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Initiation of Apoptosis and Autophagy by the Bcl-2 Antagonist HA14-1

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Abstract

L1210 murine leukemia cells exposed to an LD₉₀ concentration of the Bcl-2/Bcl-x_L antagonist HA14-1 rapidly undergo apoptosis but also develop numerous intracellular vacuoles with double membranes, exhibit enhanced labeling by monodansylcadaverine, and convert the cytosolic protein LC3-I to LC3-II. These are hallmarks of autophagy. Autophagic vacuoles develop rapidly, preceding the appearance of an apoptotic nuclear morphology and can be observed in both non-apoptotic and apoptotic cells. Inhibition of autophagy by the PI 3-kinase inhibitor wortmannin promoted apoptosis; conversely inhibition of caspase-3/7 with zDEVD-fmk promoted autophagy. Neither process was dependent on calcium translocation. These results indicate that pharmacological suppression of Bcl-2 function can mimic the induction of autophagy that can occur following the down-regulation of Bcl-2 expression by molecular approaches.

Keywords

apoptosis; autophagy; Bcl-2; HA14-1; vacuoles; L1210

1. Introduction

The drug HA14-1 (ethyl 2-amino-6-bromo-4(1-cyano-2-ethoxy-2-oxoethyl)-4Hchromene-3-carboxylate) was initially identified as a non-peptidic ligand for the surface pocket of the anti-apoptotic protein Bcl-2 with pro-apoptotic activity [1]. Additional studies with HL-60 cells suggested that HA14-1 facilitated the release of Ca²⁺ from the endoplasmic reticulum (ER), contributing to mitochondrial dysfunction and the release of cytochrome c [2]. Initiation of apoptosis by HA14-1 could therefore be antagonized by either co-incubation with the calcium chelating agent EGTA or Ru360 [2], a ruthenium complex that inhibits Ca²⁺ uptake by the mitochondrial ‘uniporter’ [3].

Macroautophagy (hereafter termed ‘autophagy’) is a process whereby cytosol and organelles become enclosed in double membrane structures termed autophagosomes. The latter ultimately fuse with lysosomes and a subsequent proteolytic process permits the engulfed material to become available for recycling [4,5]. Although originally described as a survival response

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evoked by nutrient deprivation, it is now clear that autophagy can also serve as a cell death pathway [6–8].

Antisense-mediated down-regulation of Bcl-2 expression has been reported to induce autophagy in HL-60 cells [9]. Similarly, siRNA-mediated down regulation of Bcl-2 in MCF-7 cells dramatically enhanced the induction of autophagy by nutrient deprivation [10,11]. Conversely, Bcl-2 over expression has been reported to suppress the induction of autophagy by nutrient deprivation in several cell types [10]. In this study we determined if a functional inactivation of Bcl-2/Bcl-X_L, mediated by HA14-1, also causes the induction of autophagy. As a test system we used murine leukemia L1210 cells. We also assessed the role of Ca²⁺ on HA14-1-initiated responses in this cell line. We found that HA14-1 promoted the development of both autophagy and apoptosis in L1210 cells, and that these processes were Ca²⁺ independent.

2. Materials and methods

2.1 Chemicals and biologicals

Amino acids, tissue culture media, wortmannin, monodansylcadaverine (MDC), ruthenium complex Ru360, and the caspase inhibitor zDEVD^FFMK were obtained from Sigma-Aldrich (St. Louis, MO). Sterile horse serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Höchst dye HO33342 (HO342), tetramethylrhodamine methyl ester (TMRM), and the cell-permeable calcium-chelating agent BAPTA-AM were obtained from Invitrogen (Molecular Probes; Carlsbad, CA). The Bcl-2 antagonist HA14-1 was provided by Ryan Scientific Inc. (Isle of Palms, SC). Since this reagent slowly loses activity in the presence of water, solutions were made up in anhydrous dimethyl sulfoxide, and stored in small aliquots at –20°C under nitrogen.

2.2 Fluorescent probes

Nuclear morphology/condensed chromatin, mitochondrial membrane potential and enhanced lysosomal/endosomal activity were probed by staining cells with HO342, TMRM and MDC, respectively, as previously described [12]. After labeling, cells were washed once with fresh growth medium and examined by phase-contrast and fluorescence microscopy. Excitation wavelengths were 360–380 nm (HO33342, MDC) and 510–560 nm (TMRM). Interference filters were used to isolate the emission fluorescence of HO342 (400–450 nm), MDC (520–560 nm) and TMRM (550–650 nm).

