of 50% neuroprotection afforded by 0.1 μM ITH4012 against Aβ-induced apoptosis.

At 0.3 μM and 3 μM, tacrine failed to show antiapoptotic effects against $A\beta_{25-35}$-induced cell death (data not shown).

Effects of ITH4012, tacrine and Bay K8644 on veratridine-induced cytotoxicity

It is widely accepted that Ca^{2+}-overload plays a crucial role in causing cell death (Choi et al., 1988). Veratridine causes cell depolarization by delaying Na^{+}-channel inactivation (Ohta et al., 1973), which leads to $[Ca^{2+}]_c$ oscillations, calcium-overload and cell death (Maroto et al., 1994). To induce cell death in bovine chromaffin cells, we treated cells for 24h with 30 μM veratridine; under these conditions, cell death, measured as LDH released to the extracellular medium, increased from 10±0.4% (basal conditions) to 45.5±1.8%.

Compound ITH4012 achieved cytoprotection against veratridine-induced cytotoxicity in a concentration-dependent manner; significant protection was obtained at 0.1 μM, 1 μM and 10 μM (Fig. 5a). When basal death (about 10%) was subtracted, it was calculated that 0.1 μM ITH4012 afforded 30% protection, 1 μM 40% protection and 10 μM 50% protection. Neither the calcium promotor Bay K8644 nor tacrine provided significant protection at any of the concentrations tested (1 nM-1000 nM for Bay K8644 and 0.1 μM - 10 μM for tacrine) (Fig. 5b,c).

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Effect of ITH4012 on cytosolic Ca^{2+} concentrations, $[Ca^{2+}]_c$

Figure 6a shows the changes in $[Ca^{2+}]_c$ induced by ITH4012 in single fura-2-loaded chromaffin cells superfused with Krebs-Hepes solution. Once stable basal $[Ca^{2+}]_c$ was achieved (47±6 nM, n= a total of 12 individual cells from 4 different primary cultures), the cell was superfused with ITH4012 at concentrations of 1 nM, 10 nM and 100 nM for about 2 min. In the example cell given in figure 6a, 1 nM ITH4012 did not increase the basal $[Ca^{2+}]_c$ levels of about 60 nM. At 10 nM, a slow-developing increment of $[Ca^{2+}]_c$ was seen, which reached a plateau at around 250 nM and remained stable, with slight oscillations, for 3 min. Addition of 100 nM ITH4012 above this plateau did not further enhance the level of $[Ca^{2+}]_c$. Figure 6b shows pooled results from 12 cells; a plateau of $[Ca^{2+}]_c$ of 216±19 nM was induced by 10 nM ITH4012. This increment above basal $[Ca^{2+}]_c$ was statistically significant.

The cytoprotective effect of ITH4012 is related to protein synthesis: implication of Bcl-2

When protein synthesis was blocked by cycloheximide, ITH4012 (1 μM and 10 μM) lost its cytoprotective effects against veratridine-induced cytotoxicity (Fig. 7a). Therefore, we looked at proteins involved in neuroprotection, such as those of the antiapoptotic Bcl-2 family. Evidence indicating that the protective actions of ITH4012 could be related to upregulation of Bcl-2 are shown in the experiments of figure 7b, where HA14-1, a drug that blocks Bcl-2´s antiapoptotic action, was used. HA14-1 alone slightly increased basal cell death, although this increase was not significant and did not protect

against veratridine-induced toxicity. However, when HA14-1 was co-incubated with ITH4012 at 1 μM or 10 μM, the protective effect of the latter was completely lost.

Immunofluorescent staining with anti-Bcl-2 antibodies was performed in bovine chromaffin cells pre-treated for 24-48 h with 1 μM ITH4012; under these conditions, higher fluorescence was observed in ITH4012 cells, as compared to untreated cells, indicating higher amounts of Bcl-2 protein in the treated cells (Fig. 8a). Western blot analysis of Bcl-2 in control cells and in cells incubated 24–48 h with 1 μM ITH4012 also showed higher protein levels in treated cells; Bcl-2 increased nearly 3-fold with respect to basal levels (Fig. 8b, c).

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DISCUSSION

The results of this study show that ITH4012, at concentrations ranging from 0.1 μM to 10 μM, can afford protection against a variety of toxic stimuli such as thapsigargin, free radicals, $A\beta_{25-35}$ and veratridine. This neuroprotective effect is related to a mild, sustained increase in $[Ca^{2+}]_c$ and to the de nova synthesis of proteins, such as Bcl-2.

There is evidence in the literature that apoptosis, induced in various neuronal cell types deprived of essential growth factors, afferent innervation, or exposed to Aβ, is inhibited by a mild, chronic depolarization evoked by increasing the potassium concentration of the incubation medium (Franklin, 1972; Koike et al., 1989; Balazs et al., 1992; Pike et al., 1996). This neuroprotective effect has been related to a mild and sustained elevation in $[Ca^{2+}]_c$ which, in turn, would induce synthesis of proteins related to cell survival (Koike et. al., 1989). This mild, sustained $[Ca^{2+}]_c$ elevation was nicely mimicked by ITH4012 (Fig. 6); this makes attractive the idea that ITH4012 may be enhancing cell survival by a mechanism similar to that described for potassium depolarization. Suprisingly, the L-type calcium-channel agonist Bay K8644 was not as effective as ITH4012 in protecting cells against the different toxic stimuli used in this study. One interpretation of these results is that the calcium signal afforded by Bay K8644 is different from that induced by ITH012. Bay K8644 needs a certain level of depolarization (i.e., a slight increase in the concentration of extracellular K^+) so that L-type Ca^{2+} channels remain open longer to enhance Ca^{2+} entry into bovine chromaffin cells (García et al., 1984). In contrast, ITH4012 seems to generate an appropriate $[Ca^{2+}]_c$ signal on its own. In addition,

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Bay K8644 is a powerful activator of Ca^{2+} entry through non-inactivating L-type Ca^{2+} channels (Michelena et al., 1993); this property could be generating an excessive $[Ca^{2+}]_c$ increase beyond the Ca^{2+} signal that affords neuroprotection (Pike et al., 1996).

The fact that ITH4012 provided protection against a wide variety of stimuli with different mechanisms of action (calcium-overload induced by veratridine, endoplasmic reticulum stress elicited by thapsigargin, free-radical generation by H_2O_2 or Aβ), together with its calcium-promotor activity, indicates that this compound could be exerting this effect on the cascade of apoptosis/cell death beyond the particular mechanism of each toxic agent. Therefore, we thought about the possibility that ITH4012 acts by inducing the expression of proteins implicated in cell survival. Experiments with cycloheximide, which reversed the protective effects of ITH4012, already suggested that new protein synthesis was required in order for the protective effects of ITH4012 to be manifested. Two pieces of evidence point to the antiapoptotic protein Bcl-2 as a target for ITH4012. On the one hand, the neuroprotective effects of ITH4012 could be blocked by a compound (i.e. HA-14-1) that binds to the active pocket of Bcl-2 (Wang et al., 2000). On the other hand, compound ITH4012 was itself able to almost triple the amount of Bcl-2 protein in cells chronically treated with the compound (Fig. 8).

Recently, we have shown that galantamine has an interesting antiapoptotic effect, linked to α7 nicotinic receptors and to an overexpression of Bcl-2 (Arias et al., 2004). Since increased numbers of neurons suffering apoptosis have been described in post-mortem brains of AD patients (Su et al., 1994; Dragunow et al., 1995), the induction of the antiapoptotic protein Bcl-2 could be a mechanism for preventing apoptosis and affording neuroprotection. ITH4012 is in this line, since it exhibits an antiapoptotic profile similar to

that of galantamine and, hence, might have useful therapeutic applications in treating AD patients.