2.3 Cells and maintenance

Murine leukemia L1210 cells were grown in sealed flasks, using an approximation of Fisher's Medium consisting of α -MEM (GIBCO-BRL, Grand Island, NY) supplemented with MgCl₂ (45 mg/l), methionine (75 mg/l), phenylalanine (30 mg/l), valine (30 mg/l), folic acid (9 mg/l), 1 mM glutamine, 1 mM mercaptoethanol, gentamicin, and 10% horse serum. Cells were maintained in suspension culture at 37°C in a 5% CO₂ atmosphere.

Western blots for LC3—The conditions used for cell lysis and western blot analyses have been described [12]. Protease inhibitors (10 μ g/ml each of E64d and pepstatin A) were added to inhibit degradation of LC3-II [13,14]. Samples containing 40 μ g of protein were used for these assays. Immune complexes were detected with Vistra ECF western blot reagent (Amersham Biosciences Corp., Piscataway, NJ) using the 'Storm' imaging system (Molecular Dynamics, Sunnyvale, CA). A rabbit polyclonal antibody raised against the microtubule-associated protein LC3 was the gift of Dr. Masahiro Shibata, Osaka University Graduate School of Medicine, Japan.

2.4 Phase/fluorescence microscopy

Images were acquired using a Nikon Eclipse E600 microscope and a SenSys CCD camera (Photometrics, Tucson, AZ), and further processed using MetaMorph software (Universal Imaging, Downingtown, PA). A Uniblitz shutter was used to control exposure to the excitation source. This was configured to open and close with the camera shutter, thus minimizing photobleaching.

2.5 Electron Microscopy

For these studies, L1210 cells were fixed with glutaraldehyde and osmium tetroxide, treated with uranyl acetate + lead citrate for enhanced protein and lipid staining, and then dehydrated in ethanol. The cell pellets were embedded in epon resin and cut with an ultramicrotome to a 70 nm thickness.

2.6 HA14-1 treatment protocols

L1210 cells were treated with LD₅₀ or LD₉₀ doses of HA141, previously determined to be 20 and 40 μ M, respectively [15], for specified times at 37°C. In some studies, cultures were co-treated with the caspase 3/7 inhibitor zDEVD-fmk (50 μ M) or the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin (100 nM). To assess the role for translocation of cytosolic calcium in HA14-1 initiated responses, some incubations were carried out in the presence of BAPTA-AM (10 μ M) plus the ruthenium complex Ru360 (5 μ M). After incubation, cells were labeled with HO342, TMRM or MDC, as described above. Because of the lack of spectral overlap, it was feasible to label cells simultaneously with HO342 and TMRM.

3. Results and discussion

Exposure of L1210 cultures to 40 μ M HA14-1 for 60 min caused a partial loss of mitochondrial membrane potential ($\Delta\Psi_m$) in some cells, and the total loss in others (as indicated by TMRM staining, Fig. 1). HO342 staining indicated that many of the latter also exhibited condensed chromatin (Fig. 1). Many HA14-1 treated cells, including some with condensed chromatin, also increased in size and developed numerous intracellular vacuoles. Monodansylcadaverine (MDC) staining, commonly used as a marker of autophagic vacuoles and lysosomes [16], was primarily punctate in control cultures (Fig. 1, top row). HA14-1 treated cultures exhibited enhanced levels of punctate MDC staining, as well as diffuse vacuole staining (Fig. 1, second row). PI 3-kinase activity is required for formation of autophagic vacuoles [4,17]. Vacuole formation was strongly suppressed in cultures co-treated with HA14-1 and the PI 3-kinase inhibitor wortmannin, whereas the formation of condensed chromatin was not suppressed by inclusion of wortmannin (Fig. 1, third row). Conversely, inclusion of the caspase-3/7 inhibitor zDEVD strongly suppressed HA14-1-induced development of condensed chromatin, but potentiated the formation of vacuoles whose periphery stained with MDC (Fig. 1, fourth row).

A structural feature of autophagosomes that distinguishes them from other vacuoles is a double walled membrane [14]. An examination of L1210 cells 60 min after exposure to 40 μ M HA14-1 by electron microscopy confirmed the appearance of cells with multiple vacuoles (Fig. 2 top) that were enclosed in a double membrane (Fig 2, bottom).