In conclusion, ITH4012 is a new tracrine derivative that maintains its AChE-blocking activity (Marco et al., 2001) and, additionally, has neuroprotecive properties against a variety of stimuli that have been implicated in the pathophysiology of AD. Whatever its ultimate neuroprotective mechanism might be, our data suggest that a mild "calcium promotor" that maintains a significant, yet modest, AChE inhibitory activity, could be a new type of therapeutic target for treating patients of Alzheimer's disease. The fact that its parent compound, tacrine, does not provide protection suggests that the neuroprotective effect of ITH4012 may go beyond simple inhibition of AChE. This is the case of donepezil, another AChE inhibitor that has been shown to afford protection through a mechanism linked to nicotinic receptors (Akasofu et al., 2003; Takeda et al., 2003).

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REFERENCES

Abad F, Maroto R, López MG, Sanchez-García P and García AG (1995) Pharmacological protection against the cytotoxicity induced by 6-hydroxydopamine and H_2O_2 in chromaffin cells. *Eur J Pharmacol* 293:55-64.

Akasofu S, Kosasa T, Kimura M and Kubota A (2003) Protective effect of donepezil in a primary culture of rat cortical neurons exposed to oxygen-glucose deprivation. *Eur J Pharmacol* 472:57-63.

Arias E, Alés E, Gabilan NH, Cano-Abad MF, Villarroya M, García AG and López MG (2004) Galantamine prevents apoptosis induced by beta-amyloid and thapsigargin: involvement of nicotinic acetylcholine receptors. *Neuropharmacology* 46:103-114.

Balazs R, Resink A, Hack N, Van der Valk JB, Kumar KN and Michaelis E (1992) NMDA treatment and K^+-induced depolarization selectively promote the expression of an NMDA-preferring class of the ionotropic glutamate receptors in cerebellar granule neurones. *Neurosci Lett* 137:109-113.

Choi DW (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 11:465-469.

Collins F and Lile JD (1989) The role of dihydropyridine-sensitive voltage-gated calcium channels in potassium-mediated neuronal survival. *Brain Res* 502:99-108.

De los Rios C, Marco JL, Carreiras MD, Chinchón PM, García AG and Villarroya M (2002) Novel tacrine derivatives that block neuronal calcium channels. *Bioorg Med Chem* 10:2077-2088.

Dragunow M, Faull RL, Lawlor P, Beilharz EJ, Singleton K, Walker EB and Mee E (1995) In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. *Neuroreport* 6:1053-1057.

Franklin JL and Johnson EM Jr (1992) Suppression of programmed neuronal death by sustained elevation of cytoplasmic calcium. *Trends Neurosci* 15:501-508.

García AG, Gandía L, López MG and Montiel C (2000) Calcium channels for exocytosis: Functional modulation with toxins. In: Seafood and Freshwater Toxins. Pharmacology, Physiology and Detection. (Ed. Luis M. Botana. Marcel Dekker Inc. New York).

García AG, Sala F, Reig JA, Viniegra S, Frías J, Fonteríz R and Gandía L (1984) Dihydropyridine BAY-K-8644 activates chromaffin cell calcium channels. *Nature* 309:69-71.

Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440-3450.

Guttman R, Altman RD and Nielsen NH (1999) Alzheimer disease. Report of the Council on Scientific Affairs. *Arch Fam Med* 8:347-353.

Jellinger KA and Stadelmann C (2001) Problems of cell death in neurodegeneration and Alzheimer´s disease. *Journal of Alzheimers Disease* 3:31-40.

Koike T, Martin DP and Johnson EM Jr (1989) Role of Ca^{2+} channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal Ca^{2+} determine nerve growth factor dependence of sympathetic ganglion cells. *Proc Natl Acad Sci U S A* 86:6421-6425.

Marco JL, De Los Ríos C, Carreiras MDC, Baños JE, Badía A and Vivas N (2001) Sínthesis and acetylcholinesterase/butyrylcholinesterase inhibition activity of new tacrine-like analogues. *Bioorg Med Chem* 9:727-732.

Maroto R, De la Fuente MT, Artalejo AR, Abad F, López MG, García-Sancho J and García AG (1994) Effects of Ca^{2+} channel antagonists on chromaffin cell death and cytosolic Ca^{2+} oscillations induced by veratridine. *Eur J Pharmacol* 270:331-339.

Michelena P, Garcia-Perez LE, Artalejo AR and García AG (1993) Separation between cytosolic calcium and secretion in chromaffin cells superfused with calcium ramps. *Proc Natl Acad Sci USA* 90: 3284-3288.

Moro MA, López MG, Gandía L, Michelena P and García AG (1990) Separation and culture of living adrenaline- and noradrenaline-containing cells from bovine adrenal medullae. *Anal Biochem* 185:243-8.

Nath R., Raser KJ, Hajimohammadreza I and Wang KK (1997) Thapsigargin induces apoptosis in SH-SY5Y neuroblastoma cells and cerebrocortical cultures. *Biochemistry and Molecular Biology International* 43:197-205.

Neher E (1989) Combined fura-2 and patch-clamp measurements in rat peritoneal mast cells. In: Sellin, L.P., Libelius, R., Thesleff, S. (Eds.), *Neuromuscular Junction* Elsevier, Amsterdam, pp. 65-76.

Ota M, Narahashi T and RF Keeler (1973) Effects of veratrum alkaloids on membrane potential and conductance of squid and cray-fish giant axons, *J Pharmacol Exp Ther* 184:143-154.

Pike CJ, Balazs R and Cotman CW (1996) Attenuation of amyloid-beta neurotoxicity “in vitro” by potassium-induced depolarization. *J Neurochem* 67:1774-1777.

Robinson JP, Darzynkeiwicz Z, Dean PN, Orfao A, Rabinovitch PS, Stewart CC, Tanke H.J and Weeless LL (1997). In: Current protocols in cytometry. John Wiley & Sons, Inc. New York.

Schroder H, Giacobini E, Struble RG, Zilles K and Maelicke A (1991) Nicotinic cholinoceptive neurons of the frontal cortex are reduced in Alzheimer's disease. *Neurobiol Aging* 12:259-262.

Standaert DG and Young AB (1996) In: Goodman and Gilman´s The Pharmacological Basis of Therapeutics, 9th Edition, pp 39-556; McGraw-Hill, Mexico.

Su JH, Anderson AJ, Cummings BJ and Cotman CW (1994) Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* 5:2529-2533.

Takada Y, Yonezawa A, Kume T, Katsuki H, Kaneko S, Sugimoto H and Akaike A (2003) Nicotinic acetylcholine receptor-mediated neuroprotection by donepezil against glutamate neurotoxicity in rat cortical neurons. *J Pharmacol Exp Ther* 306:772-777.

Wang JL, Liu D, Zhang ZJ, Shan S, Han X, Srinivasula SM, Croce CM, Alnemri ES, and Huang Z (2000) Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells. *Proc Natl Acad Sci U S A.* 97:7124-7129.

Wie H, Wei W, Bredensen DE and Perry DC (1998) Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. *J Neurochem* 70:2305-2314.

Footnotes

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LEGENDS FOR FIGURES

Figure 1: Chemical structures of ITH4012 (a), tacrine (b) and Bay K8644 (c).

Figure 2: Effects of ITH4012, Bay K8644 and tacrine on thapsigargin-induced cell death. Bovine chromaffin cells were exposed for 24h to increasing concentrations of ITH4012 (a), Bay K8644 (b) or tacrine (c); then they were incubated for another 24h with 5 μM thapsigargin in the presence of one of the drugs mentioned above. Cell viability was assessed by measuring the amount of LDH released to the extracellular media. Data correspond to the mean ± SEM of 16 wells of 4 different primary cultures. * $p \leq 0.05$ and ** $p \leq 0.01$, compared to thapsigargin-treated cells in the absence of drugs.

Figure 3: Effect of ITH4012 on H_2O_2-induced cell death. The top part of the figure shows microphotographs of primary cultures of untreated bovine chromaffin cells (a), cells treated for 24 h with 500 μM H_2O_2 alone (b), and cells preteated 24h with 1 μM ITH4014 before H_2O_2 exposure (c). Cells were observed with a 20X objective. The bottom of the figure (panel d) represents quantitative data of bovine chromaffin cells exposed for 24h to increasing concentrations of ITH4012 (0.01 μM to 10 μM) and then exposed for another 24h to 500 μM H_2O_2 in the presence of the drug. Cell viability was assessed by measuring the amount of LDH released to the extracellular media (ordinate). Data are means ± SEM of 16 wells of 4 different batches of cells. ** $p \leq 0.01$, compared to H_2O_2-treated cells in the absence of ITH4012.

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Figure 4: Effect of ITH4012 on apoptosis elicited by $A\beta_{25-35}$ in SH-SY5Y. ITH4012 was preincubated for 24h in the presence of fetal calf serum and then serum-free $A\beta_{25-35}$ was added in the presence or absence of ITH4012. Control apoptosis was elicited by only serum withdrawal for 24 h. Apoptosis was evaluated by flow cytometry (see Material and Methods section). Data are means $\pm$ SEM of 5 different batches of cells. ** $p \leq 0.01$, compared to $A\beta_{25-35}$-treated cells in the absence of ITH4012.

Figure 5: Effects of ITH4012, Bay K8644 and tacrine on cell death elicited by veratridine. Bovine chromaffin cells were exposed for 24 h to increasing concentrations of ITH4012 (a), Bay K8644 (b) or tacrine (c); then they were incubated for another 24h with 30 μM veratridine, still in the presence of the drugs mentioned above. Cell viability was assessed by measuring the amount of LDH released to the extracellular media, as a fraction of total LDH present in cells at the beginning of the experiment (ordinate). Data are means $\pm$ SEM of 16 wells of 4 different batches of cells. ** $p \leq 0.01$ and *** $p \leq 0.001$, compared to veratridine-treated cells in the absence of drugs.

Figure 6: Effect of ITH4012 on basal $[Ca^{2+}]_c$. (a) An original recording of $[Ca^{2+}]_c$ in a single bovine chromaffin cell loaded with fura-2 AM and superfused with increasing concentrations of ITH4012 (arrows). (b) Quantative data of the mean peak of $[Ca^{2+}]_c$ induced by 10 nM ITH4012. *** $p \leq 0.001$, compared to control cells.

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Figure 7: Reversion of the cytoprotective effect of ITH4012 by inhibition of protein synthesis or blockade of Bcl-2 protein. (a) The effect of the protein synthesis blocker cycloheximide (10 μM) and (b) the effect of the Bcl-2 blocker HA14-1 (20 μM) on the cytoprotective effects of ITH4012 (1 μM and 10 μM) gainst veratridine-induced toxicity, measured as LDH release. Data are means ± SEM of 16 wells from 4 different batches of cells; * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$, compared to veratridine-treated cells in the absence of drugs; † † $p \leq 0.01$ and ††† $p \leq 0.001$, compared to ITH4012-plus-veratridine-treated cells in the presence or absence of either 10 μM cycloheximide or 20 μM HA14-1. N.S. – not significant.

Figure 8: Effect of ITH4012 on the induction of Bcl-2 protein. (a) Immunofluorecence against Bcl-2 in bovine chromaffin cells treated for 48 h with DMEM alone (left) or 1 μM ITH4012 (right). (b) Western blots against Bcl-2 in control cells or in cells incubated for 24 h or 48h with 1 μM ITH4012. (c) Densitometric measurements of bands expressed as percentages with respect to control bands. The results are corrected with respect to protein charge, measured in the β-actin bands that were taken as controls.

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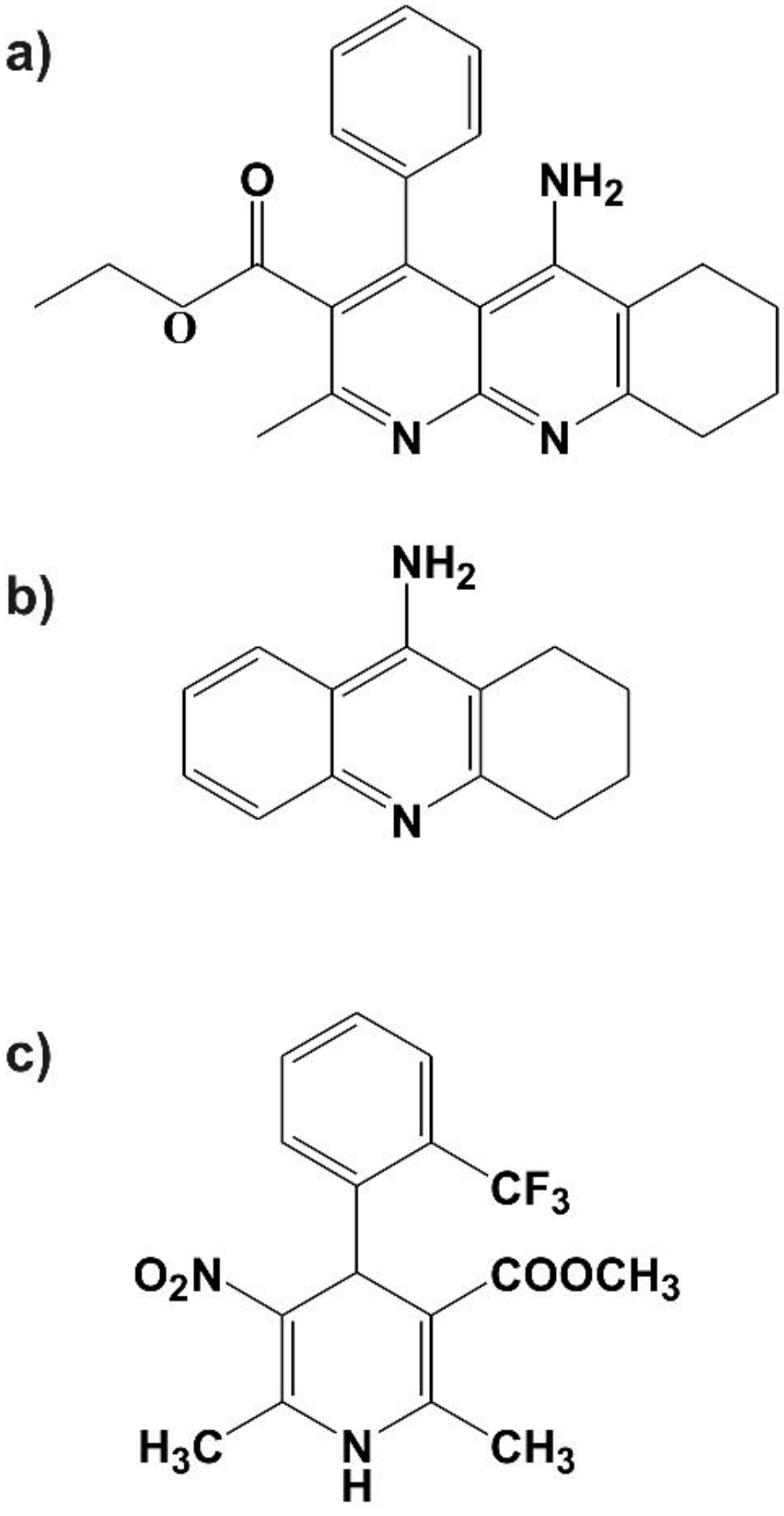