Upon the induction of autophagy, phosphatidylethanolamine is covalently linked to the cytosolic protein LC3-I to yield LC3-II, which then associates with the autophagosome [14, 18]. This conversion is commonly used as a marker for autophagy [14]. Exposure of L1210 cells to HA14-1 caused the conversion of LC3-I to LC3-II (Fig. 3). This effect was enhanced by the presence of the caspase inhibitor zDEVD-fmk, and antagonized by the PI 3-kinase inhibitor wortmannin (Fig. 3). The effects of these inhibitors on LC3-1 processing in HA14-1 treated cultures are consistent with the morphological effects reported in Fig. 1: suppression

or enhancement of vacuolization by wortmannin or zDEVD-fmk, respectively. Collectively, the data presented in Figs. 1–3 suggest that HA14-1 treatment of L1210 cultures triggers both autophagy and apoptosis. Furthermore, the two processes are not mutually exclusive. Cells that eventually undergo apoptosis, may or may not exhibit features of autophagy. Similar observations have been reported for L1210 cells following PDT with the photosensitizer CPO [12].

Data shown in Fig. 4 demonstrate the formation of autophagic vacuoles prior to the appearance of an apoptotic nuclear morphology. Specifically, exposure to an LD₅₀ concentration of HA14-1 (20 μM) for 60 minutes caused significant vacuolization, without loss of ΔΨ_m, or the development of condensed chromatin (Fig. 4, top row). At an LD₉₀ concentration (40 μM) vacuoles were apparent within 15 min of treatment (Fig. 4, bottom row). Although the latter conditions promoted a coordinate loss of ΔΨ_m, no chromatin condensation had occurred. Hence, the processes whereby autophagosomes are generated following HA14-1 exposure are faster than those involved in activation of the endonuclease responsible for chromatin fragmentation.

An et al. [2] reported that the induction of apoptosis in HL-60 cells by HA14-1 could be inhibited by co-incubation with either the acetoxymethyl ester of EGTA, a Ca²⁺ chelator, or by inhibition of mitochondrial Ca²⁺ uptake by Ru360. Co-treatment with the calcium chelator BAPTA-AM and the mitochondrial uniporter antagonist Ru360 did not, however, impair HA14-1 induced chromatin condensation or vacuole formation in L1210 cells (Fig. 5). In other studies, we found that co-treatment with these agents did not affect the lethality of HA14-1 (not shown). Thus, unlike the situation in HL-60 cells, Ca²⁺ does not appear to play a role in the mechanism of HA14-1-induced apoptosis or autophagy in L1210 cultures.

Autophagy was originally described as an adoptive survival response to nutrient deprivation [4,5]. Lemasters has proposed that cells utilize autophagy to rid themselves of damaged organelles [19]. Whereas the above situations envision autophagy as a survival mechanism, autophagy can also lead to cell death under some circumstances [6–8]. In the current study it is unclear as to whether the observed autophagic response results in death or survival. However, autophagic morphological features were expressed in over 90% of L1210 cells following exposure to an LD₅₀ concentration of HA14-1. Hence, a large percentage of cells undergoing autophagy did not die.

In summary, our studies indicate that the pharmacological inhibition of Bcl-2 function, like molecular approaches that suppress Bcl-2 expression [9–11], is associated with the both apoptosis and autophagy in L1210 cells. Our findings are also pertinent in terms of recent developments in the field of photodynamic therapy (PDT). We, as well as others, have reported that Bcl-2 becomes rapidly inactivated/lost following irradiation of photosensitized cells where the targets for photodamage include the endoplasmic reticulum [20] or the ER + mitochondria [21]. Both apoptosis and autophagy are induced in L1210 cultures following ER photodamage [12]. Presumably, these responses are triggered by the rapid loss of Bcl-2 following irradiation [20].

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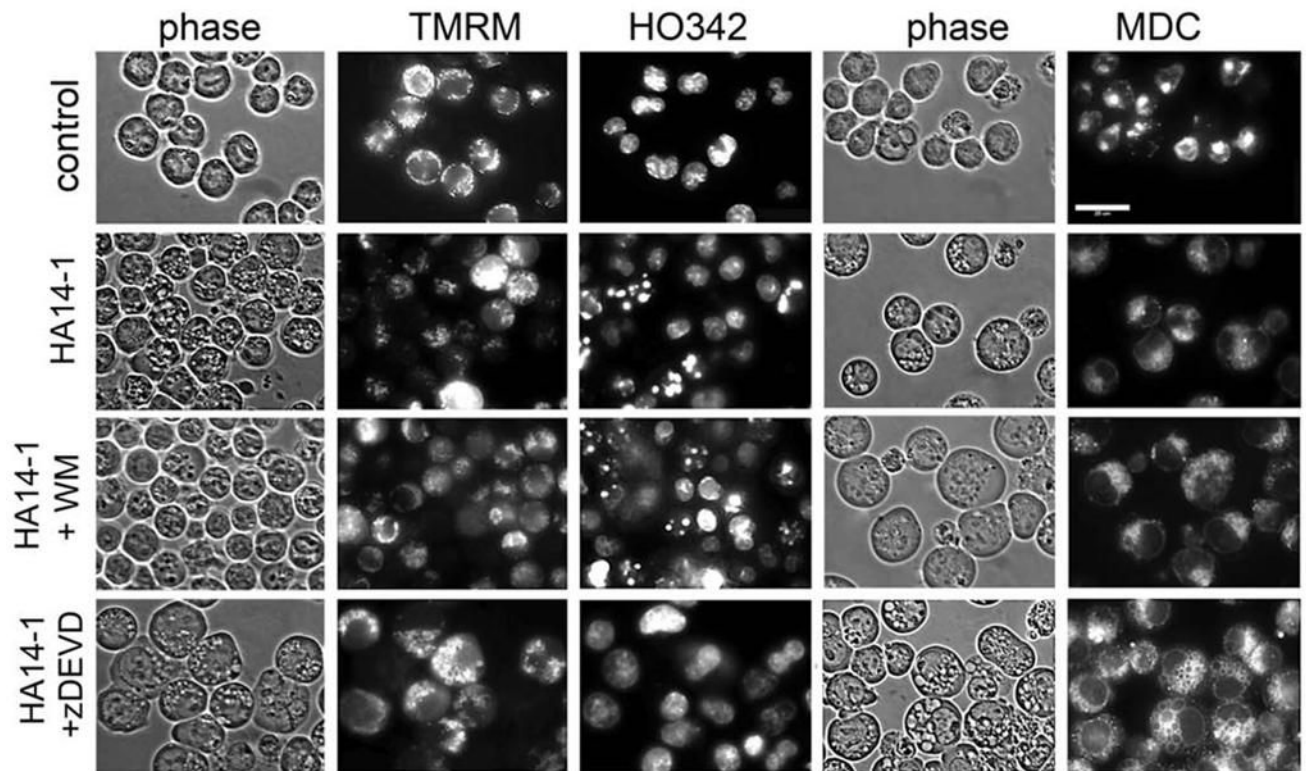


Fig 1. Loss of mitochondrial membrane potential and formation of condensed chromatin in L1210 cultures treated with HA14-1. Phase-contrast (first and fourth columns) and fluorescence images of L1210 cells stained with TMRM, HO342 and MDC. Row headings indicate additions of HA14-1 (40 μ M), zDEVD-fmk (50 μ M) and/or wortmannin (WM, 100 nM). All incubations were for 60 min. The first three columns are from the same field. The MDC labeling studies (columns 4 and 5) represent a separate experiment. White bar in top right panel = 20 μ .