Figure 1

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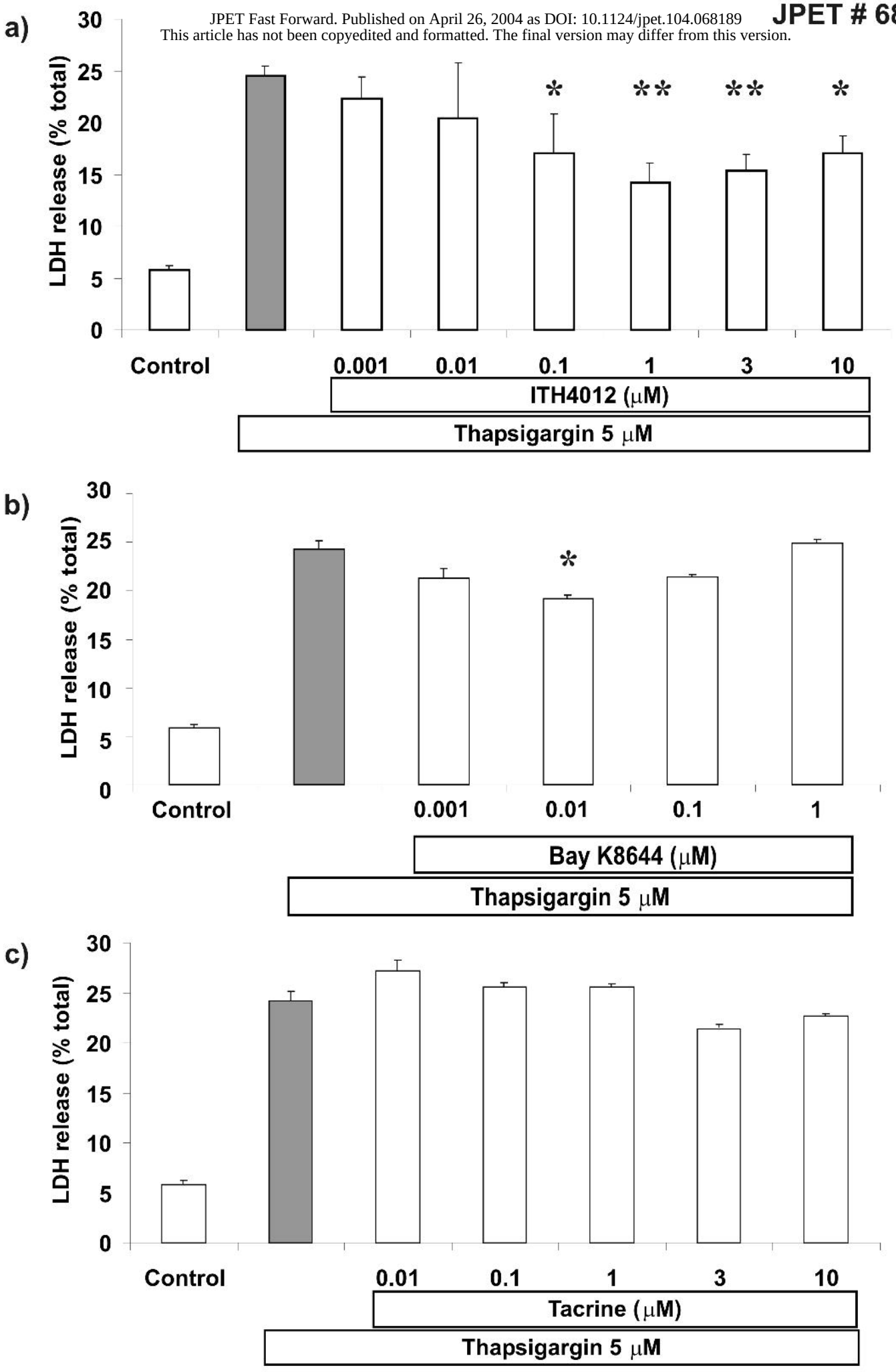

Figure 2

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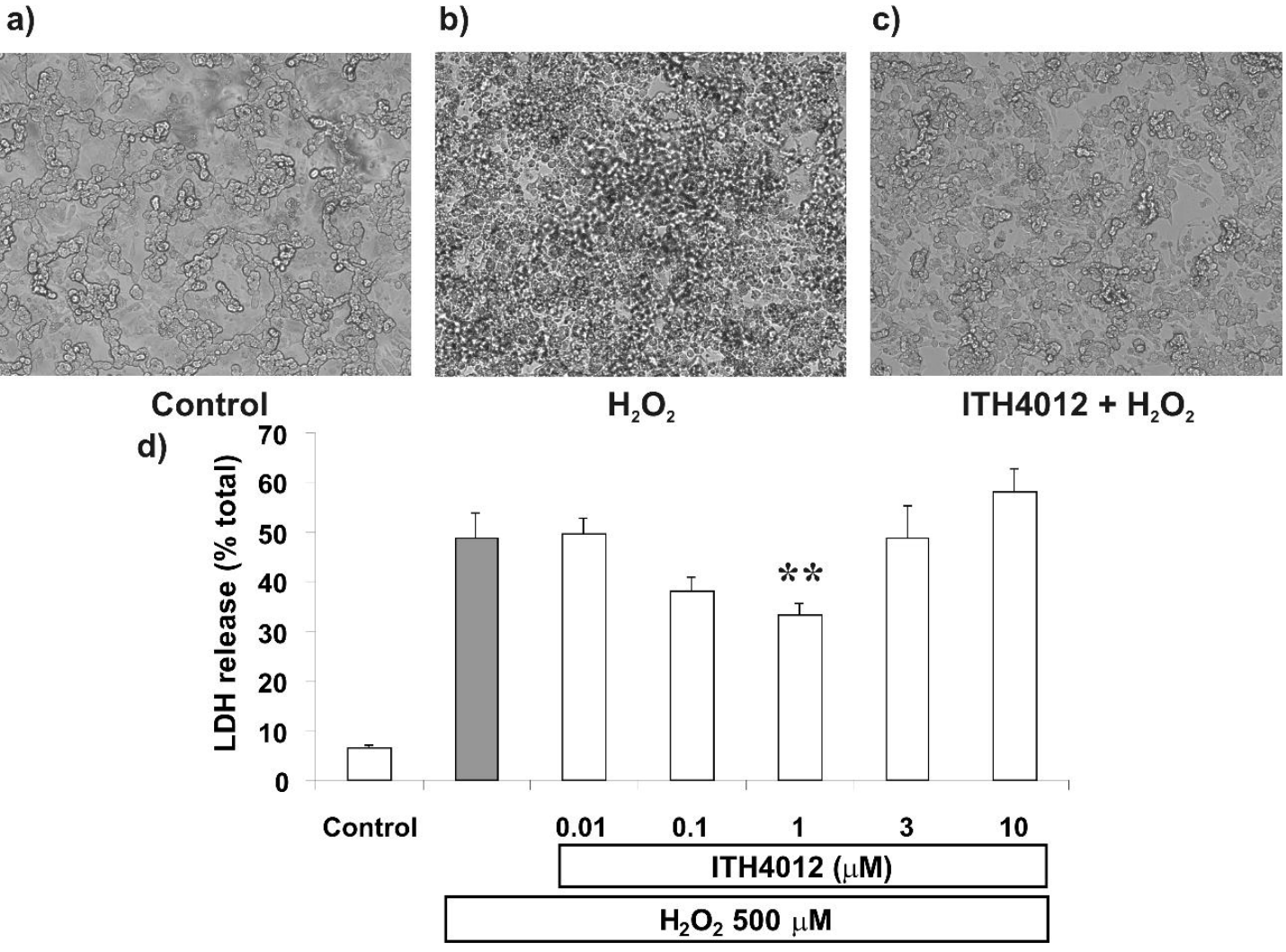