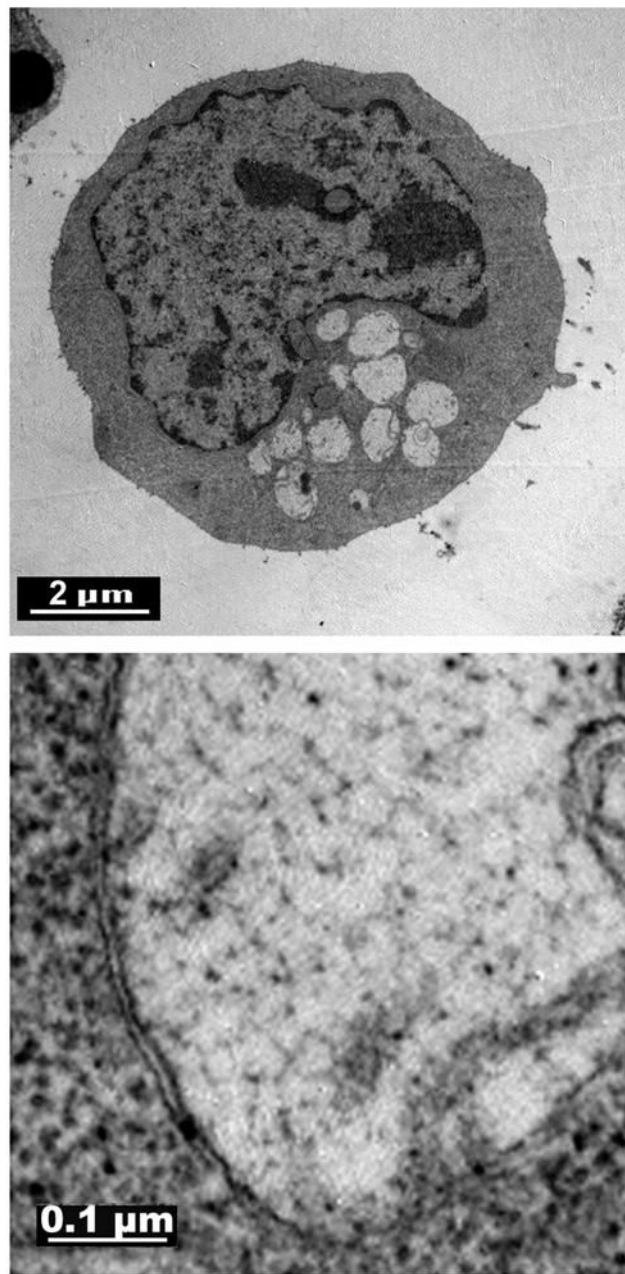


Fig 2. Electron micrographs of an L1210 cell after a 60 min exposure to an LD₉₀ concentration of HA14-1. The bottom panel depicts a typical vacuole. Top = 5,000 x, bottom = 30,000 x.

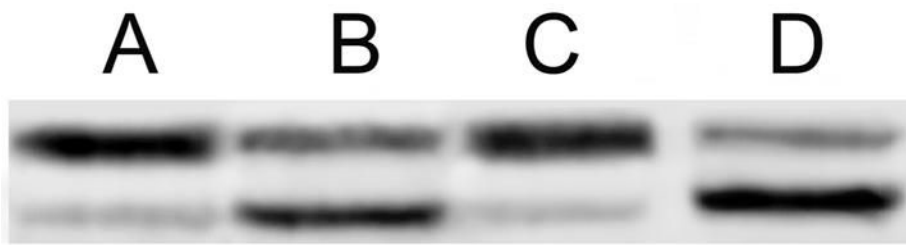


Fig 3.

Conversion of LC3-I to LC3-II in L1210 cultures treated with HA14-1. A = Controls, B = cells treated with an LD₉₀ concentration of HA14-1 for 60 min, C = same as B but with 100 nM wortmannin present, D = same as B with 50 μM zDEVD-fmk present.

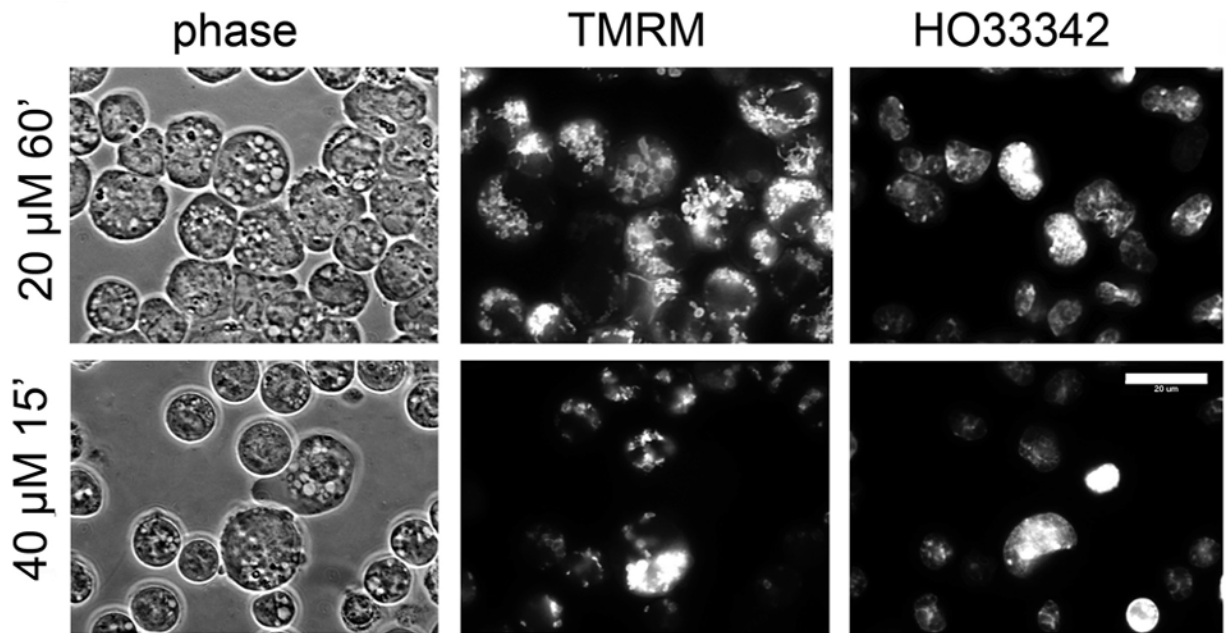


Fig 4. Rate of appearances of autophagic vacuoles and apoptotic nuclear morphology, and loss of $\Delta\Psi_m$, after treatment with HA14-1. Cultures were treated with 20 μM HA14-1 for 60 min (top row) or 40 μM HA14-1 for 15 minutes before being stained with TMRM or HO342 and photographed. White bar in bottom right panel = 20 μ .

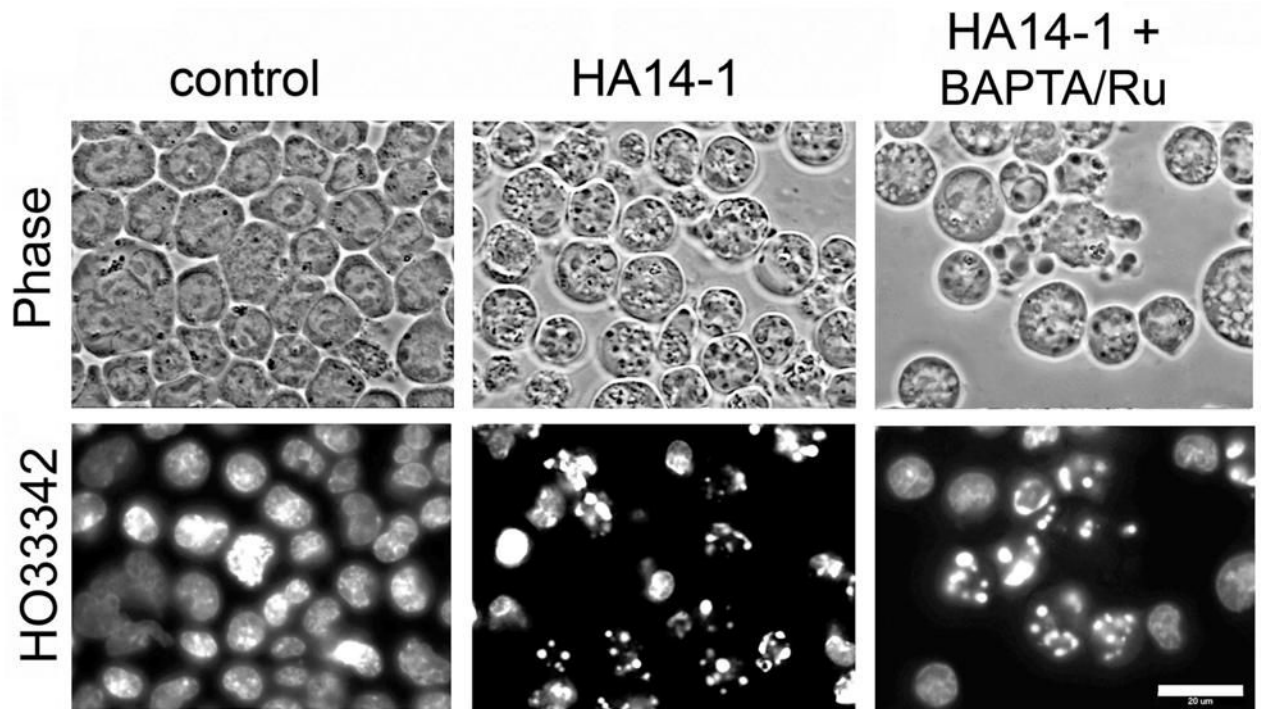


Fig 5. Role of calcium in HA14-1 induced apoptosis and autophagy. L1210 cultures were treated with 40 μM HA14-1 or HA14-1 + BAPTA-AM (10 μM) + Ru360 (5 μM) for 60 min prior to being stained with HO342 and photographed. White bar in bottom right panel = 20 μ .