Figure 3

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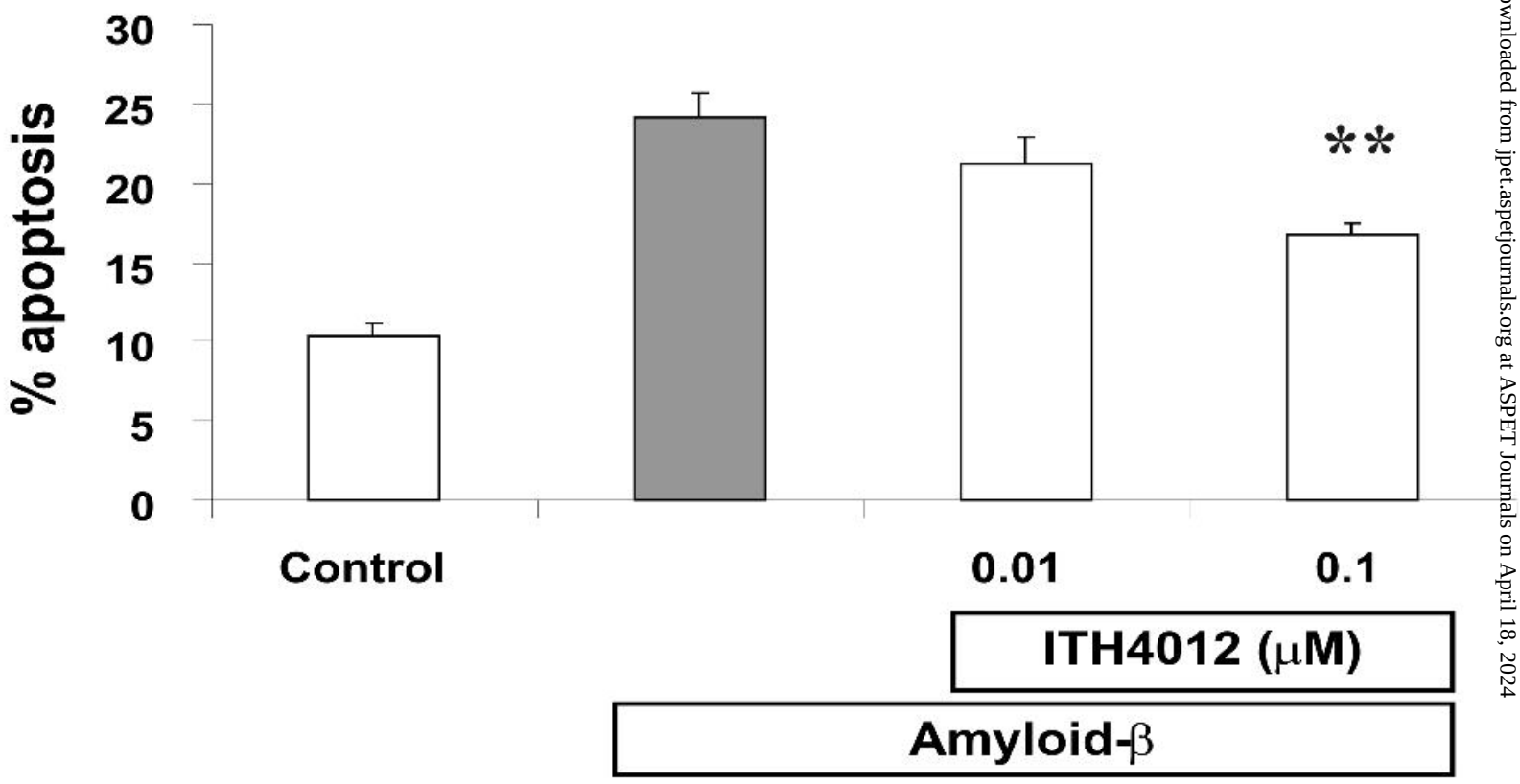

Figure 4

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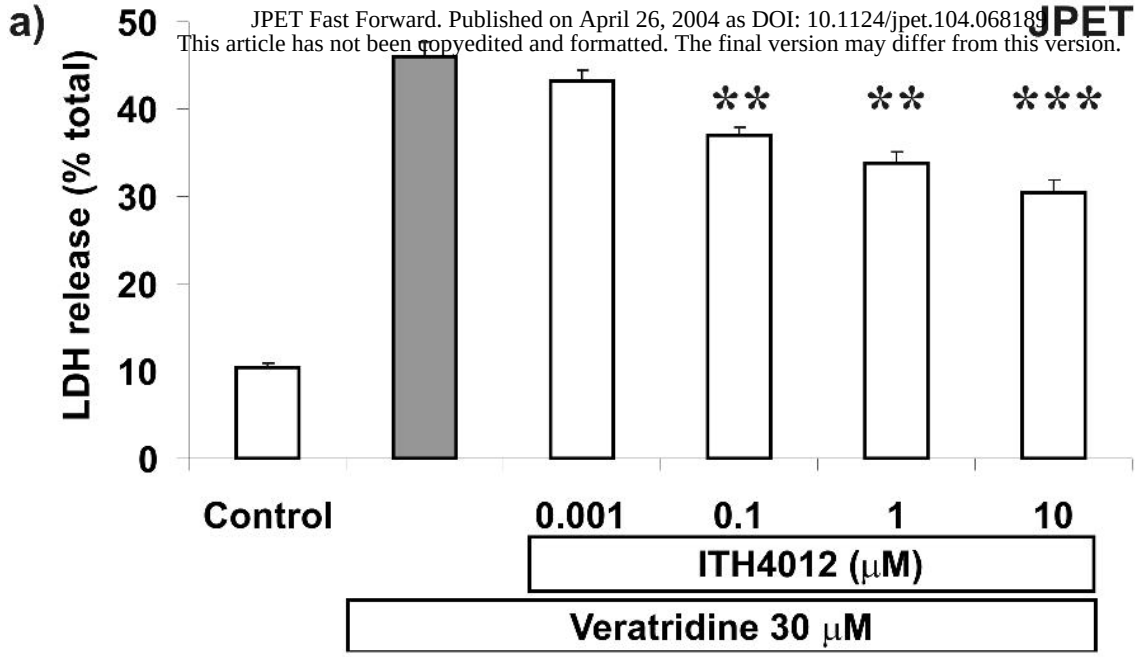

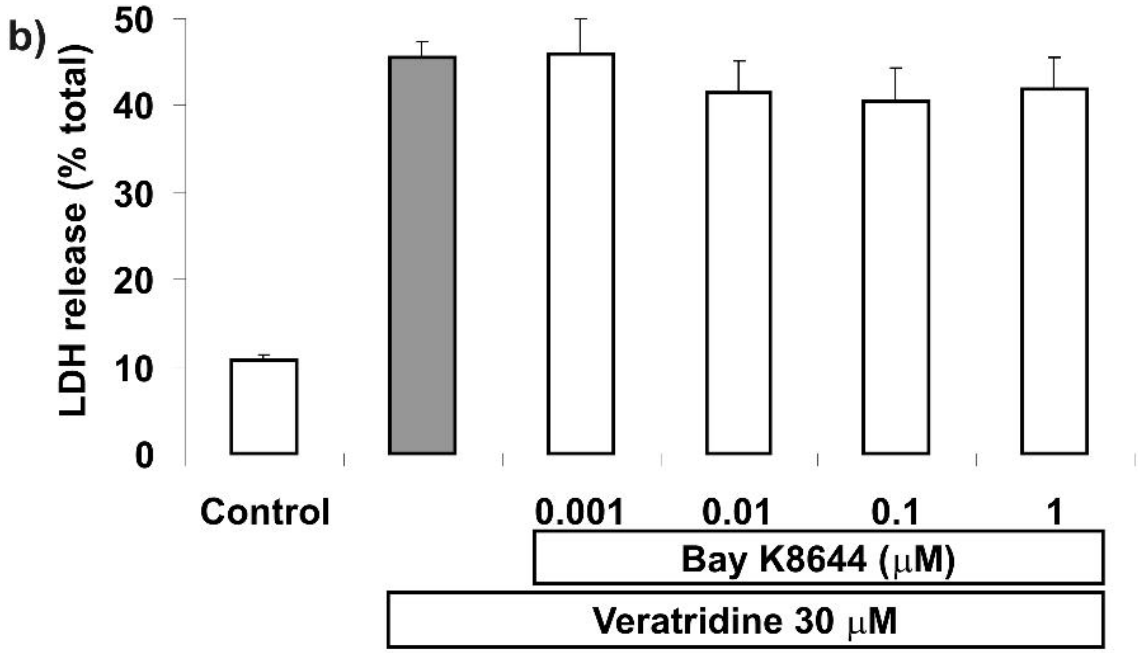

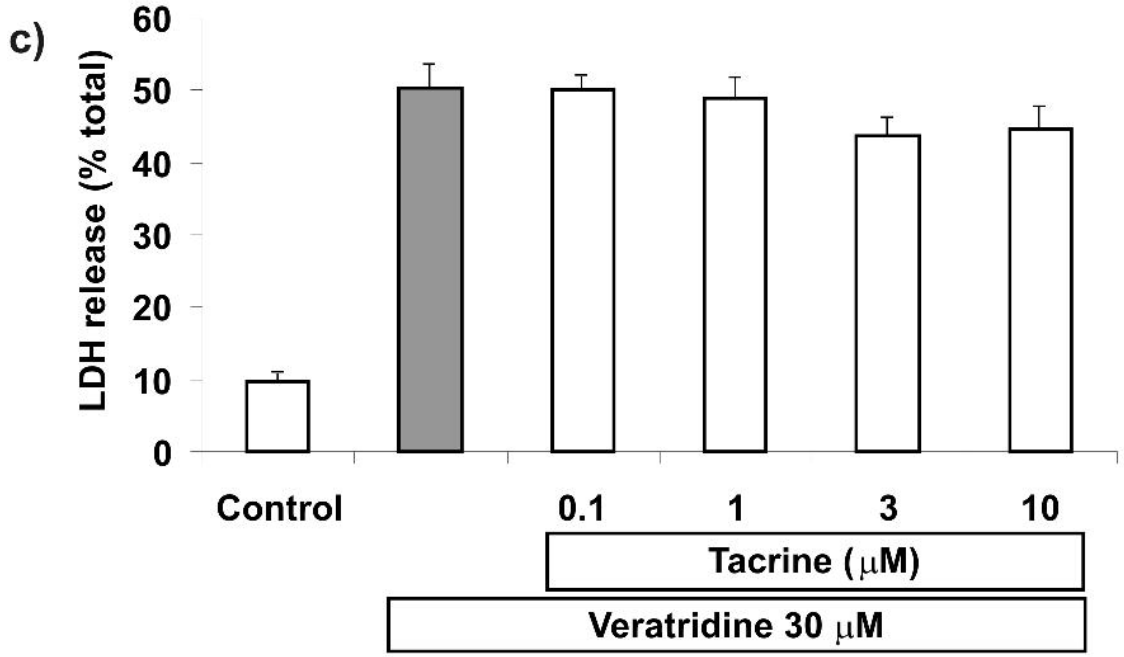

Figure 5

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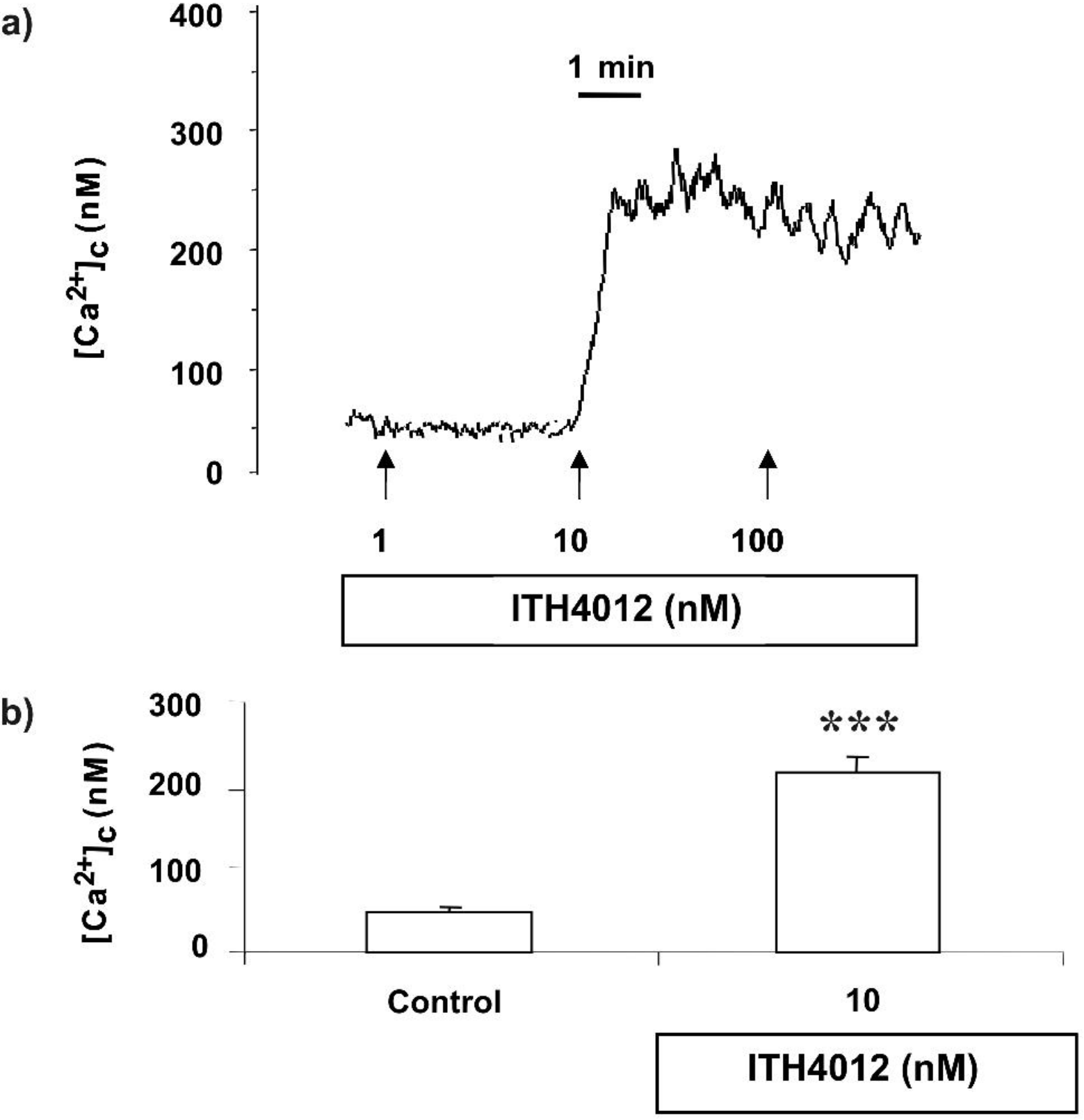

Figure 6

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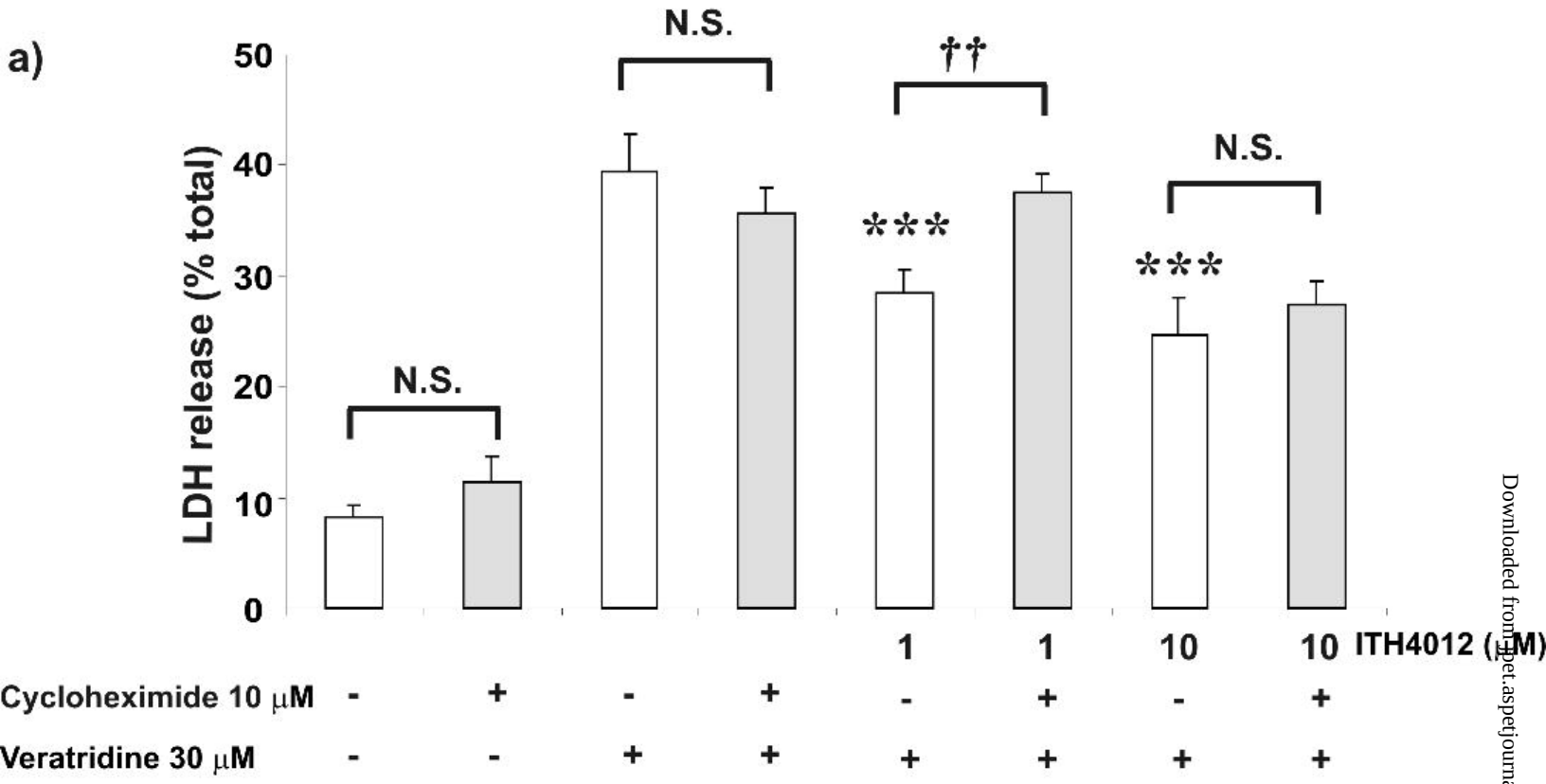

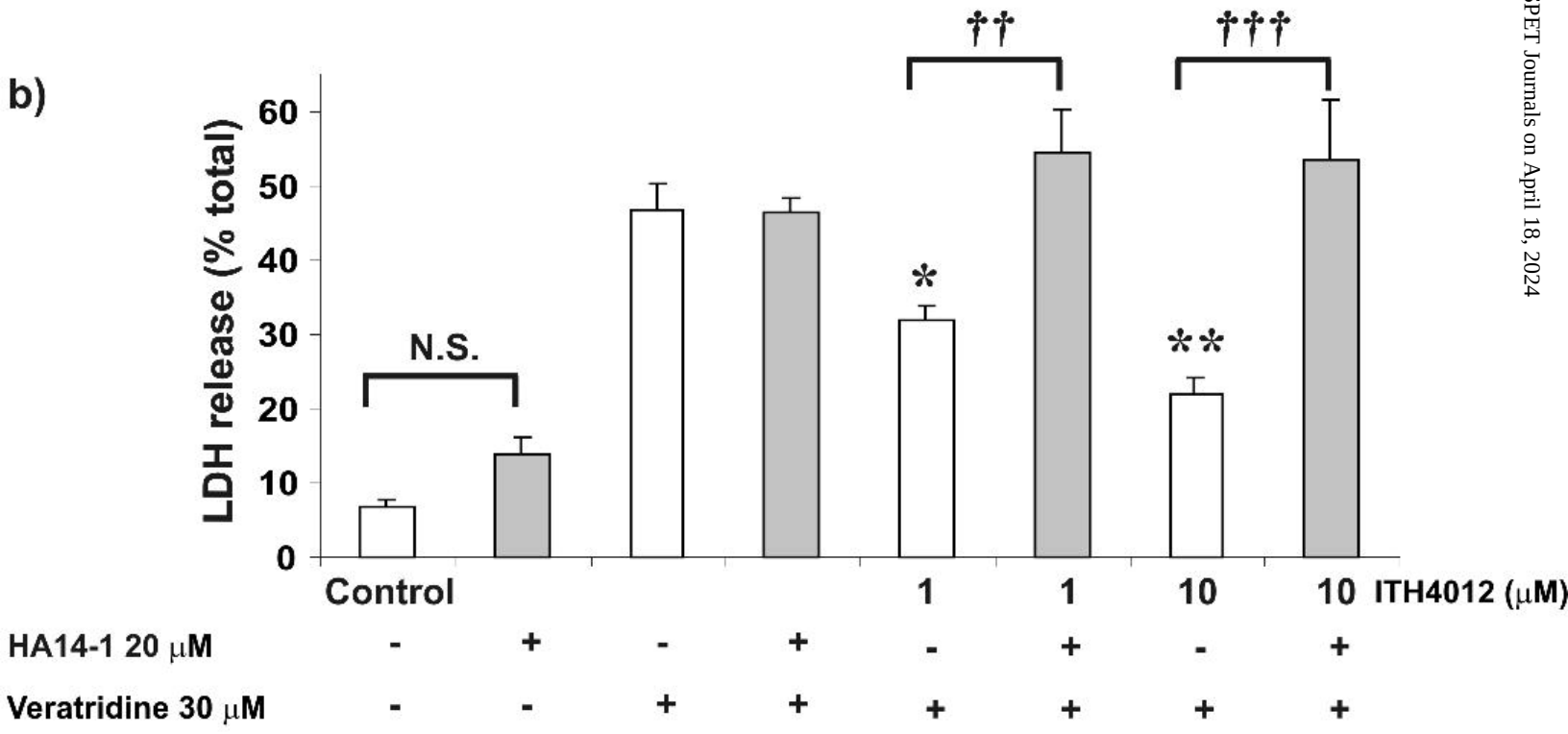

Figure 7

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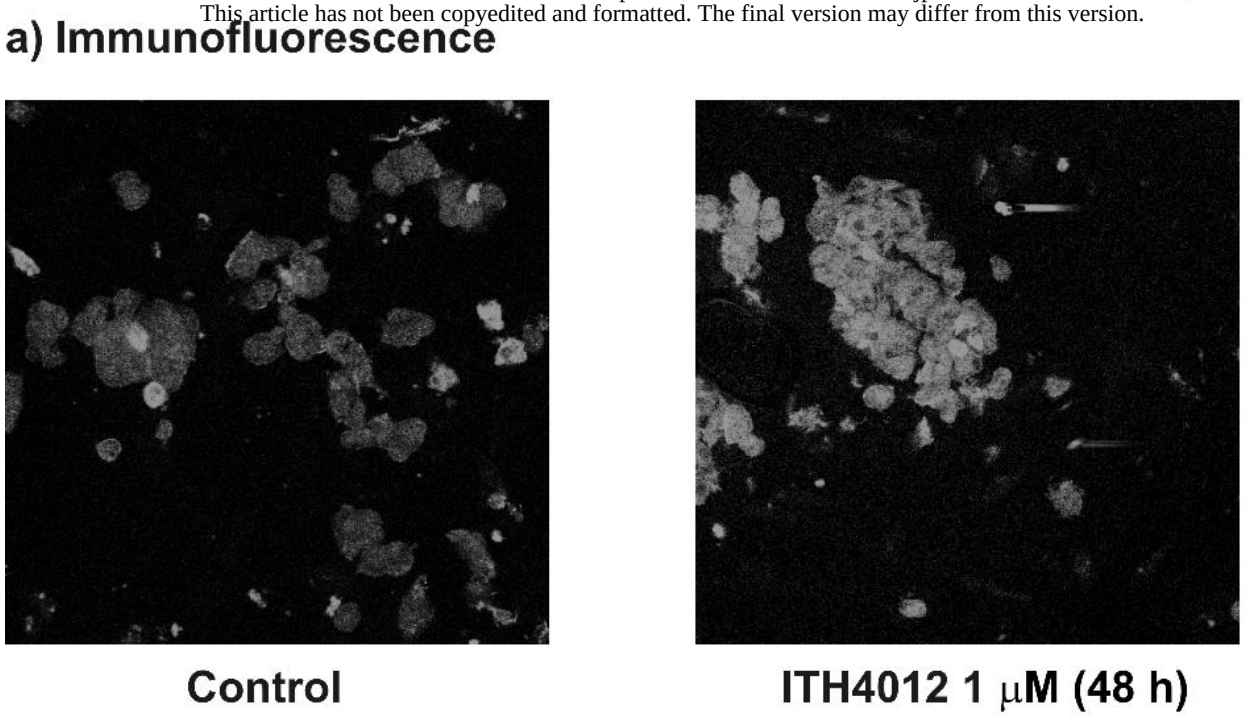

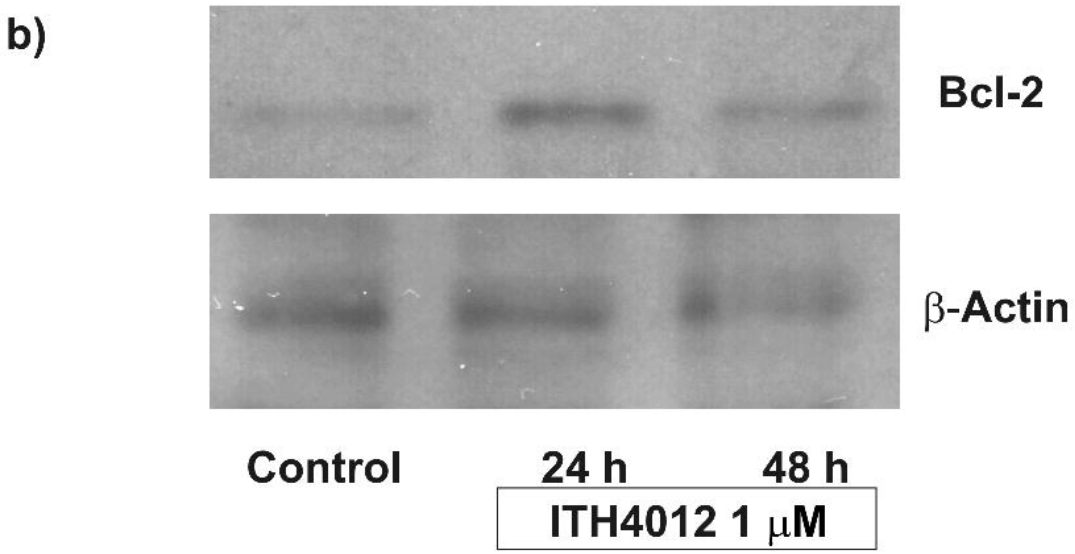

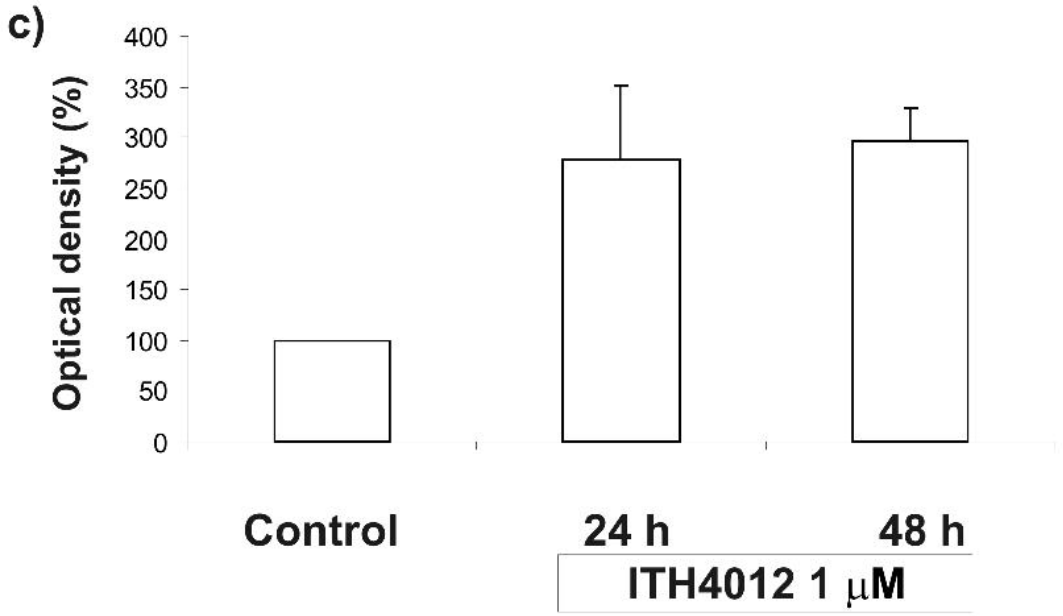

Figure 8